Investigating the Role of Heme Oxygenase and Oxidative Stress in Oesophagogastric Cancer

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ABSTRACT

Background: Molecular mechanisms underlying gastric and oesophageal cancer include alterations in growth factors, cytokines and cell adhesion molecules. Heme oxygenase (HO) enzyme catalyses the degradation of heme and generates bilirubin and carbon monoxide that have antioxidant, anti-inflammatory and anti-apoptotic activities. HO enzyme is implicated in the biology of cancer by its effects on cell growth and resistance to apoptosis. The roles of HO-1 and HO-2 enzymes in cancer cell growth are poorly understood, with reports suggesting both anti-inflammatory, anti-tumour effects and tumour-protective mechanisms mediated by HO activity. The role of HO-2 in inflammation and cancer is largely unexplored. Further understanding the influence of the HO enzyme system may provide improved novel targets for oesophagogastric cancer therapy.

Materials and Methods: The primary objective of the thesis was to characterise the role of the heme oxygenase pathway and modulation of HO activity in upper gastrointestinal cancer cell growth in vitro. Cell culture techniques included MTT growth assay, Western blotting protein analysis, pharmacological modulation of HO activity and targeted knockdown of HO mRNA. Apoptosis was studied with flow cytometry techniques. Enzymatic HO activity was measured by specific colorimetric assay.

Results: Results showed that specific knockdown of HO-2 protein activity with induction of HO-1 enzyme caused a reduction in oesophageal and gastric cancer cell growth with cell cycle effects and an increase in apoptosis. Treatment with downstream products of HO activity carbon monoxide and bilirubin and the anti-inflammatory agent N-acetyl cysteine had similar effects and increased the response of cancer cells to chemotherapeutic agents.

Conclusion: These studies further our understanding of the role of heme oxygenase in cancer, providing evidence that modification of HO activity in oesophagogastric cancer cells leads to reduced cell proliferation. The findings support future potential clinical applications of carbon monoxide therapy that require further experimental animal model and clinical studies in the context of oesophagogastric cancer.
Declaration of Originality:

I hereby declare that I am the sole author of this thesis and that all work within it is my own. Any individuals who carried out work in collaboration with the author are appropriately credited and all reviewed literature appropriately referenced.

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LIST OF ABBREVIATIONS

HO (1, 2) Heme oxygenase enzyme (1, 2 isoforms)
HMOX (1, 2) Heme oxygenase genes (1, 2 isoforms)
NADPH Nicotinamide adenine dinucleotide phosphate
HRM Heme regulatory motifs
HSP Heat shock protein
ROS Reactive oxygen species
CAD Coronary artery disease
GIST Gastrointestinal stromal tumours
Gn Guanine thymine repeats
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
UV Ultraviolet
NAC N-acetyl cysteine
RPM Revolutions per minute
ZnPP Zinc protoporphyrin
cGMP Cyclic guanosine monophosphate
sGC Soluble Guanylate Cyclase
ATP Adenosine triphosphate
CD4\(^+\)/8\(^+\) Cluster of differentiation 4/8
LPS Lipopolysaccharide
TNF Tumour necrosis factor
IL (n) Interleukin (n)
MAPK Mitogen-activated protein kinase
NF-κB Nuclear factor-kappa B
Nrf2 Nuclear factor E2-related factor 2
VEGF Vascular endothelial growth factor
EGF Epidermal growth factor
ALA δ-Aminolevulinic acid
iNOS Inducible nitric oxide synthase
FBS Foetal bovine serum
PBS Phosphate buffered saline
DMEM Dulbecco’s modified Eagle’s medium
BEBM Bronchial epithelial basal medium
EDTA Ethylenediaminetetraacetic acid
DMSO Dimethyl sulfoxide
SDS Sodium dodecyl sulfate
BSA Bovine serum albumin
MOPS 3-N-morpholino-propane sulfonic acid
5-FU Fluorouracil
IC\((\%))\) (Percentage of) maximal inhibitory concentration
PCR Polymerase chain reaction
RT-PCR Reverse transcriptase polymerase chain reaction
C\(_T\) Threshold cycle
DNA Deoxyribonucleic acid
cDNA Complementary deoxyribonucleic acid
RNA Ribonucleic acid
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short interfering ribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleoside triphosphates</td>
</tr>
<tr>
<td>NUC</td>
<td>Negative universal control (scrambled RNA duplex)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>FAM</td>
<td>6-Carboxyfluorescein</td>
</tr>
<tr>
<td>TAMRA</td>
<td>6-Carboxytetramethylrhodamine</td>
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<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidyl serine</td>
</tr>
<tr>
<td>BrdU (TP)</td>
<td>Brominated deoxyuridine (triphosphate)</td>
</tr>
<tr>
<td>TdT</td>
<td>Terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>JC-1</td>
<td>5,5′,6,6′-tetrachloro-1,1′, 3,3′-tetraethylbenzimidazolcarbocyanine iodide</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>CORM</td>
<td>Carbon monoxide releasing molecule</td>
</tr>
<tr>
<td>iCORM</td>
<td>Inactivated carbon monoxide releasing molecule</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon monoxide</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
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<td>Nitric oxide</td>
</tr>
<tr>
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<td>Ferrous iron</td>
</tr>
<tr>
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<td>Hydrogen sulfide</td>
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<tr>
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<td>Hydrogen peroxide</td>
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<tr>
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<td>MgCl₂</td>
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CHAPTER 1: INTRODUCTION

1.1 Oesophagogastric cancer – the clinical problem

1.1.1 Oesophageal carcinoma

The epidemiology of oesophageal cancer in the Western world has undergone an impressive change over the last 30 years. Previously a rare tumour, adenocarcinoma of the oesophagus has the fastest increasing incidence of any cancer in the United States. Comparable trends are seen in Europe, with the highest reported incidence (9 per 100,000) in the United Kingdom\(^1\).

Oesophageal carcinoma is diagnosed in approximately half a million people worldwide every year. It is the eighth leading cause of cancer death worldwide\(^2\). The global incidence of oesophageal cancer has increased by 50% over the past 20 years, from 316,000 people diagnosed in 1990 to 482,300 new cases recorded in 2008\(^3\). This global increase reflects an interesting pattern in the epidemiology of oesophageal cancer in the Western world. Historically, oesophageal cancer has predominantly been of squamous cell pathological type with adenocarcinoma accounting for as little as 4% of the disease burden in the 1970s\(^5\). There has been a rapid rise in the incidence of oesophageal adenocarcinoma in Western countries over the last three decades with a reported increase in American white males of 463% (from 1.01 per 100,000 person-years in 1975 – 1979 to 5.69 per 100,000 person-years in 2000 – 2004)\(^6\). A similar rapid increase has also been reported among white females in whom the adenocarcinoma rate increased 335% over the same time period\(^7\). Although the greater part of this increase has occurred in white males, significant increases have also been seen in females and across racial categories\(^8\)\(^9\).

These rapid increases have meant that in the United States and Northern Europe adenocarcinoma represents the predominant histological subtype\(^10\)\(^12\). Adenocarcinoma has replaced squamous cell carcinoma as the most common oesophageal cancer in the Western world even though squamous cell carcinoma is still the most common pathological subtype of oesophageal cancer worldwide\(^10\)\(^13\). The rate of increase in the incidence of oesophageal adenocarcinoma in the United States has been higher than any
other cancer and this development parallels the increased prevalence of both obesity and gastroesophageal reflux disease\textsuperscript{6}. The patterns observed may be associated with changes in the exposure to potential aetiological risk factors for oesophageal carcinoma such as smoking, alcohol consumption, overweight and obesity and inadequate dietary intake of fruit and vegetables\textsuperscript{14, 15}. Oesophageal adenocarcinoma in particular is linked with excessive body mass index and long-term gastroesophageal acid reflux.

The U.S. National Cancer Institute reported 13,900 new cases and 13,000 deaths from oesophageal carcinoma in 2003 and anticipated 17,460 new cases and 15,070 deaths in 2012\textsuperscript{16}. The latest cancer statistics identify oesophageal cancer as the 7\textsuperscript{th} leading cause of cancer deaths in males in the U.S. (12,040) and the 5\textsuperscript{th} leading cause of cancer deaths in males aged 40 – 79 years old\textsuperscript{16}. The UK has one of the highest incidences of oesophageal cancer in Europe with 8,477 new cases diagnosed in 2010. It is the 9\textsuperscript{th} most common malignancy in the UK and the 8\textsuperscript{th} most common in males with a male: female ratio of approximately 2:1. It is more common with advanced age with an average of 70\% of cases diagnosed in men and women aged 65 years and over between 2008 and 2010\textsuperscript{17, 18}. Scotland has the highest rate of oesophageal carcinoma in the UK and Europe\textsuperscript{19} with only 33\% of Scottish patients being found to be suitable for curative resection\textsuperscript{20}.

Complex surgical resection in the form of radical oesophagectomy offers the best chance for survival for patients with locally advanced disease\textsuperscript{21}. If oesophageal cancer is deemed to be resectable then the only curative option is surgical resection with radical lymphadenectomy, usually after neoadjuvant chemotherapy or chemoradiotherapy\textsuperscript{22-24}. Peri-operative mortality rates for oesophageal resection should be less than 5\%, but the operation has a significant morbidity with a relatively high complication rate and impact on the quality of life during patient convalescence\textsuperscript{25}.

Oesophageal cancer has a relatively poor overall outlook. This is reflected in a high mortality-to-incidence rate ratio of 0.83 compared to that of 0.52 in colorectal cancer\textsuperscript{26}. Upper gastrointestinal cancers are often diagnosed at an advanced stage with improved survival a consistent feature of disease that presents early. For oesophageal cancer confined to the mucosa the 5-year survival rate can exceed 80\% after surgery.
Adenocarcinoma is a highly aggressive disease with a poor prognosis: reported 5-year survival rates are typically about 10%\textsuperscript{27}. Prognosis depends on clinical stage. Patients with cancer restricted to the mucosa commonly have an 80% survival rate, which drops to <50% with submucosal involvement, 20% with extension to the muscularis propria, 7% with extension to adjacent structures, and less than 3% with distant metastases.

Oesophagogastric cancer still carries a relatively poor prognosis with little significant change in overall survival in recent years\textsuperscript{28}. American Cancer Society 2009 cancer statistics demonstrate an overall 5-year survival of only 17% with improved survival for local (33.7%) or regional (16.9%) compared to distant (2.9%) disease at the time of diagnosis\textsuperscript{29}. Advances in the diagnosis, staging, and management of oesophageal cancer have led to small but significant improvements in survival for patients with resectable disease and a modest improvement in the overall 5-year survival rate over the last decade\textsuperscript{30}. Surgical resection is a fundamental approach to potentially curative therapy for early-stage and locally advanced oesophagogastric cancer. In a series of patients undergoing resection for oesophageal cancer an improvement in the 5-year survival rate from 18.8% to 42.3% (P < 0.001) was demonstrated from 1980 to 2004\textsuperscript{31}. Unfortunately, advanced disease or co-morbidities mean that only 30-40% of patients diagnosed with oesophageal cancer are suitable for the complex surgery required for curative resection\textsuperscript{32}. Early diagnosis is the key to survival with a reported 5-year survival of 95 – 100% in patients presenting with early-stage lesions including cancer-in-situ (high-grade dysplasia) or T1 disease confined to the mucosa or submucosa\textsuperscript{33}.

Improvements in the outcomes from oesophageal cancer over the last 30 – 40 years are supported by reported data of age-standardised survival rates in adults with oesophageal carcinoma. There has been a significant improvement (p < 0.05) in the 5-year relative survival rate in the United States between 2001 and 2007 (19%) when compared to the same survival rate between 1975 and 1977 (5%)\textsuperscript{34}. Similar data are available from the United Kingdom with a published 5-year standardised relative survival of 13.8% for adults diagnosed with oesophageal cancer during the period 2006 – 2010 and followed up to 2011\textsuperscript{35}. This represents an absolute increase in 5-year age-standardised relative survival of 10% when compared to adults diagnosed between 1971 and 1975\textsuperscript{36}. The latest available survivorship statistics from the UK demonstrate an improvement from a 5-year
survival rate for adults diagnosed between 1996 and 2000 of 7.2% to an equivalent rate of 13.8% for patients diagnosed in the period 2006 – 2010. These moderate improvements in outcome data may be associated with a conscious effort towards increasing specialisation in surgical services and centralisation of the management of oesophageal cancer in specialist units over the last 20 years.

Oesophageal adenocarcinoma is believed to develop via a sequential change in the nature of oesophageal epithelium from squamous to Barrett’s metaplasia and low-grade dysplasia and high-grade dysplasia within the Barrett’s epithelium progressing to invasive carcinoma. The change from squamous epithelium to columnar epithelium with glandular metaplasia (Barrett’s oesophagus) is considered to be due to chronic gastroesophageal acid and biliary reflux. An increased risk of adenocarcinoma is associated with a high dietary fat intake, overweight and obesity (body mass index above 25) and excessive alcohol consumption. Recent estimates suggest that Barrett’s oesophagus is prevalent in 2% of the UK population although only 1 in 20 of these is diagnosed. The prevalence of Barrett’s oesophagus may be as high as 8 – 10% in Western countries. The lifetime risk of developing adenocarcinoma in patients with Barrett’s oesophagus has been estimated to be 12.5 – 14.5 % from a recent UK registry study. Although this represents a 10 – 30-fold increased risk over the general population for oesophageal adenocarcinoma most patients with Barrett’s oesophagus die from other causes.

Early tumour detection may improve outcome in this defined subgroup of patients. Major challenges in the management of Barrett’s oesophagus include the efficacy and cost-effectiveness of endoscopic surveillance strategies, evaluation of other risk factors for cancer such as obesity and acid reflux, and the identification of potential biomarkers indicating disease progression.

A key area of current research concerns the possibility of sensitive and specific tests that predict the risk of malignancy for an individual with an established diagnosis of Barrett’s oesophagus. During the development of oesophageal cancer there is an established sequential progression from specialised intestinal metaplasia to low-grade dysplasia through to high-grade dysplasia and adenocarcinoma. The natural history of Barrett’s...
oesophagus is highly variable along all stages of the sequence described. Most published series report an incidence of dysplasia around 5%\textsuperscript{45-47}. Both low-grade and high-grade dysplasia can remain static, regress or progress to invasive cancer. The majority of patients with low-grade dysplasia do not progress to cancer in the short term\textsuperscript{47}. When high-grade dysplasia is evident, however, up to 50% will have a focus of invasive adenocarcinoma at the time of diagnosis\textsuperscript{48}.

The overall risk of adenocarcinoma has been investigated by various authors in recent years and was thought to be around 1% per year. However, a recent meta-analysis of small positive studies suggests that this risk has been overestimated, particularly as a result of publication bias, and that a more reliable annual risk is nearer 0.5%\textsuperscript{48,49}.

The conversion rate of non-dysplastic Barrett’s oesophagus to high-grade dysplasia or invasive oesophageal adenocarcinoma has recently been estimated at 0.47 – 0.86% per year\textsuperscript{49,50}. There is a low rate of progression toward adenocarcinoma in Barrett’s oesophagus with low grade dysplasia but the conversion rate of high-grade dysplasia to adenocarcinoma is much higher: 16 – 60% patients have invasive carcinoma at up to 8 years of follow up\textsuperscript{51-55}. Overall the conversion rate of Barrett’s oesophagus without dysplasia is 0.3 – 0.5% per person year\textsuperscript{50,56-58}. In view of the limited resources available to perform regular endoscopy and the lack of clear evidence to support screening protocols, the management of Barrett’s oesophagus varies throughout the UK\textsuperscript{59}. Current surveillance strategies are relatively ineffective, costly and somewhat cumbersome.

Progression of established metaplasia to carcinoma occurs as a consequence of molecular events triggered by the direct action of locally produced cytokines and exposure of the distal oesophagus to bile acids in refluxed gastric juice. Attempts at predicting this transformation include studies of biomarkers detectable by immunohistochemistry. Tumour suppressor genes, cell adhesion molecules and genes regulating cell apoptosis have all been investigated\textsuperscript{60}. Expression of p53, cyclin D1, the mucins MUC1 and MUC4 and increased staining of Ki-67 all appear to correlate with dysplasia. Reduced expression of E-cadherin and p16 and loss of heterozygosity of adenomatosis polyposis coli gene seem to be associated with neoplastic progression. Cytokines identified as having an important role in carcinogenesis include cyclooxygenase-2 and telomerase\textsuperscript{61}.
Available data indicate that no single marker accurately and reliably predicts the malignant potential of Barrett’s oesophagus. Further research is recommended to verify the application of surrogate cancer markers within the domain of clinical practice.
1.1.2 Gastric carcinoma

The incidence of gastric cancer has been in steady decline in the industrialised world over the past five decades\textsuperscript{62}. In the United States and Europe the incidence of gastric cancer has been decreasing with rates 11% lower in the year 2000 compared with 1990\textsuperscript{39}. Cancer statistics from the US estimate 21,320 new diagnoses of gastric cancer in 2012 and anticipate 10,540 deaths due to gastric carcinoma\textsuperscript{3}. Gastric cancer was the 12\textsuperscript{th} most commonly diagnosed cancer in the United Kingdom between 2008 and 2010 with 7,544 cases diagnosed. It was the 8\textsuperscript{th} leading cause of cancer-related death in the UK being responsible for 5,056 deaths over the same time period\textsuperscript{63}. Despite now being the fourth most common cancer worldwide with an estimated 934,000 cases diagnosed per year, gastric carcinoma results in 700,000 deaths annually\textsuperscript{64}. Gastric cancer remains the 2\textsuperscript{nd} leading cause of cancer mortality worldwide\textsuperscript{62,65-67}. There is a marked geographical variation in incidence with particularly high-risk areas being Japan, China and Eastern Europe. The disease presents a major clinical challenge with poor overall prognosis and limited survival for patients with advanced disease\textsuperscript{68,69}.

Overall survival from stomach cancer is limited by the fact that most patients present with advanced inoperable disease. In the last 20 years survival from stomach cancer has improved in the United Kingdom\textsuperscript{70}. Survival in those patients who undergo potentially curative resection is approximately 50% at five years\textsuperscript{71-73}. The risk of cancer recurrence and survival prognosis is determined by several clinic-pathological features including involvement of resection margins, tumour stage and status of resected lymph nodes\textsuperscript{74-76}.

The majority of gastric cancer is of the adenocarcinoma histological subtype. The pathogenesis of gastric cancer is multifactorial and complex with potential predisposing conditions including chronic atrophic gastritis, gastric epithelial intestinal metaplasia and dysplasia, gastric polyps and chronic peptic ulcer. Lauren’s classification of gastric cancer describes intestinal type with glandular structures (53%), diffuse carcinoma without structure that secrete mucin (33%) and mixed-type tumours (14%)\textsuperscript{77}. Intestinal-type gastric cancer is strongly linked to environmental factors such as nitrate ingestion. The Correa hypothesis describes the pathway for malignant transformation of gastric
epithelium including the influence of socio-economic factors, dietary influences and exposure to carcinogens that are especially relevant for intestinal-type cancer\textsuperscript{78-80}.

Chronic atrophic gastritis can be caused by \textit{Helicobacter pylori} infection, duodenal reflux, ingested chemicals or autoimmune-mediated damage in pernicious anaemia. Atrophic gastritis can be present in up to 40\% of normal patients over the age of 60 years\textsuperscript{80-82}.

There is an established link between \textit{H. pylori} infection and consequent inflammatory damage with particular strains including \textit{cagA} (cytotoxin-associated gene A), \textit{vacA} and \textit{iceA}. The International Agency for Cancer Research has classified \textit{Helicobacter pylori} as a group I carcinogen for gastric cancer. Environmental and host response factors are important in the pathogenesis of malignant transformation from atrophic gastritis to intestinal-type gastric adenocarcinoma\textsuperscript{83-85}.

Hereditary diffuse gastric cancer (HDGC) is a syndrome of familial gastric cancer that is associated with an inactivating germ-line mutation in the E-cadherin tumour suppressor gene (CDH-1) in 30 – 40\% of affected families. The remaining 60 – 70\% of HDGC families may have other susceptibility genes that have yet to be identified, including those coding for cell adhesion molecules β-catenin and γ-catenin. Carriers of the CDH1 mutation have a 70\% or more lifetime risk of developing diffuse gastric cancer with its attendant poor long-term survival. Counselling of individuals from HDGC families raises difficult questions as to the most appropriate management options that are essentially either prophylactic total gastrectomy or endoscopic surveillance\textsuperscript{86-89}.

The location of the primary tumour in gastric cancer has changed in tandem with the observed changes in histological subtype for oesophageal carcinoma. Distal oesophageal cancer and proximal gastric cancer are now the dominant sites of disease in the western world\textsuperscript{10 13}. Over the past 20 years there has been an annual increase in incidence of adenocarcinoma of the oesophagogastric junction in the UK\textsuperscript{39}. The incidence of adenocarcinoma of the oesophagogastric junction has been increasing at 5–10\% annually since the mid-1970s and represents the most rapidly increasing cancer in many Western countries\textsuperscript{90}. There have been parallel increases in adenocarcinoma of the gastric cardia, which now accounts for up to 50\% of all gastric cancers\textsuperscript{39}. Both oesophageal and gastric cancer accounted for 7.4\% of all cancer deaths in the European Union in 2006 ranking
joint third with breast cancer. The close relationship between adenocarcinoma tumours involving the gastric cardia, oesophagogastric junction and distal oesophagus has led some authors to suggest an equivalent approach to their diagnosis, staging and treatment.

There is a persistent demand on the disciplines of basic science research to evaluate novel therapeutic options in oesophagogastric cancer. Development of tumour chemoresistance or the unpredictable responses to chemotherapy and radiotherapy present a challenge to the clinical effectiveness of treatment strategies. Alternative targeted therapies are required to augment chemoresponsiveness or overcome chemoresistance as well as providing improved options for situations in which curative surgical resection is contra-indicated for clinical reasons. A central aim of the studies included in this thesis is the evaluation of the heme oxygenase system and oxidative stress responses affecting the growth of oesophagogastric cancer cells. These investigations may provide valuable information for future clinical applications.

1.2 The Heme Oxygenase System

1.2.1 The physiological role of heme oxygenase

The primary physiological role of heme oxygenase (HO) is the degradation of heme, a prosthetic group of various types of proteins including hemoglobin, myoglobin and respiratory cytochromes. Heme is essential for diverse biological processes such as oxygen transport, electron transfer and energy production, but unbound heme is highly toxic to cellular structures. Heme synthesis and breakdown is tightly regulated by feedback mechanisms that maintain intracellular heme at a precise level.

Microsomal heme oxygenase is the rate-limiting enzyme of heme catabolism. It catalyses the oxygen-dependent cleavage of the tetrapyrrole heme ring to generate biliverdin, with the concurrent release of equimolar amounts of carbon monoxide (CO) and free iron (Fe^{2+}). Biliverdin is then reduced to bilirubin by the action of cytosolic biliverdin reductase. Bilirubin is poorly soluble and transported in the circulation bound to albumin. It is subsequently conjugated with glucuronic acid in the liver to form pigments that are excreted into the bile. Ferrous iron produced from heme breakdown rapidly up regulates
the synthesis of both ferritin and a transporter protein that removes Fe\textsuperscript{2+} from the cell\textsuperscript{98}. Ferritin is a high-capacity iron-chelating protein that binds most intracellular iron and limits the generation of free radicals. Under normal conditions, the amount of free iron that can lead to the generation of reactive oxygen species (ROS) is kept at very minute levels, and ferritin is an efficient indicator of intracellular iron levels\textsuperscript{99}.

The HO system has a vital role in iron homeostasis. It is responsible for the recycling of iron from senescent red blood cells and cells of the reticuloendothelial system, and HO has a high basal activity in spleen and bone marrow tissues. It is estimated that 80% of the total body iron in humans is in the form of heme bound to proteins, with the rest bound to ferritin. Up to 85% of the bilirubin formed \textit{in vivo} is derived from hemoglobin released from damaged or aging erythrocytes\textsuperscript{100}.

\textbf{1.2.2 Iron, Heme and Ferritin}

\textbf{A Brief Overview of Iron Chemistry}

Iron exists in three primary forms in nature: firstly in its elemental form with no net charge (Fe) and the other two as ferrous and ferric ionic compounds depending on the valence state of the Fe atom. Loss of two electrons leaves the iron atom in a charged state as ferrous ion (Fe\textsuperscript{2+}) and loss of a further electron a ferric ion (Fe\textsuperscript{3+}). Ferrous and ferric compounds involving iron in a charged state are physiologically important in human health and disease. A prime example is the iron carried in the blood that must be in the ferrous form Fe\textsuperscript{2+} in order to bind to oxygen and carry the oxygen to the body tissues. Transport and delivery of oxygen is a vital physiological function of human blood. If the iron in the blood is changed to the Fe\textsuperscript{3+} state the bonds to oxygen are broken and oxygenated hemoglobin becomes deoxyhemoglobin.

Oxidation formally refers to the process of losing electrons, increasing the positive charge state of an ion or ionic compound. A typical example of oxidation is the change of iron from the Fe\textsuperscript{2+} state (ferrous) to the Fe\textsuperscript{3+} (ferric) state. Common oxidizing agents include the hydroxyl radical, ozone, peroxides and bleaches, and natural oxidisers exist within the human body. Oxidative stress refers to the process whereby oxidation produces free radicals that are highly reactive molecules capable of wreaking havoc within cell
homeostatic systems. The structure of molecules and atoms contains two electrons spinning in opposite directions in the outermost orbit. Free radicals are molecules or molecular fragments containing one or more unpaired electrons in their outermost orbital. The unpaired electron of a free radical is unstable and highly reactive. They are represented in nomenclature by a superscript dot \( (R^\cdot) \). Important biological free radicals include the hydroxyl radical \((\text{OH}^\cdot)\), the superoxide anion \((\text{O}_2^-)\), peroxide \((\text{O}_2^{2-})\) and nitric oxide \((\text{NO}^\cdot)\).

Molecular oxygen \((\text{O}_2)\) is stable as each oxygen atom shares two of the electrons in the outermost orbit. A single oxygen atom (singlet oxygen) is unstable and highly reactive as a strong oxidising agent but does not have unpaired electrons in its outer orbit and is therefore by definition not a free radical. There are two forms of bonding between atoms that occur in conventional chemistry: ionic and covalent bonding. Ionic bonding means that electrons are transferred from one atom to another. Covalent bonding means that the electrons are shared between atoms with symmetrical forces applied to the shared electrons, as in molecular oxygen. Polar covalent bonding is a variation of covalent bonding when different types of atoms join together producing an asymmetry of forces between shared electrons, e.g. two hydrogen atoms and one oxygen atom, \(\text{H}_2\text{O}\).  

The change in oxidation state of iron from the \(\text{Fe}^{2+}\) state (ferrous) to the \(\text{Fe}^{3+}\) (ferric) state in sufficient numbers within the blood can be detrimental to human health. Fenton's reaction involves the combination of iron in the \(\text{Fe}^{2+}\) state (ferrous sulfate) and hydrogen peroxide\(^{101}\):  

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^\cdot + \text{OH}^-
\]

Note that the combination of iron in the ferrous state with hydrogen peroxide produces three things: iron ions in the ferric state \((\text{Fe}^{3+})\), the highly reactive free radical hydroxyl radical \((\text{OH}^\cdot)\) and the hydroxide radical \((\text{OH}^-)\). Hydroxyl radical is the strongest oxidizing agent known.

These products can trigger cellular stress and sensitise a variety of cell types to undergo apoptosis (programmed cell death) in response to inflammatory agonists.
Heme

Heme is a ubiquitous molecule in aerobic cells and is essentially a prosthetic group composed of a ferrous iron ion contained in the centre of a large organic heterocyclic ring called a porphyrin. Porphyrins are aromatic compounds consisting of four modified pyrrole subunits joined at their α-carbon atoms via methine bridges. The majority of metalloproteins containing a porphyrin ring have heme as their prosthetic group and contain iron in the oxidised Fe\(^{2+}\) state surrounded by four nitrogen atoms bound with coordinated covalent bonds. These heme proteins or hemoproteins enable the protein to carry out a function that it cannot do alone via the ability of ferrous iron to react with various molecules. Heme (iron-protoporphyrin IX complex) is a regulatory molecule that affects transcription and translation depending on its intracellular localisation and concentration\(^ {103} \). Heme is a critical component of heme proteins that include cytochromes for mitochondrial respiratory chain electron transfer, oxidases and peroxidases, catalases and synthases such as nitric oxide synthase\(^ {104} \)\(^ {105} \). Heme biosynthesis occurs in all cells, especially erythroid cells and hepatocytes\(^ {104} \)\(^ {106} \).

The majority of iron in the body is present in the form of heme as hemoglobin, myoglobin and cytochromes while up to two-thirds of Western dietary iron intake is derived from dietary heme\(^ {107} \)\(^ {108} \). Gaseous molecules including oxygen (O\(_2\)), carbon monoxide (CO), nitric oxide (NO) and hydrogen sulfide (H\(_2\)S) readily bind to the Fe\(^{2+}\) ion in heme proteins. The structure and activity of the heme protein is modulated by the binding of reactive molecules to the prosthetic heme group with resulting changes in cellular signal transduction pathways.

The structural and functional characteristics of heme proteins are well studied due to their diverse range of biological functions. The Heme Protein Database [http://hemeprotein.info/heme.php] provides a global web resource uniting data from studies of the relationships between heme protein structure and function. The heme proteins hemoglobin and myoglobin have a high affinity to oxygen that enables reversible binding and transport of oxygen in humans. The heme prosthetic moiety functions as an essential electron donator in oxidation – reduction cycles and is thereby of cardinal significance for electron transfer reactions. Important proteins involved in aerobic
metabolism such as respiratory chain cytochromes and numerous cytochrome P450 isoenzymes are tightly bound to heme groups that serve as the catalytic site in protein activity. The heme molecule functions as a prosthesis for proteins involved in vital biological oxidation reactions in cellular homeostasis including signal transduction pathways, mitochondrial respiration, steroid biosynthesis, oxygen transport and cytoprotective antioxidant mechanisms. In mammals the majority of heme is utilised for the oxygen transport protein hemoglobin. Hemoglobin heme is synthesised in erythrocytes and ultimately degraded in the reticuloendothelial system where it accounts for 80% of bilirubin production in humans.

Heme is present in large amounts in many cells but is also inherently cytotoxic especially when it escapes from intracellular sites as it readily enters the hydrophobic domain of biological plasma membranes. Unbound heme is highly reactive due to the ability of the Fe$^{2+}$ ion contained within its protoporphyrin IX ring to undergo the Fenton reaction and catalyse the rapid and exponential production of free radicals. The reactivity of heme is controlled by insertion of the prosthetic group into the heme pockets of heme proteins to maintain cellular homeostasis. Free cellular heme derived from heme proteins acts as a potentially cytotoxic pro-oxidant via the production of free radicals from the Fenton reaction.

Oxidative reactions catalysed by intracellular iron are particularly potent and important damaging events. Generation of free radicals derived from oxygen can be driven by free iron and cause damage to cellular components by oxidation of proteins, carbohydrates, nucleic acids and lipoproteins as well as initiation of chain-propagating lipid peroxidation. Oxidative damage to vital cellular components may have deleterious effects at cellular and tissue levels leading to programmed cell death, tissue necrosis and degenerative diseases or cell phenotype changes causing neoplasia. Molecules with the ability to sequester iron may play a crucial role in the cellular antioxidant strategy.

Intracellular levels of free heme are vanishingly small and tightly regulated in most cells and tissues. Under conditions of oxidative stress some heme proteins can release their heme prosthetic groups with the consequent generation of reactive oxygen and nitrogen species including the superoxide anion, hydroxyl radical and nitric oxide free radicals and...
other oxidising agents like hydrogen peroxide. These reactive species cause damage to living systems by reacting with several biomolecules including lipids, proteins and nucleic acids. Oxidation of polyunsaturated fatty acids in the plasma cell membrane and the mitochondrial membrane causes direct cellular damage by altering membrane permeability and indirect damage via the production of toxic products and initiation of a chain reaction to produce other hydroperoxy free radicals. Loss of the mitochondrial membrane potential causes programmed cell death (apoptosis) in eukaryotic cells. Oxidation of the —SH groups of proteins by free radicals changes the biological function of the protein throughout physiological systems. Direct damage to nucleic acids by free radicals can caused DNA mutations that can be carcinogenic. The hydroxyl radical is the most toxic reactive oxygen species targeting protein and nucleic acids. The reaction of hydroxyl radicals with the nucleic acid base 8-hydroxyguanine (8-OHG) is highly correlated with teratogenic and carcinogenic consequences of oxidative stress.

A variety of cell types undergo apoptosis when exposed to pro-inflammatory agonists. This adverse effect is believed to play an important role in the pathogenesis of certain inflammatory diseases such as malaria.

In addition to the regulation of intracellular iron levels heme has a variety of roles in other fundamental cellular processes including ion-channel functions, circadian rhythm and the regulation of microRNA processing. Heme is also capable of inducing cell differentiation in mammalian adipose, neuronal and erythroid cells via the promotion of specific gene expression patterns. Free heme is lipophilic and potentially cytotoxic due to the promotion of lipid peroxidation and the production of ROS resulting in membrane damage and cell apoptosis. Therefore to facilitate the regulatory functions of heme while avoiding its toxicity the intracellular levels of free heme must be tightly controlled and are estimated at less than 0.1 μM.

Tight control of intracellular free heme levels is a feature of homeostasis. Regulation of heme biosynthesis is achieved through the modulation of δ-aminolevulinic acid synthase (ALA synthase) activity. Enzymatic heme degradation is controlled by microsomal heme oxygenase isoenzymes that catalyse the initial rate limiting step in heme catabolism.
Heme oxygenase catalyses the oxidative cleavage of the α-mesocarbon bridge of heme molecules to yield equimolar quantities of biliverdin-IXα and carbon monoxide with the concurrent release of divalent ferrous iron. Cellular levels of free heme regulate the activities of both ALA synthase and heme oxygenase\textsuperscript{130}. Heme degradation appears to be a highly conserved evolutionary cytoprotective response to oxidative stress. The stress-responsive heme oxygenase-1 isoenzyme (HO-1) is rapidly induced in cells exposed to free radicals and other stressful stimuli. The increase in heme oxygenase activity enables an exponential capability of the cells to degrade heme and thereby avoid the deleterious effects of free heme.

**Ferritin**

Recent studies have suggested a protective role for the iron storage protein ferritin against the toxic effects of iron overload in cells. Ferritin has also been demonstrated to protect endothelial cells from oxidised low-density lipoprotein (LDL) and from oxidative stress induced by UV light exposure\textsuperscript{131,132}. Ferritin has cytoprotective antioxidant activity in endothelial cells due to antiapoptotic effects\textsuperscript{133-135}. Increased transcription of ferritin mRNA has been shown in the nuclei of cells harvested from the livers of rats intoxicated with iron as compared with control animal nuclei\textsuperscript{136}. \textit{In vitro} studies demonstrate a dose-dependent cytoprotective antioxidant effect of ferritin in endothelial cells loaded with iron\textsuperscript{133}. There is a dichotomous time-dependent effect of heme exposure in endothelial cells. Brief heme exposure of up to 2 hours causes an increase in oxidative stress and oxidant-mediated cytotoxicity. By contrast, prolonged heme exposure of 16 hours causes significant cellular resistance to oxidative damage in endothelial cells. This time lag effect observed correlates with the endothelial response to heme by induction of heme oxygenase and the concurrent rapid synthesis of the iron-binding protein ferritin. Cells respond to oxidant stimuli by increasing heme oxygenase activity and the concomitant production of large amounts of ferritin. Studies demonstrate an inversely proportional relationship between ferritin content of endothelial cells and their susceptibility to oxidant stress damage under a wide range of experimental conditions\textsuperscript{125,133,137}.

Some reports suggest that heme oxygenase activity does not directly correlate with cytoprotection but functions mainly to provide free intracellular ferrous iron released
from heme breakdown. This increase in intracellular iron up regulates an iron-transporter pump to remove intracellular Fe\(^{2+}\) but also induces ferritin expression. Synthesis of ferritin protein is promoted thereby generating a primary protectant against oxidant damage. As intracellular chelatable iron levels increase the synthesis of the iron-sequestering protein ferritin is up-regulated by a post-transcriptional mechanism\(^{138}\). The potential for Fe\(^{2+}\)-driven catalysis of oxidative reactions is thereby limited by the sequestration of intracellular iron by ferritin\(^{139}\). Ferritin synthesis may be directly enhanced by heme itself via an increase in RNA translation\(^{140}\). Further studies demonstrate that over-expression of heavy-chain ferritin protein protects cultured endothelial cells from undergoing apoptosis and protects against the ischemia-reperfusion injury associated with liver transplantation\(^{135}\).

Ferritin binds free intracellular Fe\(^{2+}\) that would otherwise rapidly participate in the Fenton reaction to promote the generation of ROS. The oxidative stress created by intracellular Fe\(^{2+}\) is particularly relevant to mitochondrial dysfunction and is limited by up-regulation of ferritin\(^{106}\)\(^{141}\). A direct link between HO-1 induction and ferritin synthesis is not universally established. Recent studies have demonstrated equivalent levels of ferritin in cells overexpressing HO-1 and in HO-1−/− cells\(^{98}\). A similar recent study noted a lack of ferritin synthesis despite induction of HO-1 protein when cultured cell lines were exposed to oxidising reagents. Ferritin synthesis was however demonstrably increased in these cells after treatment with extracellular heme\(^{142}\). These findings suggest that the known level of endogenous intracellular heme may be so low as to be insufficient as a substrate for HO-1 enzyme. Under conditions of oxidative stress all available iron may be shunted directly to the mitochondria for biosynthesis of heme to increase the availability of antioxidant heme proteins without a concurrent increase in ferritin synthesis.

Studies have demonstrated that heme also functions as an important substrate for heme oxygenase activity to produce end products with beneficial antioxidant properties. Many organisms deliberately dispose of free intracellular heme by enzymatic breakdown. It has been shown that a notable proportion of newly-synthesised heme is rapidly broken down to biliverdin in cultured rat hepatocytes to generate bilirubin\(^{143}\). There are numerous published observations of bile pigment antioxidant activity in vitro\(^{144-149}\). These studies support the idea that a central physiological function of enzymatic heme breakdown by
HO enzyme is to produce the bile pigments biliverdin and its metabolite bilirubin specifically to exploit the intrinsic antioxidant properties of these species at a cellular level\textsuperscript{150}.

1.2.3 Characterisation of the Heme Oxygenase Enzyme

1.2.3.1 The catabolism of heme

Initial studies by Nakajima and colleagues obtained an enzyme from liver and kidney homogenate that converted heme to a possible precursor of biliverdin. This soluble enzyme system was partially purified and characterised as heme α-methenyl oxygenase, requiring NADPH, ferrous iron, and a liver cell nucleus extract as activating co-factors\textsuperscript{151}. The system was unusually substrate-specific for pyridine hemochromogen, hemoglobin-haptoglobin complex and myoglobin with no activity using hematin, oxyhemoglobin or methemoglobin\textsuperscript{152}. Furthermore, tissues presumed to be active in hemoglobin degradation such as spleen and bone marrow were almost devoid of enzyme activity. Subsequent work failed to confirm the existence of this soluble heme α-methenyl oxygenase system, attributing the previous findings to a heat-stable factor of low molecular weight functioning as a reducing agent\textsuperscript{153, 154}.

Studies of heme catabolism suggest that both the heme group bound to hemoglobin and unbound hemin are degraded by the same mechanism, especially as when oxidised, the prosthetic heme group of hemoglobin is easily detached from the native globin. Labelled hemoglobin or hemin administered to rats is converted to bile largely in the liver, with both injected hemin and endogenous hemin formed in the liver being concentrated in the hepatic microsomal fraction\textsuperscript{155, 156}.

Tenhunen and colleagues reported an enzymatic reaction that catalysed the oxidative cleavage of heme at the α-methene bridge of the ferriprotoporphyrin IX ring to yield biliverdin IXα which is then converted to bilirubin in the presence of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent biliverdin reductase. The iron atom contained within the centre of the heme porphyrin ring is released as the free ferrous iron Fe\textsuperscript{2+}. The reaction product formed under the specified conditions of incubation was identified as bilirubin by chemical, chromatographic and spectrophotometric methods.
The action of biliverdin reductase was not identified as a rate-limiting step within the complete enzyme system. Important characteristics of the reaction include its heat-labile nature, its absolute requirement for NADPH and its inhibition by carbon monoxide in the presence of oxygen. This enzymatic conversion of the heme moiety to bilirubin was localised specifically to endoplasmic reticulum microsomes concentrated and separated from cellular debris by differential centrifugation\(^{128}\)\(^{157}\).

The tissue distribution and kinetics of the microsomal heme oxygenase system were characterised in 1969, suggesting a major physiological role for heme oxygenase in the breakdown of heme and hemoproteins to bile pigment. Cleavage of the ferriprotoporphyrin ring at the α-methene bridge results in the formation of the α-configuration linear tetrapyrrole biliverdin IXα, converted to bilirubin IXα by biliverdin reductase and identified as the product reaction by all analytical criteria employed by Tenhunen and co-workers. Bilirubin IXα is the only physiological isomeric form of bilirubin\(^{129}\).

Microsomal heme oxygenase has an absolute and stoichiometric requirement for molecular oxygen and NADPH and generates carbon monoxide in equimolar amounts to bilirubin. Three moles of oxygen are consumed per mole of bilirubin formed. The enzyme system is capable of catalysing the oxidative cleavage of the heme moiety of the α and β chains of hemoglobin and other hemoproteins including methemoglobin, methemalbumin, protohemin IX and the hemoglobin-haptoglobin complex. Unbound heme also provides a substrate for the enzyme; heme easily dissociates from its native globin as well as from altered or denatured proteins. Hemoproteins that are not acted upon by the enzyme include oxyhemoglobin, carboxyhemoglobin, myoglobin and free porphyrins\(^{158}\).

Heme oxygenase enzyme is strongly inhibited by carbon monoxide. Heme oxygenase activity was measured by specific colorimetric assay to quantify bilirubin production from hemin substrate in spleen microsomes. The assay was performed under specific conditions with cuvettes gassed for 2 minutes before and during the assay with 50% carbon monoxide, 46% nitrogen and 4% oxygen. These conditions inhibited enzymatic heme oxygenase activity\(^{129}\).
CO can activate soluble guanylyl cyclase (sGC) with the resultant production of cyclic GMP and also modulates mitogen-activated protein kinase (MAPK) signalling pathways\textsuperscript{159}. Carbon monoxide has a high affinity for reversible binding to hemoglobin to form carboxyhemoglobin. CO can also form complexes with reduced forms of metalloproteins including cytochrome P-450, cytochrome-c oxidase, inducible nitric oxide synthase and other heme proteins and metabolic oxidases. CO is a small gaseous ligand that can bind to heme oxygenase and to the heme oxygenase-heme complex\textsuperscript{160-162}. The interaction of CO with cytochrome P-450 or cytochrome-c oxidase has been demonstrated to cause inhibition of enzymatic activity\textsuperscript{163 164}. The findings of Tenhunen and colleagues suggest that cytochrome P-450 is involved in the oxidative cleavage of the ferriprotoporphyrin ring and has an essential role in the enzymatic degradation of heme\textsuperscript{157}.

Heme oxygenase enzyme is inhibited by sodium dodecyl sulfate, trypsin, sodium azide, potassium cyanide, lipase and phospholipase. The rate-limiting step in heme degradation is the oxidation of heme at the α-methene bridge catalysed by microsomal heme oxygenase enzyme. The enzyme requires mixed function oxidation with cytochrome P450 as the terminal oxidase\textsuperscript{165}, converting heme to biliverdin with subsequent reduction of biliverdin to bilirubin by soluble biliverdin reductase in the cytosol\textsuperscript{166}.

Heme oxygenase enzyme activity measured in experimental rat models correlates with the physiological sequestration and breakdown of red blood cells. Enzyme activity is highest in the spleen, liver and bone marrow tissues responsible for this important function. The heme oxygenase system is capable of a regulatory adaptive response to substrate alterations, with increased hepatic heme oxygenase activity observed following splenectomy or induced haemolysis.

Landaw and colleagues studied the production of bilirubin and carbon monoxide (CO) from the heme oxygenase enzyme reaction. The carbon atom at the α-methene bridge of the ferriprotoporphyrin ring is oxidised to carbon monoxide as the ring is cleaved at this site. For each mole of heme degraded, 1 mole of CO and 1 mole of bilirubin are formed, providing the source of endogenous CO production.

The production of bilirubin and CO from hepatic heme catabolism can be measured by an indirect approach in experimental animal models by controlled intravenous
administration of isotopically labelled hematin. The rate of formation of radioactive bilirubin and CO is subsequently measured and compared to the total amount of isotopic tracer excreted in the bile. Heme compounds can also be pulse-labelled with glycine-2-14C to produce labelled bilirubin and CO and isotopic bile pigment from hepatic heme degradation. In the experiments reported by Landaw et al^{167} concurrent production of bilirubin-14C and 14CO was determined in 16 rat subjects of whom 8 were injected with glycine-2-14C, 5 with hematin-14C prepared from glycine-2-14C, 2 with bilirubin-14C, and 1 with glycine-1-14C. The results demonstrated an equimolar relationship between heme breakdown and reaction product production, with virtually equivalent quantities of isotopic bilirubin and CO measurable from rats given an infusion of hematin-14C. Although heme catabolism pathways exist that result in alternative end products, in these studies only a minor fraction of labelled hematin was metabolised to compounds other than bilirubin and carbon monoxide. Chemical modification of the heme molecule or a change in metabolic conditions may encourage these alternate pathways of heme turnover. Treatment with the porphyrogenic drug allylisopropylacetamide (AIA) enhanced the production of non-bilirubin pyrrolic derivatives and shifted the ratio of CO:bilirubin production in favour of carbon monoxide. Pre-treatment with phenobarbital to enhance liver metabolism of heme resulted in a proportional increase in the production of CO and bilirubin without altering the equimolar relationship. These findings suggest that the physiological pathway of heme breakdown is decided by the nature of the initial attack on the heme moiety tetrapyrrole ring. Microsomal heme oxygenase enzyme catalyses the cleavage of the ferriprotoporphyrin ring at the α-methene bridge producing equimolar amounts of CO and bilirubin. If the tetrapyrrole ring is opened by a different mechanism then alternative pathways produce other metabolites without endogenous CO production.

Studies reported by Pimstone et al^{168-170} provide direct evidence that alveolar and peritoneal macrophages derived from monocyte precursors have the potential for heme oxygenase (HO) enzyme activity. Although native macrophages have very low HO activity, exposing these cells to heme pigments in vivo stimulates enzyme activity. This demonstrates that heme triggers the activity of its own breakdown pathway in macrophages that are not physiologically responsible for hemoglobin degradation. Cells
of the reticuloendothelial system including splenic macrophages and the Kupffer cells of
the liver have equivalent heme oxygenase enzyme apparatus that performs at a high
physiological activity to remove old and damaged red blood cells from the circulation\textsuperscript{170}.

The characteristic colour change observed over time in subcutaneous bruises provides a
useful picture of the successive steps in enzymatic hemoglobin breakdown. Extravasated
blood stimulates movement of local tissue macrophages to the site of injury and
phagocytosis (digestion) of red blood cells trapped in the tissue. The initial appearance of
the bruise is dark red due to ingested hemoglobin. Hemosiderin deposits in tissue
macrophages give a dark brown appearance. The process of phagocytosis includes
induction of lysosomal and cellular microsomal enzymes including heme oxygenase.
Activity of heme oxygenase breaks down the hemoglobin into bilirubin, ferrous iron and
amino acids that are gradually released into the interstitial fluid. As the breakdown
products appear the bruise changes colour in sequence to dark brown (methemoglobin or
hematin), green-blue (biliverdin) and eventually to yellow (bilirubin).

By contrast, the dark purple or black colour of subungual haematomas (bleeding
underneath a toenail or fingernail) or intraepidermal blood blisters can persist for several
weeks. This can be explained by the fact that these tissue injuries do not elicit much in
the way of a cellular response. There is not a major process of phagocytosis by
macrophages and the extravasated hemoglobin-heme complex remains intact and
essentially unchanged until the protective layer of skin or nail above the trapped blood is
shed.

1.2.4 Heme oxygenase isoenzymes

Heme oxygenase activity has been observed in all systemic organs. Two recognised
isoforms of the heme oxygenase enzyme have been isolated and characterised in
humans. HO-1 has a molecular mass of 32 kilo Daltons (kDa) and is inducible by a variety
of stimuli including oxidative stress, tissue injury and nitric oxide (NO).

The two isoforms of HO share approximately 40\% of amino acid sequence homology\textsuperscript{171,172}. HO-1 is the product of one transcript whereas HO-2 is encoded by two transcripts of
the single gene which differ in the use of the polyadenylation signal\textsuperscript{173}. HO-2 is a
constitutively synthesised protein with molecular weight 36 kDa, responsible for providing basal HO activity for cell homeostasis\textsuperscript{174}. There is wide variability in the tissue distribution of HO isoforms and the basal levels of constitutively expressed HO-2 protein with the highest levels of HO-2 detectable in the brain. HO-2 is also expressed at lower levels in testes, endothelial cells, distal nephron segments, liver, and gut myenteric plexus\textsuperscript{175-177}. HO-1 seems to be constitutively expressed in some unique types of cells including liver Kupffer cells, renal medullary cells, and CD4\textsuperscript{+} regulatory T-cells\textsuperscript{178-180}. The basal level of HO-1 is rather weak in mammalian tissues apart from the liver and spleen where the gene is strongly expressed\textsuperscript{181}.

HO-1 can be readily and strongly up-regulated in many tissues in response to cellular stress caused by a wide spectrum of stimuli including heme, heavy metals, UV irradiation, ROS, nitric oxide (NO), inflammatory cytokines, heat shock, ethanol, and prostaglandins\textsuperscript{182-188}. The expression of a third isoform, HO-3, has been demonstrated at mRNA level in the rat brain but this has negligible heme breakdown activity and its presence has not been established in human tissues\textsuperscript{189}. The isoform described in rats termed HO-3 is more likely to reflect a processed pseudogene derived from an HO-2 transcript\textsuperscript{190}.

HO-1 and HO-2 enzymes have the same mechanism of heme oxidation, with equivalent co-factor and substrate specificity and susceptibility to inhibition by metalloporphyrins. Heme oxygenase isoforms HO-1 and HO-2 have the same enzymatic activity catalysing the breakdown of heme into carbon monoxide (CO), ferrous iron (Fe\textsuperscript{2+}) and biliverdin\textsuperscript{128}. Both isoenzymes require molecular oxygen (O\textsubscript{2}) and NADPH (nicotinamide adenine dinucleotide phosphate) as cofactors for effective function\textsuperscript{189}. HO-1 and HO-2 proteins have distinct immunological characteristics, dissimilar molecular weights and substantial differences in their regulation and expression pattern\textsuperscript{191}. Their structure differs in terms of available heme and cysteine binding-sites, and their postulated function is different. While constitutive HO-2 is believed to be responsible for basal HO activity contributing to cell homeostasis, inducible HO-1 expression is relatively low in most tissues and it has functional significance only in response to injurious stimuli\textsuperscript{191-195}.
The significant increases in heme oxygenase enzymatic activity observed in models of inflammatory and oxidative stress are mediated by an increase in HO-1 gene transcription rates\textsuperscript{179, 196, 197}. Constitutive HO-2 protein does not appear to be inducible by stressful stimuli although the HO-2 gene promoter contains a glucocorticoid response element and administration of glucocorticoids stimulates HO-2 transcriptional activity in neuronal cells\textsuperscript{198}. Distinct genes encode the HO isoforms located on chromosomes 22q12 (HMOX1) and 16q13.3 (HMOX2) in the human genome\textsuperscript{199, 200}. Expression of HO-2 protein is found in various tissues under physiological conditions especially the testis and the brain. Constitutive expression of HO-1 isoform is relatively low with the exception of the spleen\textsuperscript{201, 202}.

The constitutively expressed HO-2 enzyme isoform is not yet as widely researched as HO-1. HO-2 activity is believed to be essential for normal cellular function both in maintaining intracellular levels of the pro-oxidant heme moiety and in the tonal production of biliverdin and CO that contribute to endogenous cellular antioxidant capacity and regulation of signalling pathways. Although the role of HO-2 in inflammation is largely unexplored, the enzyme has an equal ability to produce CO and bilirubin and so should be equally capable of exerting anti-inflammatory and antioxidant effects as HO-1. The relatively constant expression levels of HO-2 protein may be suitable for its regulatory role in heme homeostasis. The enzymatic activity of HO-2 may be dynamically regulated via posttranslational mechanisms that involve phosphorylation of cellular proteins\textsuperscript{203}.

The HO-2 isoform molecule contains three cysteine residues each of which can function as the heme-binding site\textsuperscript{204}. HO-2 protein may thereby play an important regulatory role by sequestering heme to maintain the intracellular heme level\textsuperscript{204}. Some reports suggest that HO-2 functions as an oxygen sensor\textsuperscript{205, 206}. Studies in HO-2 knockout mice have demonstrated several important cellular functions of the HO-2 enzyme particularly in the regulation of endothelial cell homeostasis. Endothelial cells from HO-2\textsuperscript{−/−} mice display active inflammation and increased levels of oxidative stress\textsuperscript{207}. Deficiency of HO-2 protein leads to an exaggerated inflammatory response after antigen-induced peritonitis with an impaired acute inflammatory and reparative response to epithelial injury\textsuperscript{174}. HO-2\textsuperscript{−/−} mice are not able to compensate for the loss of HO-2 by increasing HO-1 expression as the
induction of HO-1 protein is impaired by HO-2 deficiency, suggesting that HO-2 protein may be critical for HO-1 expression\textsuperscript{208}.

Inducible HO-1 is widely expressed in response to stimuli in organs and tissues such as liver, spleen, pancreas, intestine, kidney, heart, retina, prostate, lung, skin, brain, spinal cord, vascular smooth muscle cells and endothelial cells. HO-2 is expressed at high levels and is the predominant form of HO protein expressed in the brain and testis of mammals where it is suggested to have a vital neuroprotective function\textsuperscript{177,209,210}. In a rat model of ischaemic stroke, neural damage following middle cerebral artery occlusion and reperfusion was significantly worsened in HO-2 knockout mice\textsuperscript{177,210}. HO-2 is reported to be critical for protection of cerebral microvascular endothelial cells against apoptotic changes induced by oxidative stress and cytokine-mediated inflammation\textsuperscript{209}.

A characteristic feature of HO-1 expression appears to be its rapid induction in response to increased cellular oxidative stress and then subsequent regression to low levels. The HO-2 isoform is by contrast constitutively expressed in all cells. The HO-2 gene promoter responds solely to glucocorticoids to cause up-regulation of HO-2 protein\textsuperscript{109}.

A unique function for HO-2 protein is suggested by the presence of heme-binding Heme Regulatory Motifs (HRMs) on the N-terminus of the HO-2 molecule. These ancient HRMs consist of short amino acid sequences and function as heme–oxygen sensors in bacteria, yeast and mammals and suggest a role for HO-2 as an intracellular sink, binding and regulating the heme gaseous ligands nitric oxide, carbon monoxide and molecular oxygen\textsuperscript{117,211-213}. This role is supported by the finding that HO-2 co-localises with nitric oxide synthase and binds the vasodilator nitric oxide (NO) with high affinity\textsuperscript{214}.

Recent studies have indicated a potential role for HO-2 as an oxygen sensor. HO-2 protein binds to the potential vasodilator carbon monoxide (CO) and oxygen (O\textsubscript{2}) with less affinity than it binds to NO. The interaction with CO and the increased proximal production of CO from heme breakdown mediated by increased HO-2 activity stimulates the calcium-sensitive potassium channels that mediate the excitatory responses of the carotid body to hypoxia\textsuperscript{215}. Studies in HO-2\textsuperscript{\textminus}\textsuperscript{\textminus} mice show that HO-2\textsuperscript{\textminus}\textsuperscript{\textminus} mice have mild hypoxemia. Although the carotid bodies of HO-2\textsuperscript{\textminus}\textsuperscript{\textminus} mice are enlarged the chemoreceptors respond normally to acute hypoxia and the mice have only partially
impaired acute ventilatory responses\textsuperscript{216, 217}. The HO-2 knockout mice phenotype had an increased susceptibility to the lethal effects of hyperoxia. The principal drawback in the interpretation of studies arises from the observation of a higher total lung HO enzymatic activity and increased HO-1 protein levels in mice with the HO-2\textsuperscript{-/-} genotype and HO-2 protein deficiency. Therefore it is not clear if the observed sensitivity to hyperoxia was directly related to HO-2 deficiency or a combination of HO-2 deficiency and increased HO-1 activity.

The acute response of central and peripheral chemoreceptors to hypoxia may be regulated by alternative mechanisms other than HO-2 to cause inhibition of membrane ion channels\textsuperscript{218}. Changes in CO signalling in HO-2\textsuperscript{-/-} mice may be responsible for possible chronic pulmonary ventilation – perfusion mismatches\textsuperscript{206}. Furthermore, the up regulation of HO-1 demonstrated in the pulmonary epithelium of HO-2\textsuperscript{-/-} mice perhaps functions as an alternative compensatory source of CO production\textsuperscript{116, 219}.

In a murine model of antigen-induced peritonitis, HO-2-null mice exhibited a phenotype of exaggerated inflammation and reduced neutrophil function. Using a well-characterised model of the reparative response to epithelial injury, HO-2-null mice demonstrated an acute inflammatory response that failed to resolve, evolving into augmented and uncontrolled inflammation with impaired wound closure and neovascularisation. These findings suggest a crucial role for HO-2 in providing an ordered and reparative inflammatory response to cellular injury\textsuperscript{174}.

1.3 The consequences of heme breakdown

1.3.1 Heme oxygenase enzyme products

Heme oxygenase enzyme catalyses the rate-limiting step in the breakdown of heme, a reaction that produces biliverdin IX\textalpha, carbon monoxide (CO), and ferrous iron (Fe\textsuperscript{2+}), at the expense of molecular oxygen and NADPH. The cytosolic enzyme biliverdin reductase then rapidly reduces biliverdin IX\textalpha to the hydrophobic pigment bilirubin IX\textalpha\textsuperscript{220}. Circulating bilirubin-IX\textalpha is bound to albumin and has antioxidant activity at physiological concentrations\textsuperscript{144, 221}. Accumulated serum bilirubin is conjugated to water-soluble
glucuronide derivatives by hepatic microsomal glucuronyl transferase enzymes and secreted into the bile with subsequent elimination via the faecal routes\textsuperscript{222}.

The heme oxygenase system has generated substantial scientific interest over the last decade with numerous studies of human physiology. The role of the HO pathway and the products of heme breakdown in diabetes, inflammation, heart disease, hypertension, transplantation, and pulmonary disease are all areas of recent research. Research has included investigation of several genetic and metabolic processes that are mediated by HO activity and heme metabolism. It has become increasingly recognized that inducible HO-1 has cytoprotective activities including antioxidant, anti-inflammatory, anti-apoptotic and pro-angiogenic properties\textsuperscript{201}. The reaction catalysed by heme oxygenase enzyme (Figure 1.3.1) has significant cellular and physiological consequences. The HO system interacts with cell signalling mechanisms and participates in the cell response to injury. Heme oxygenase enzyme has kinase and transcription factor activities as well as its vital function for the intracellular transport of Fe\textsuperscript{2+} and heme. Biliverdin reductase has recently been identified as a key regulator of HO-1 expression\textsuperscript{223-225}.

By generating bilirubin and carbon monoxide, HO has antioxidant, anti-inflammatory and anti-apoptotic activities. The simple degradation of heme is an important component of anti-oxidant activity as heme itself is an effective catalyst for the formation of ROS\textsuperscript{226}. Although free iron is produced from heme breakdown, heme oxygenase also facilitates cellular storage and export of free iron by up-regulating synthesis of ferritin and iron-transport proteins\textsuperscript{227}. Biliverdin and bilirubin are both reducing molecules that have been demonstrated to possess potent antioxidant activities\textsuperscript{228}. Numerous studies have demonstrated that bilirubin at intracellular physiological (nanomolar) concentrations is a potent antioxidant able to protect cells from up to a 10,000-fold excess of H\textsubscript{2}O\textsubscript{2}\textsuperscript{210 221 229 230}. Bilirubin is able to scavenge hydroxyl radicals, singlet oxygen and superoxide anions. In providing this antioxidant activity bilirubin is oxidised back to biliverdin which is then recycled back to the reducing agent bilirubin via the action of cellular signalling pathways that activate biliverdin reductase enzyme\textsuperscript{229 231 232}. In neuronal cells this reaction depends upon the substrate biliverdin being supplied by HO-2 isoform rather than HO-1 enzyme\textsuperscript{177 210 233}. The water-soluble glutathione system is a major intracellular antioxidant that protects water-soluble proteins from targeted damage by ROS. Recent studies indicate
that bilirubin similarly protects lipophilic proteins in plasma cell membranes from oxidative damage\textsuperscript{230}.

The cytoprotective activity of biliverdin and bilirubin is largely based on inhibition of lipid and protein peroxidation\textsuperscript{229}. Bilirubin is also capable of anti-inflammatory activity. It reduces leucocyte adhesion and rolling and inhibits the complement cascade\textsuperscript{234, 235}. Carbon monoxide (CO) functions as an important cellular signal molecule\textsuperscript{236} in a number of pathways. CO stimulates cyclic GMP to affect vascular tone and neurotransmission, binds to potassium channels to produce vasodilatation, increases angiogenesis and has anti-inflammatory and anti-apoptotic activity\textsuperscript{237}.

While the removal of free intracellular heme by heme oxygenase is certainly an important aspect of cellular survival the products of HO enzymatic activity are the more likely mechanisms underlying the cytoprotective effects mediated by HO protein. Administration of biliverdin or bilirubin or delivery of exogenous carbon monoxide either directly as a gas or via carbon monoxide-releasing molecules mediates the majority of protective functions ascribed to HO activity\textsuperscript{238}. Exogenous treatments with bile pigments and/or carbon monoxide are similarly beneficial for cell survival as isolated induction of HO-1 enzyme and this strategy can substitute for the lack of HO-1 activity in cell and animal models\textsuperscript{239, 240}. The potentially toxic effects of iron, carbon monoxide and bile pigments in human physiology are likely to limit their beneficial actions to a narrow threshold of overexpression\textsuperscript{241, 242}.

Up-regulation of HO-1 and increased HO activity may produce both pro- and anti-oxidant effects as part of the cellular response to stress. Available evidence to date suggests that that HO-1 is neither exclusively cytoprotective nor exclusively cytotoxic. Published reports suggest that induction of HO-1 could be harmful to the cell under certain circumstances\textsuperscript{224}. The release of ferrous iron previously chelated with heme provides a potential for the production of Fe\textsuperscript{2+}-driven free radicals such as the hydroxyl radical via the Fenton reaction to cause subsequent cell damage. An increase in HO-1 activity will have an overall benefit provided the released iron is either sequestered by storage within ferritin or exported out of the cell by the plasma membrane exporter protein\textsuperscript{243, 244}.
There is a large body of literature that supports a seminal role for the HO system in protecting cells from oxidative stress, in particular the inducible HO-1 enzyme. The cytoprotective effects of HO activity depend upon limited heme degradation and preservation of cellular heme available for the continued production and function of other critical cell heme proteins. Published evidence indicates that HO-1 activity exerts an important anti-inflammatory action and regulates inflammation in vivo. Characteristic features of a progressive chronic inflammation are displayed in HO-1 knockout mice as compared to wild-type mice with a higher peripheral blood leucocyte count, inflammatory cell adherence and infiltration in hepatic endothelium, splenomegaly due to follicular hyperplasia and high splenic and lymph node CD4⁺:CD8⁺ T-cell ratios with numerous activated CD4⁺ T-cells. Peritoneal macrophages from HO-1-knockout mice also exhibit distinctly increased pro-inflammatory cytokines such as MCP-1 (monocyte chemotactic protein-1) and interleukin-6 (IL-6). Administration of exogenous carbon monoxide or induction of HO-1 protein inhibits the production of LPS (lipopolysaccharide)-induced pro-inflammatory cytokines such as TNF (tumour necrosis factor)-α, interleukin-1 (IL-1) and macrophage inflammatory proteins. Expression of the anti-inflammatory cytokine IL-10 induced by bacterial LPS was increased by HO-1 induction.

HO-1 is considered to have a valuable role in vascular biology due to its cardioprotective effects. HO-1 activity has been shown to limit endothelial dysfunction by reducing the production of pro-inflammatory cytokines such as macrophage colony-stimulating factor. The interaction between capillary endothelial cells and leucocyte adhesion is diminished by induction of HO-1 via the activity of bilirubin product. Up-regulation of HO-1 enzyme can inhibit the production of E-selectin and VCAM-1 vascular cell adhesion molecules mediated by the pro-inflammatory cytokine TNF-α.

The importance of HO-1 protein in protection against cardiovascular disease has been evaluated by numerous in vivo models. Increased HO-1 expression in human carotid endarterectomy specimens have been shown to be associated with reduced plaque vulnerability. The effects of altered HO enzymatic activity in the cardiovascular system can be related to the levels of bile pigment products generated by heme breakdown.
High bilirubin levels have been shown to be associated with reduced prevalence and incidence of coronary artery disease and ischaemic stroke\textsuperscript{257, 258}. Low bilirubin levels meanwhile can be associated with endothelial dysfunction indicated by flow-mediated dilatation and an increase in carotid intima-media thickness\textsuperscript{259}.

Features of human iron overload syndrome such as splenomegaly, tissue iron deposition, hepatomegaly, hepatic fibrosis, growth retardation and premature death are demonstrated in HO-1 knockout mice\textsuperscript{245, 246}. Mice with a phenotype deficient in both HO-1 and ApoE (apolipoprotein E) have increased atherosclerosis and vein graft stenosis compared with mice not lacking in HO-1 protein suggesting a specific role of HO-1 in protection against atherosclerosis\textsuperscript{260}. Cultured endothelial cells deficient in HO-1 protein demonstrate impaired angiogenesis \textit{in vitro}\textsuperscript{261}. HO-1 knockout mice have diminished angiogenesis \textit{in vivo} and worse neovascularization during tissue healing as compared with their wild-type counterparts in a cutaneous wound injury model. Skin-specific overexpression of HO-1 produced an enhanced process of neovascularization during wound healing in transgenic mice\textsuperscript{261, 262}. If the observed differences in HO-1 knockout mice can be assumed to be exclusively due to the absence of HO-1 protein these studies indicate a critical role for HO-1 activity in angiogenesis.

The activity of HO-1 is of central importance for effective reutilisation of iron in both rodents and humans even under physiological conditions. Deficiency of HO-1 protein has been shown to result in iron deposition and cause harmful inflammatory sequelae secondary to impaired iron recycling\textsuperscript{245, 246, 263}. The first reported case of human HO-1 deficiency described a case of a 6-year-old boy with severe growth retardation characterised by marked intravascular haemolysis and hyperlipidaemia. Extensive endothelial injury was evident on electron microscopy of the renal glomeruli with cell swelling and detachment and subendothelial deposition of iron. Atherosclerotic fatty streaks and fibrous plaques were present in the aorta at post mortem examination\textsuperscript{263, 264}. Production of bilirubin was impaired in this patient, perhaps reducing the bilirubin-mediated capacity to reduce the oxidation of LDL low-density lipoprotein and leading to oxidative vascular endothelial injury\textsuperscript{149, 221, 265}. 

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The notion that HO-1 enzyme has a fundamental physiological purpose is supported by the fact that both the enzyme and its heme substrate are highly conserved and ubiquitous across evolutional life forms. The molecular processes and mechanisms by which HO-1 activity provides cellular and tissue protection remain only partially understood. The majority of studies investigating HO-1 or CO-dependent cytoprotection suggest the involvement of inflammatory response mechanisms including down-regulation of pro-inflammatory cytokines as well as HO-1-mediated regulation of apoptosis and cell proliferation.

A number of cell signaling molecules and transcription factors have been identified to be involved in regulating HO-1 expression including mitogen-activated protein kinases (MAPKs), nuclear factor-κB (NF-κB), biliverdin reductase, activating transcription factors, nuclear factor E2-related factor 2 (Nrf2), phosphatidyl inositol 3-kinase / Akt and protein kinase C. Expression of HO-1 protein is most likely to result from complex coordinated interactions between these various molecules.
1.3.2 Heme oxygenase and inflammation

Heme oxygenase (HO) is present as two functionally active isoforms in humans that are encoded by specific genes: HO-1 and HO-2. A pseudo gene variant of HO-2 isoform termed HO-3 has been described in the rat brain. Heme oxygenase isoforms share the function of heme degradation but otherwise have dissimilar molecular and biochemical properties.

HO-1 enzyme is a highly inducible heat shock protein up-regulated by a variety of stimulants including hypoxia or hyperoxia, depletion of intracellular glutathione, endotoxin, inflammatory cytokines, hyperthermia, ultraviolet irradiation, increased heme levels, and various heavy metals including sodium arsenite, cobalt and selenium. HO-1 is known to be activated by a greater number of stimuli than that of any other gene with the cellular response depending on the cell type. HO-1 is induced by oxidative stress and reactive species including H$_2$O$_2$ and nitric oxide (NO) and its up-regulation in response to heat shock conferred its alternative name of heat shock protein (HSP) 32.

Several in vitro studies have suggested that heme oxygenase activity may potentiate rather than attenuate oxidant toxicity under certain conditions. Treating epithelial cells with the competitive inhibitor of heme oxygenase enzyme zinc protoporphyrin IXa reduced the cytotoxic effects of H$_2$O$_2$ treatment at 24 hours. Similar studies showed...
that co-incubation of cultured astroglial cells with metalloporphyrin inhibitors of heme oxygenase activity protected against the cytotoxic effects of H$_2$O$_2$ treatment$^{277}$. Studies using transfection models of heme oxygenase-1 (HO-1) expression suggest that HO-1 is capable of both pro- and anti-oxidant activity. Rabbit endothelial cells that over-expressed HO-1 were resistant to heme toxicity$^{278}$. Up-regulation of HO-1 in human pulmonary epithelial cells caused growth arrest and increased cellular tolerance to hypoxia$^{279}$. Protection against hypoxia-mediated apoptosis, protein oxidation and lipid peroxidation was afforded by overexpression of HO-1 in rat foetal pulmonary epithelial cells, whereas HO-1 overexpression during hyperoxia increased glutathione depletion and plasma membrane damage$^{241,280}$.

Increased enzyme activity confers protection against oxidative stress conditions in vitro and in vivo through anti-oxidative, anti-apoptotic and anti-inflammatory actions. These include inhibition of inflammatory adhesion molecules and leucocyte recruitment and suppression of cytokine and chemokine expression. When cells are exposed to non-heme insults, it is assumed that the cellular stress response also promotes release of sufficient amounts of substrate for the induced HO-1 enzyme. A study of heme catabolism found that a noticeable proportion of newly-synthesized heme was converted rapidly and directly to biliverdin without incorporation into hemoproteins, suggesting that heme substrate is synthesized purely for the products of its breakdown by heme oxygenase$^{143}$.

It is apparent that an increase in HO activity is a positive cellular response to potentially damaging stimuli, with anti-inflammatory and immunosuppressive effects. HO activity augments the endogenous cellular antioxidant reserve and its effects are achieved by influencing redox-sensitive signal transduction pathways and cell to cell interactions such as leukocyte migration and inflammatory cytokine production. Moreover, evidence suggests that HO-1 activity is essential for the survival of organisms. In murine models of HO-1 deficiency, mice lacking the gene are at increased risk of adverse consequences of oxidative stress and show poor overall survival, commonly dying in utero or within 1 year of birth. HO-1$^{-/-}$ adult mice generate increased amounts of pro-inflammatory cytokines including interferon-γ (IFN-γ) and interleukin-6 (IL-6) and develop progressive inflammatory disease characterised by hepatic and renal inflammation, splenomegaly, lymphadenopathy and leukocytosis$^{281,282}$. Young adult HO-1$^{-/-}$ mice exposed to endotoxin
were vulnerable to hepatic necrosis and had an increased mortality when compared to their HO-1+/+ counterparts\textsuperscript{246}.

The first human case report of HO enzyme deficiency was reported in 1999 describing a 6-year-old boy diagnosed with HO-1 deficiency\textsuperscript{263}. He suffered from growth failure, anaemia, tissue iron deposition, lymphadenopathy, leucocytosis and increased sensitivity to oxidant injury. The enzyme deficiency ultimately led to his premature death\textsuperscript{189,264}. The same phenotype is observed in HO-1\textsuperscript{−/−} mice and emphasises the importance of HO-1 for cellular homeostasis\textsuperscript{246}. There has been only one further case of HO-1 deficiency in humans reported to date so the expression of HO-1 protein seems to be an indispensable component of human physiology\textsuperscript{283}.

The principal functions of HO-1 in cellular homeostasis include regulation of the oxidative load, anti-inflammatory activities and modulation of the cell cycle and programmed cell death\textsuperscript{284}. The widespread effects on cell function encompass the beneficial effects of HO-1 and the products of heme breakdown observed in inflammatory responses including septic shock, ischemia-reperfusion injury, angiogenesis, carcinogenesis and degenerative diseases\textsuperscript{201}. HO-1 activity has a role in many pathophysiological conditions, including ischaemic stroke and potential graft failure following organ transplantation. Abnormal HO activity has been demonstrated in angiogenesis and in stress-induced neurodegenerative disorders such as Alzheimer’s disease. HO-1 expression has a potent cytoprotective effect in experimental models of cardiac ischemia and reperfusion injury, pulmonary inflammation and hypertension\textsuperscript{285-287}.

The mechanisms that mediate the anti-inflammatory effects of the HO have not been fully elucidated. The major contribution may be from CO-mediated signalling, with the antioxidant properties of biliverdin/bilirubin and the sequestration of iron by ferritin combined towards cell protective effects of heme oxygenase activity\textsuperscript{248,288-291}. Carbon monoxide is reported to have important anti-inflammatory effects in models of tissue injury. In lipopolysaccharide (LPS)-stimulated macrophages, CO caused a significant reduction in the generation of inducible nitric oxide synthase (iNOS)-derived NO and production of pro-inflammatory tumour necrosis factor (TNF) and IL-6\textsuperscript{249,292}. Under the
same conditions, CO increased production of the anti-inflammatory cytokine, interleukin-10 (IL-10).

HO-1 expression has been demonstrated to reduce vascular constriction and prevent excessive vascular smooth muscle cell proliferation in mouse and pig models of arterial injury\(^{293}\). Increased HO-1 activity produces CO that readily diffuses into adjacent smooth muscle cells and activates soluble guanylyl cyclase (sGC). This results in elevated intracellular levels of cyclic GMP (cGMP), leading to smooth muscle relaxation and vasodilatation. HO-1 expression inhibits the proliferation of vascular smooth muscle cells \textit{in vitro} and \textit{in vivo} by causing up-regulation of p21 gene and cell cycle G1/S growth arrest. This effect is most likely mediated by cyclic GMP-dependent transcriptional regulation of p21 subsequent to cGMP activation by CO. HO-1\(^{-/-}\) mice subjected to arterial wire injury demonstrated greater intimal hyperplasia and smooth muscle cell proliferation compared to HO-1\(^{+/+}\) controls on examination of the injured arteries after two weeks\(^{293}\). These findings indicate protective effects of HO-1 in arterial trauma that are relevant to vascular diseases in which vasoconstriction and cell proliferation are a pathological feature, including atherosclerosis and graft failure.

Biliverdin and bilirubin inhibit nuclear factor-κB (NF-κB) activation in leucocytes, causing reduced expression of various adhesion molecules such as E-selectin and vascular cell adhesion molecule-1 (VCAM-1), thereby reducing cell adhesion to vascular endothelium and inhibiting a recognised component of acute inflammation\(^{234}\). Biliverdin and bilirubin have also been demonstrated to reduce the chemotactic response when monocytes are exposed to oxidized low-density lipoprotein (LDL)\(^{294}\).

The role of HO-1 in inflammation appears to be of particular importance in the lungs. In rodent lung models of hyperoxia- and endotoxin-mediated acute lung injury, researchers have found that HO-1 expression and levels of activity increase after such injury and that HO-1 inhibition results in increased susceptibility to lung injury\(^{279, 295-297}\). This suggests that HO-1 has a protective role against these forms of injury\(^{298}\). In acute and chronic lung transplant rejection, increased expression of HO-1 in alveolar macrophages has been found in human transplanted lungs, whilst HO-1 has been shown to induce cytokine expression following transplantation in rat allograft lung models\(^{299, 300}\). Several groups
have also found that HO-1 overexpression protects against ischaemia-reperfusion injury, which commonly causes graft failure. A recent study found that HO-1 induction is protective against pancreatic microcirculatory derangements following ischaemia-reperfusion injury.

Published studies of the functional roles of HO-1 and HO-2 in vivo indicate that deficiencies of either HO-1 or HO-2 influence oxidant sensitivity and cause disruption in the homeostatic flow of intra and extracellular iron. Cellular oxidative stress causes modification of cell proteins by ROS to make the proteins more susceptible to proteolytic degradation. Increased breakdown of heme proteins may result in a transient increase in free intracellular endogenous heme levels. Oxidative stress conditions also temporarily decrease the cellular content of the reduced form of glutathione. Up-regulation of inducible HO-1 protein is triggered by both a rise in intracellular free heme and a disruption of intracellular thiol equilibrium. The increase in HO-1 activity compensates for the brief rise in intracellular free heme by removing the heme from the cell in exchange for free ferrous iron. The free Fe$^{2+}$ stimulates ferritin protein synthesis with consequent iron sequestration restoring the cellular iron equilibrium. Increased ferritin synthesis depends upon the intracellular accumulation of free reactive Fe$^{2+}$ that becomes temporarily available as a catalyst for harmful oxidative reactions via Fenton chemistry. An initial competition therefore arises between iron sequestration and reutilization pathways and reactions with Fe$^{2+}$ to generate free radicals. Once the iron equilibrates to the ferritin pool it is transiently sequestered in an inert oxidised form with a restricted reactivity. This sequestered iron may still however be available for catalysis of oxidative reactions under conditions that promote its release.

The cytoprotective antioxidant effects of heme oxygenase only occur when both the free intracellular heme has been degraded and the free intracellular Fe$^{2+}$ has been either completely sequestered in the ferritin pool or transported out of the cell cytoplasm. Enzymatic HO activity can therefore be regarded as an intermediate in cell antioxidant defence. The protection of cells against oxidative stress cannot be exclusively attributed to the enzymatic function of HO-1. HO-1 protein may be additionally involved in cellular signalling and regulation of gene transcription to activate cell protective
pathways involving up-regulation of catalase and glutathione peroxidase expression and an increase in glutathione content\(^\text{305}\). The multifunctional nature of heme oxygenase in the regulation of cell proliferation, differentiation, oxidative status and apoptosis renders its significance much greater than simply elimination of cellular heme. Beneficial effects of heme oxygenase activity have been demonstrated via the inhibition of inflammatory, apoptotic and proliferative processes in lung inflammation, lung and vascular transplantation, sepsis, and pulmonary hypertension models.

Numerous studies indicate a protective role for HO-1 during microbial sepsis. HO-1-deficient mice had an increased level of free circulating heme and suffered higher mortality rates after caecal ligation and puncture to induce peritonitis as compared to HO-1-sufficient mice\(^\text{306-309}\). Administration of exogenous carbon monoxide (CO) at low doses with associated HO-1 expression selectively inhibited the expression of pro-inflammatory cytokines TNF\(\alpha\) and IL-1\(\beta\) caused by a concomitant sublethal dose of bacterial lipopolysaccharide (LPS) in mice and cell culture models\(^\text{181 249 297}\). The p38 mitogen-activated protein kinase (MAPK) pathway was demonstrated to be important for the CO-mediated effect observed in cultured macrophages\(^\text{249 297}\).

Overexpression of HO-1 and low-dose administration of CO have been shown to afford protection against hyperoxic lung injury and increased cell survival from hyperoxia-related stress in both rodent models and cultured epithelial cells\(^\text{279 295}\). Animal models of ventilator-induced lung injury collectively support a potential role for low-dose CO administration with mechanical ventilation to protect from lung injury. Rodent models of lung ischaemia and reperfusion injury describe a therapeutic potential for HO-1-derived carbon monoxide. Homozygous HO-1 knockout mice (HMOX1\(^{-/-}\)) were more sensitive to the lethal effects of lung ischaemia/reperfusion injury than their wild-type counterparts, with an increased survival phenotype achieved by inhalation of exogenous CO to compensate for the HO-1 deficiency\(^\text{287}\).

Recent evidence suggests an additional mechanism mediated by heme oxygenase activity: the regulation of cellular autophagy and the preservation of mitochondrial homeostasis. The autophagy process is a regulated cellular pathway targeting cytosolic material such as damaged organelles or denatured proteins. This material is engulfed by
double-membrane vesicles that fuse with lysosomes to facilitate its enzymatic degradation. Important precursor molecules including amino acids and fatty acids are thereby regenerated for use in anabolic pathways and ATP production. This aspect of the autophagy mechanism may help to prolong cellular survival during starvation. Autophagy assists in the immune response to infection by augmenting the intracellular degradation of pathogenic microorganisms and may also contribute to adaptive immune mechanisms.

The role of autophagy in human diseases is complex with both protective and deleterious effects in response to various stimuli. Autophagy contributes to the stress response by reducing the activation of potentially lethal signal transduction cascades, enabling the elimination of cytotoxic protein aggregates and maintaining crucial levels of ATP to facilitate protein synthesis. Cellular sensitivity to cell cycle-dependent toxins may be influenced by autophagy since progression of the cell cycle is affected by the induction of the process. Cell death via an autophagy process may occur during development and the neonatal period to maintain cellular energy homeostasis and cell survival and also during homeostasis in adulthood but there is no conclusive evidence for the existence of a specific mechanism for cell death via autophagy.

Translocation of HO-1 protein to the mitochondria has been observed in human alveolar and bronchial epithelial cells after treatment with hemin and exposure to LPS and cigarette smoke. The HO-1 response causes down regulation of apoptosis and autophagy signalling pathways to confer cytoprotection and reduce the cellular stress caused by cigarette smoke. Up-regulation of HO-1 occurs in the liver in response to sepsis and LPS exposure and is associated with induction of HO-1-dependent autophagy signalling pathways that lead to reduced hepatocellular injury and increased protection against cell death. Anti-inflammatory effects produced by HO-1-dependent autophagy signalling have also been demonstrated by a reduction in cytokine production in macrophages stimulated by LPS.

Conversely, induction of autophagy signalling by HO-1 activity has been demonstrated to increase cell death, with activation of mitochondrial autophagy by HO-1 up-regulation producing characteristic iron-laden cytoplasmic inclusions in Alzheimer’s and Parkinson’s
diseases\textsuperscript{324}. Limited available evidence suggests that the role of HO-1 in the regulation of autophagy is variable and specific to the cell type and stimulus applied. Both HO-1 induction and the process of autophagy represent an adaptive cellular response to stress with a key aim to restore cellular homeostasis\textsuperscript{325-327}. Apoptosis and autophagy may share a common regulatory pathway and while the two processes can occur simultaneously the phenomenon of autophagy only seems to occur in cells that cannot die by conventional apoptotic mechanisms\textsuperscript{328}. The detailed interaction between autophagy and apoptosis pathways requires further investigation.

1.3.3 Heme oxygenase and cancer

There are a host of molecular mechanisms underlying gastric and oesophageal cancer including alterations in growth factors, cytokines and cell adhesion molecules which contribute to the activation of complex signalling pathways leading to cell cycle modulation. Abnormalities of oncogenes, tumour suppressor genes and genetic instability influence the development of carcinoma. Understanding of the molecular pathways that characterize cell growth, cell cycle kinetics, apoptosis, angiogenesis and invasive biology may provide improved novel targets for cancer therapy.

There is a recognised link between inflammation and carcinogenesis, but the precise role of inflammation in tumourigenesis is not fully understood. Leucocyte infiltration into tumour tissue is recognised as a negative prognostic indicator of clinical outcomes\textsuperscript{329}. Tumours are infiltrated by many types of leucocytes that have conflicting influences on cell proliferation and survival, angiogenesis and tumour migration. Macrophages can promote tumour growth by producing angiogenic factors such as interleukin-8, vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF)\textsuperscript{330}. Macrophages can also promote tumour cell migration by the release of proteolytic enzymes that breakdown the extracellular matrix. At the same time, an important role of macrophages is the ability to destroy tumour cells directly by releasing lysosomal enzymes, TNF or macrophage-activating factor (MAF)\textsuperscript{331}. They also function as antigen-presenting cells and activate T lymphocytes which release inflammatory cytokines. These lymphocyte-derived cytokines are primarily responsible for the anti-tumour effects of leucocyte
infiltration into tumour tissue\textsuperscript{332}. The anti-inflammatory action of heme oxygenase may influence tumour progression and could have a potential clinical benefit.

The reported cytoprotective effects of heme oxygenase activity may be relevant to cancer cells. An increasing body of literature suggests that HO-1 expression and activity may modulate tumour induction and influence tumour growth and metastasis. HO-1 activity may affect various aspects of tumour progression including cell growth, resistance to apoptosis and angiogenesis\textsuperscript{333}. The level of HO-1 protein expression has been demonstrated to be elevated in various tumour tissues including prostate cancer, pancreatic cancer, hepatoma, malignant melanoma, glioblastoma and various sub-types of adenocarcinoma\textsuperscript{334-341}. Within the tumour tissues expressing HO-1 the protein can be localised to the malignant epithelial cells or found in infiltrating leucocytes, particularly macrophages\textsuperscript{335 340}. Expression of HO-1 has been shown to be up-regulated further by anti-cancer therapy as a response to increased oxidative stress\textsuperscript{334 342}.

The cytoprotective, anti-inflammatory and anti-apoptotic properties of HO-1 activity may confer a clinical disadvantage in cancer therapy. HO-1 has an established role in the regulation of cell growth and angiogenesis in studies of inflammation. Numerous studies indicate an important role for HO-1 activity in angiogenesis\textsuperscript{343-345}. Specific targeted knockdown of HO-1 protein in murine fibroblasts and human keratinocytes lowered the basal synthesis of VEGF, an important inducer of angiogenesis\textsuperscript{343}. Angiogenesis in human capillary endothelial cells is promoted by carbon monoxide (CO) signalling via an increased production of endogenous CO from heme oxygenase enzymatic activity\textsuperscript{344 346-348}.

The propensity of tumours to metastasise may be enhanced by the pro-angiogenic activity of HO-1\textsuperscript{349}. Angiogenesis is necessary for the continued growth, invasion, and metastasis of cancer cells. Tumours with a volume greater than 1–2 mm\textsuperscript{3} require expansion of the vasculature to facilitate metastatic spread via the bloodstream\textsuperscript{350}. HO-1 activity has been demonstrated to accelerate tumour angiogenesis in a mouse model of human pancreatic carcinoma. Increased expression of human HO-1 protein accelerated tumour growth, stimulated the early stages of angiogenesis and increased the occurrence of lung metastasis\textsuperscript{351}.
Studies in a human colonic adenocarcinoma cell line report an increase in resistance to apoptosis after induction of HO-1 protein and increased production of bile pigment products bilirubin and biliverdin. Induction of HO-1 activated Akt signalling and reduced the levels of p21 expression causing the modification of the apoptotic protein Bcl-2/Bax ratio toward cell survival\textsuperscript{352}. Increased levels of HO-1 may thereby promote tumour resistance to stress in conditions of limited nutrient supply.

On the other hand, inhibition of HO activity has been shown to improve control of angiogenesis and reduce both the growth and spread of certain tumours\textsuperscript{353,354}. Administration of the competitive heme oxygenase enzyme inhibitor zinc protoporphyrin IX has been demonstrated to suppress cancer cell growth in lung, sarcoma and hepatoma cancer models. Inhibition of total heme oxygenase enzymatic activity by zinc protoporphyrin IX significantly reduced tumour growth in a rat model\textsuperscript{355-358}. Significant reductions of tumour growth \textit{in vivo} have been reported after administration of zinc protoporphyrin that causes HO-1 inhibition and reduced production of bile pigment products with a consequent impairment in cellular antioxidative function\textsuperscript{359}.

Targeted knockdown of the HO-1 gene caused reduced HO-1 protein expression leading to significant reduction in the growth of pancreatic cancer cells. Tumour tissues with reduced HO-1 expression became significantly more sensitive to anti-cancer radiotherapy and chemotherapy\textsuperscript{334}. Inhibition of heme oxygenase activity may be a useful adjunct in anti-cancer chemotherapeutic intervention to prevent the promotion of angiogenesis and cancer cell resistance to apoptosis associated with HO-1 activity\textsuperscript{356,357,360,361}.

Studies of the effects of modification of heme oxygenase activity on cancer cell proliferation have produced contradictory results. Induction of HO-1 enzyme in rat and human breast cancer cell lines caused HO-mediated inhibition of a proliferative enzyme indoleamine dioxygenase and caused an overall reduction in cell growth\textsuperscript{362}. Studies in human pancreatic cancer cell lines and murine or rodent models of lung cancer, hepatoma and Kaposi’s sarcoma have demonstrated an increase in tumour cell proliferation associated with activation of HO-1 protein\textsuperscript{351,363}. Some reports indicate that the antioxidant properties of heme oxygenase pathway activation appear to protect cancer cells from oxidative damage. Up-regulation of the HO-1 isoform in particular has
been demonstrated when tumour cells are exposed to oxidative stress, hypoxia, serum deprivation, or toxic compounds including chemotherapeutic agents\textsuperscript{334 337 355 357}.

HO-1 is implicated in the biology and pathogenesis of cancer by its effects on cell growth, resistance to apoptosis and angiogenesis. Increased expression or activity of HO-1 has been demonstrated to correlate with cellular proliferation in tumour tissues including prostate cancer and renal cell carcinoma\textsuperscript{339 341}. HO-1 expression may represent the cellular response to an acute inflammatory precancerous condition. Initially this may serve as a protective mechanism to limit the extent of inflammation and oxidative stress. Anti-proliferative effects of HO-1 may limit neoplastic cell proliferation and progression, but if neoplastic cells become established the increased HO-1 activity may serve to protect the cancerous cells, promoting tumour growth by allowing apoptotic escapes. CO produced at increased levels with HO-1 up-regulation can readily diffuse through cell membranes and reduce inflammatory cytokine release from infiltrating leucocytes. The biliverdin/bilirubin system could protect tumour cells from oxidative stress mediators generated by activated neutrophils. Bilirubin and CO production can improve the antioxidant capacity of tumour cells, conferring tumour resistance to chemotherapies whose effect relies on intracellular oxidative stress\textsuperscript{333}. HO-1 enzyme is demonstrably up-regulated when tumour cells are exposed to oxidative stress, hypoxia, serum deprivation, or toxic compounds including chemotherapeutic agents\textsuperscript{337}. This response may contribute to tumour chemoresistance. Cancer cell phenotype may be altered by constitutive expression, induction or inhibition of HO-1. Studies in cultured human gastric cancer cell lines demonstrated that up-regulation of HO-1 caused markedly increased expression of p21. Cells with elevated p21 levels were significantly resistant to apoptotic stimuli. The resistance to apoptosis was achieved via a p38 mitogen-activated protein kinase-dependent pathway, with decreased caspase-3 activity demonstrated\textsuperscript{364}. Evidence supporting a role of the HO-1 system as an endogenous influence on cancer growth is further provided by reports of HO inhibitors causing reduced tumour cell proliferation and restoration of apoptosis\textsuperscript{355}.

HO-1 is recognised as an important regulator of cell proliferation and angiogenesis. Angiogenesis is required for the sustained growth, invasion and metastasis of tumours. Increased HO activity in human endothelial cells promotes prolactin-mediated cell
proliferation and angiogenesis via CO-signalling pathways\textsuperscript{344, 353}. HO-1 has been shown to accelerate tumour angiogenesis in human pancreatic cancer\textsuperscript{351}.

HO-1 enzyme has been investigated as a potential target for chemotherapy with varying success. Increased HO-1 expression is a characteristic feature of only certain tumours, with considerable individual variability within one particular cancer. Up-regulation of HO-1 enzyme has been shown to inhibit the growth of rat and human breast cancer cell lines by inhibiting a pro-proliferative enzyme, indoleamine dioxygenase, to give an overall antiproliferative effect\textsuperscript{362}.

Fang and colleagues demonstrated that inhibition of HO activity by zinc protoporphyrin (ZnP) significantly reduced tumour growth in an orthotopic mouse model of sarcoma. Mice were treated with polyethylene glycol-conjugated ZnP (pegylated zinc, PEG-ZnP) that is water-soluble and accumulates in tumour tissue after intravenous administration. The PEG-ZnP treatment caused tumour-selective suppression of HO activity and induction of apoptosis with suppression of implanted sarcoma tumour growth without apparent side effects. \textit{In vitro} studies using cultured SW480 human colon adenocarcinoma that express HO-1 showed an increase in apoptotic death via induced oxidative stress in cells treated with pegylated zinc protoporphyrin\textsuperscript{356-358}.

Increased expression of HO-1 mRNA and HO-1 protein has been demonstrated in human pancreatic cancer as compared to normal pancreatic tissue\textsuperscript{334}. HO-1-mediated effects on angiogenesis, tumour progression and metastasis have been studied by HO-1 gene manipulation in mouse models of pancreatic adenocarcinoma. Up-regulation of HO-1 protein caused an increase in tumour growth with accelerated angiogenesis and increased occurrence of lung metastasis, whereas inhibition of HO activity with tin mesoporphyrin (SnMP) had beneficial limiting effects on both tumour growth and metastasis\textsuperscript{351}. Targeted knockdown of HO-1 protein mediated by short interfering RNA transfection caused a significantly reduced proliferation rate in cultured pancreatic cancer cell lines, with a demonstrated reduction in bilirubin levels in transfected cells. Cell lines with an inhibited HO-1 activity were significantly sensitised to treatment with both gemcitabine chemotherapy and \textgamma-radiation (20 Gy)\textsuperscript{334}. 
A study reported by Busserolles et al showed anti-apoptotic effects of HO-1 in the human colon adenocarcinoma cell line Caco-2, with HO-1 induction resulting in resistance to apoptosis mediated by formation of biliverdin and bilirubin and activation of the protein kinase Akt pathway. Several studies of the human lung adenocarcinoma cell line A549 have described the cytoprotective effect of HO-1 expression. Constitutive over-expression of HO-1 and its associated transcription factor Nrf2 may be primarily responsible for enhancing chemoresistance in these cells. Silencing of the basal HO-1 gene caused significantly increased up-regulation of the proapoptotic tumour suppressor p53 and its downstream p21 and Bax, thereby increasing cellular vulnerability to apoptosis.

There is limited knowledge of the role of HO-1 in tumour-associated inflammation. Studies of murine melanoma show that HO-1 overexpression inhibits inflammation and reduces the leucocytic tumour infiltrate. In tumour-bearing mice with subcutaneous growing melanomas, reduced neutrophil activation improved the angiogenic potential of tumour cells. HO-1 induction increased tumour cell viability and proliferation and decreased survival in affected mice. Mice bearing melanoma cells with a high level of HO-1 had lower concentrations of tumour necrosis factor (TNF) and higher levels of soluble TNF-receptor-1 in the blood and tumour tissues, reducing TNF availability which could contribute to the observed inhibition of the inflammatory response and promote tumour angiogenesis.

The expression of HO-1 protein in gastrointestinal cancers is not well studied with one published report to date investigating HO-1 expression in ex-vivo samples of human colorectal cancer and colonic adenoma. HO-1 expression was demonstrable in 41.8% (23/55) of patients with colorectal cancer and in 36.8% (7/19) of colonic adenoma samples. There was a significantly reduced rate of lymphatic tumour invasion, fewer lymph node metastases and a significantly improved overall survival in colorectal cancer patients with tumours expressing HO-1 protein. These findings suggest an overall beneficial anti-cancer effect of HO activity that may be mediated via anti-angiogenic and anti-proliferative pathways. Consistent and definitive effects of heme oxygenase enzyme are yet to be established in studies of cancer cell biology and clinical outcomes.
Up-regulation of HO-1 enzyme has been demonstrated in human colorectal cancer cells after treatment with chemotherapeutic agents and may be induced in response to radiotherapy and photodynamic therapy. The cytoprotective effects of HO activity may be mediated via decreased levels of free intracellular pro-oxidant heme and increased levels of antioxidant bilirubin and carbon monoxide.

A dominant role for HO enzyme in the context of malignancy is yet to be established, with variable effects on apoptosis, angiogenesis and tumour progression reported in published studies to date. The properties of HO activity have implications for tumour proliferation and cell death, differentiation, angiogenesis and metastasis and tumour-related inflammation.

Increased HO-1 activity has been shown to confer resistance to apoptosis in human gastric cancer cells. The anti-apoptotic effects were independent of p53 expression and mediated via p38 MAP kinase pathways to cause reduced caspase-3 activation. Inhibition of HO-1 activity by a flavonoid Vitex fruit extract caused an increase in apoptosis in cultured gastric signet ring adenocarcinoma cells. By contrast, treating cells derived from human colonic adenocarcinoma with the flavonoid Vitex fruit extract caused induction of HO-1 enzyme and was associated with an increase in cellular apoptosis. Induction of HO-1 enzyme in alternative human colonic cancer cell lines (Caco-2) resulted in increased cell resistance to apoptosis with effects mediated via Akt signalling pathways and independent of p38 expression. Treatment with a potent competitive heme oxygenase inhibitor (pegylated zinc protoporphyrin) caused induction of apoptosis in human colon carcinoma SW480 cells when administered in vitro and reduced the growth of murine colon carcinoma in vivo.

Angiogenesis is a critical feature of cancer biology for tumour growth and metastasis. The pro-angiogenic properties of heme oxygenase enzyme may thereby facilitate tumour progression. Vascular endothelial growth factor (VEGF) has been shown to induce prolonged HO-1 expression and increased enzymatic activity in human endothelial cells. Angiogenesis driven by VEGF activity was prevented by inhibition of HO-1 protein.

Overexpression of HO-1 protein is associated with an increased occurrence of metastasis in pancreatic cancer cells and melanoma cells with effective prevention of metastasis.
achievable via inhibition of HO activity\textsuperscript{351, 366}. Studies in a mouse model of colonic carcinoma found conflicting evidence of HO-1-related effects with no effect on lung metastases and an increased frequency of liver metastases associated with inhibition of HO enzyme by zinc deuteroporphyrin\textsuperscript{372}. \textit{Ex-vivo} samples of colonic adenocarcinoma that expressed HO-1 protein demonstrated a significantly lower rate of lymphatic invasion\textsuperscript{367}.

The overall influence of the HO enzyme pathway on the growth, invasion and metastatic potential of malignant cells may depend on the specific type of cancer or other contributory factors yet to be defined.

1.3.4 HO gene promoter polymorphism

HO-1 deficiency is extremely rare within the human population but the level of HO-1 expression can be variable due to the polymorphism associated with the promoter of the HMOX1 gene. HO-1 generation is chiefly regulated at the transcriptional level and the length of the GT repeat in the HO-1 promoter is a much more common determinant of the variation of HO-1 activity in humans\textsuperscript{373}. The human HO-1 gene promoter contains a stretch of microsatellite DNA with GT repeats ranging from 11 to 40 located within the regulatory sequence approximately 250 base-pairs upstream of the transcription initiation site\textsuperscript{374}. Published studies indicate that longer (GT)\textit{n} sequences are associated with a reduction in HO-1 protein expression\textsuperscript{374-379}.

It is thought that shorter GT repeats have higher HO-1 transcriptional activity and expression compared with longer GT repeats. A recent study showed that variations in HO-1 expression and activity modulated by the number of GT repeats influenced various functions in human endothelial cells. The endothelial cells with short dinucleotide sequences (\textit{n} <25) displayed a phenotype with a beneficial intracellular glutathione ratio, increased resistance to oxidative stress, reduced synthesis of pro-inflammatory cytokines and enhanced proliferation in response to VEGF treatment\textsuperscript{375}.

Allelic variants of the HO-1 gene promoter may affect HO-1 protein expression and enzymatic activity especially in response to oxidative stress. The variability in HO-1 activity mediated by HMOX1 gene promoter polymorphism may be of clinical relevance with some studies suggesting a protective role against atherosclerosis of short, more
active alleles of the HMOX1 promoter. Short (GT)n repeats have been shown to be associated with low levels of inflammatory markers while long dinucleotide repeats were associated with increased lipid peroxidation in serum.

A protective role of HO-1 in cardiovascular disease is supported by several human studies demonstrating an association between shorter (GT)n repeats and a reduced risk of coronary artery disease and myocardial ischaemia. Some studies show that longer GT repeats are associated with a significantly higher risk of developing restenosis after coronary stenting whereas other studies have failed to find an association between GT repeat length and either coronary restenosis or CAD.

Studies examining the relationship between the genotype of (GT)n sequences and graft survival after renal and heart transplantation are contradictory. Reported studies indicate that transplanted organs with short (GT)n repeats survive better and are less susceptible to tissue injury. These findings are not universal, however, with a recent study demonstrating no influence of HMOX1 genotype and the presence of (GT)n sequences on kidney survival after transplantation, although a significant effect was observed when comparing normal and impaired graft function. The significance of (GT)n polymorphism may be related to patient ethnicity or other individual factors or may be relevant only in specific clinical conditions.

Although HO-1 may facilitate the progression of established tumours it may also protect against potential carcinogens and reduce the probability of tumour initiation. Variation of HO-1 activity associated with the HMOX1 gene promoter polymorphism may contribute to cancer risk in individuals. Patients with a lower level of HO-1 activity due to a relative lack of short (GT)n sequences but an increased proportion of longer (GT)n sequences are considered to be at an increased risk of tumour development. Published studies indicate that longer (GT)n sequences in the HO-1 gene promoter region are associated with a lower HMOX-1 transcriptional activity and a reduction in HO-1 protein expression. If one accepts that the dominant role of HO-1 activity in malignancy is to protect against carcinogenesis and tumour progression then it is feasible to consider individuals with a lower HO-1 transcriptional activity to be more susceptible to malignancy.
The dual effect of HO-1 on protection against carcinogenesis and promotion in different stages of tumour progression may indeed be related to HO-1 genetic polymorphism. Humans differ quantitatively in their ability to mount an HO-1 response, modulated by functional microsatellite polymorphisms in the HO-1 gene promoter region. The human HO-1 gene has been mapped to chromosome 22q12, and a \((GT)_n\) dinucleotide repeat has been identified as a functional polymorphism in the proximal promoter region, modulating the transcriptional activity of the gene. The length of the \((GT)\) repeat can be classified into short, medium and long component alleles. Short (<25 GT) repeats are associated with an increased HO-1 up-regulation after stimulation, and longer \((GT)_n\) repeats have been shown to exhibit lower HO-1 transcripational activity. Association of the HO-1 promoter polymorphisms has been evaluated in pulmonary disease (including the development of lung adenocarcinoma), cardiovascular disease, renal transplantation, recurrent miscarriage, and neurological disorders including Alzheimer’s and Parkinson’s disease\(^{374}\). Long \((GT)_n\) repeats are associated with increased severity of cardiovascular and pulmonary inflammatory disorders suggesting important clinical roles for HO-1 expression as an anti-inflammatory mediator.

A recent study reported that the homozygous short allele with <25 \((GT)_n\) repeats was found more frequently in patients with malignant melanoma when compared to a healthy control population. In addition, the short \((GT)_n\) genotype was significantly associated with a deeper Breslow thickness of primary tumours as compared to L-allele (>25 repeats) carriers\(^{391}\). It is possible that increased HO-1 expression confers resistance to apoptosis during the initial malignant transformation of malignant melanoma, allowing further subsequent mutations during disease progression that promote proliferation, invasion, and tumour cell migration.

By contrast, a recent study in gastric cancer suggests a higher frequency of gastric adenocarcinoma associated with the long \((GT)_n\) repeat HO-1 gene promoter polymorphism, with a lower frequency of lymphovascular invasion of gastric tumours associated with the medium \((GT)_n\) repeat allele\(^{392}\). A study in a Japanese population found that the long \((GT)_n\) repeat in the HO-1 gene promoter may also be associated with the development of lung adenocarcinoma (but not squamous carcinoma) amongst male smokers\(^{393}\). These polymorphisms reduce HO-1 protein expression, suggesting a
protective influence of HO-1 in preventing gastric and lung cancer. This supports an anti-inflammatory, anti-tumour role of HO-1 in carcinogenesis rather than a tumour-protective mechanism facilitating disease progression.

The presence of a long HMOX1 allele is recognized to be related to the increased risk of oral squamous cell carcinoma in patients chewing areca nuts\textsuperscript{394}. The risk of developing lung cancer amongst male smokers has been shown to be increased for the long allele HMOX1 genotype when compared to a non-long allele genotype\textsuperscript{393}. The incidence of long (GT)\textit{n} repeats was increased in a group of patients with lung adenocarcinoma when compared to a control group. This positive correlation is not demonstrated in other tumours, with a microsatellite polymorphism promoter associated with a modification in the relative risk for malignant melanoma\textsuperscript{391}. HMOX1 genotypes of short (GT)\textit{n} sequences were found to be more frequent in patients with malignant melanoma tumours compared to healthy subjects. There was a higher risk for malignant melanoma with higher levels of HO-1 protein and a higher likelihood of drug resistance with an increased tendency for the melanoma to be resistant to apoptosis\textsuperscript{391}.

Lymphoblastoid cell lines from patients with short allele (GT)\textit{n} repeats (less than 27) have been found to display more resistance to oxidant-induced apoptosis than those cells obtained from patients with longer (GT)\textit{n} repeats (more than 33) and therefore reduced HO-1 expression\textsuperscript{395}. This finding concurs with the observed effects of HO-1 activity on development of malignant melanoma in an animal model. The viability, proliferation and angiogenic potential of malignant melanoma cells were increased by overexpression of HO-1 protein with increased metastasis and decreased survival in tumour-bearing mice\textsuperscript{366}. Increased activity of HO-1 enzyme was associated with a detrimental outcome in this murine model of melanoma. Therefore the association of the HMOX1 promoter polymorphism with an increased risk of cancer development appears to be dependent upon the specific tumour type.

Recent studies of the relationship between the HO-1 gene promoter polymorphism and the risk of gastric cancer suggest that the long (GT)\textit{n} repeat HMOX1 genotype is associated with a higher incidence of gastric adenocarcinoma. The medium (GT)\textit{n} repeat genotype may confer some protection against gastric adenocarcinoma with a reduced
frequency of lymphovascular tumour invasion\textsuperscript{396 397}. Similar studies in gastrointestinal stromal tumours (GIST) indicate a significant association between the short (GT)\textit{n} HMOX1 allele and the incidence of high-risk tumours, higher tumour recurrence rates and metastatic spread. A significantly reduced disease-free survival and overall survival was observed in patients with GIST tumours carrying the short (GT)\textit{n} HMOX1 allele\textsuperscript{398}.

The focus of published studies to date has suggested a role for HO-1 enzyme in cancer regulation. HO-1 expression is a characteristic feature of only certain types of cancer, and the anti-tumour activity of experimental HO inhibitors may be limited to cancer cells that constitutively over-express HO-1. In the studies by Ding and colleagues\textsuperscript{399 400} of the expression profiles of HO isoforms in eight human cancer cell lines, HO-1 mRNA and protein was detected in five cell types. By contrast, HO-2 mRNA and protein was evident in all eight cell lines. In an immunohistochemical study of biopsy specimens from patients with oesophageal squamous cell carcinoma, HO-1 expression was detected in 7 out of 13 patients. There was no correlation between HO-1 expression and tumour size, stage or histological grade. The presence of HO-1 expression did however predict tumour sensitivity to radiotherapy treatment in this small clinical sample\textsuperscript{401}.

Constitutively expressed HO-2 may also play important roles in cancer cell proliferation. The potential benefits of HO inhibitors may also be applicable to cells lacking HO-1 expression. Tonic HO-2 activity is increasingly recognised to be important for cellular function presumably via basal heme breakdown and generation of HO enzyme products. HO-1 enzyme may provide an additional dynamic level of HO activity as an appropriate response to stressful situations.

1.3.5 The role of the HO pathway in cancer cell biology

The process of carcinogenesis requires eight hallmark capabilities and two facilitating characteristics to establish complete tumour formation and progression. The initial eight capabilities include the maintenance of proliferative signalling, evasion of growth suppressors, favourable reprogramming of energy metabolism, cell death resistance, promotion of replicative immortality, evasion from immune destruction, induction of angiogenesis and activation of cell invasion and metastasis. The accompanying characteristics that facilitate the process are genome instability and inflammation\textsuperscript{402 403}. 
There is various published evidence supporting an association of HO-1 activity with the majority of these features\textsuperscript{333 350}. The cytoprotective properties of HO-1 activity could tip the endogenous balance between apoptosis and proliferation towards an anti-apoptotic and pro-proliferative state\textsuperscript{404}.

The overall effects in the context of neoplasia are especially relevant to oncogenesis, maintenance of malignant cell growth, resistance to apoptosis and resistance to chemotherapy. Various reports suggest that over-expression of HO-1 protein in some human cancers provides an important contribution to resistance to chemotherapeutic agents including cisplatin, doxorubicin and gemcitabine\textsuperscript{405-407}. A potential role for HO activity in carcinogenesis has been suggested by clinical studies of the HO-1 gene promoter polymorphism. The presence of less active HMOX1 allelic variants correlates with an increased incidence of lung cancer in smokers and of squamous cell carcinoma in areca nut chewers\textsuperscript{393 394 408}.

These findings indicate a possible protective role for HO-1 activity in the induction of neoplasia. To date, only one study has investigated this possibility under controlled experimental conditions examining the effect of HO-1 on the induction of squamous cell carcinoma in mice exposed to a two-step model of chemical carcinogenesis\textsuperscript{409}. Topical application of 7,12-Dimethylbenz[a]anthracene (DMBA) followed by phorbol 12-myristate 13-acetate (PMA) was used as a chemical carcinogenesis model in mice of different HO-1 genotypes. Treatment of mice with DMBA and PMA led to the development of skin lesions resembling papilloma with gradual progression to squamous cell carcinoma. Measurement of cytoprotective genes in treated mouse livers demonstrated evidence of systemic oxidative stress in all HO-1 genotype mice.

The first carcinogen-induced skin lesions appeared 2 weeks earlier in HO-1\textsuperscript{−/−} and HO-1\textsuperscript{+/−} genotype mice than in their wild-type counterparts. The tumours contained significantly higher local concentrations of vascular endothelial growth factor and lower levels of tumour necrosis factor-\(\alpha\). The volume of nodules in the wild-type subjects increased at a much slower rate than tumours in the other genotype mice and remained small even at the last week of experiments. In HO-1\textsuperscript{+/−} mice there were low levels of HO-1 expression that produced more numerous but relatively small skin nodules. In HO-1 knockout mice
The absence of HO-1 activity led to the development of fewer often solitary tumours of increased volume that grew much larger than in the other experimental groups. Histopathological analysis of tumour specimens demonstrated that the large lesions on HO-1−/− mice displayed features of benign papilloma. By contrast, even low levels of HO-1 in HO-1+/− animals generated skin lesions with a higher grade of malignancy. Significantly more tumours displayed dysplastic features and progressed to invasive carcinoma in genotypes expressing HO-1 protein. Foci of dysplasia or typical features of infiltrating squamous cell carcinoma were found in 8 out of 9 HO-1+/− subjects and in all 10 HO-1+/+ individuals. The large tumours developed by HO-1−/− mice remained benign papillomas.

The complete absence of HO-1 expression in HO-1−/− mice was associated with the highest mortality and the smallest body weight gain over the experimental time course. Overall survival rate at the end of the experiment was 82% in HO-1+/+ mice, 69.2% in HO-1+/− animals and only 41.7% in HO-1−/− individuals. These findings indicate a level of protection against DMBA/PMA-induced skin injury and development of lesions associated with HO-1 expression but an increase in the malignant transformation of already growing tumours exposed to continued HO-1 activity. Healthy tissues may thus be protected against carcinogen-induced injury by HO-1 activity whereas malignant progression of established tumours is facilitated by HO-1 expression. It is not clear what mechanisms are responsible for the apparent facilitation of clonal promotion despite the protection against carcinogenesis associated with HO-1 activity. A deficiency of HO-1 protein may result in an increased apoptotic rate of affected cells exposed to carcinogens and thereby lead to a reduced rate of cancer initiation. Once the affected cells have transformed into malignant cells the reduced HO-1 activity may facilitate cell proliferation. The variable effects of HO-1 activity observed in cancer studies reflect the tissue-specific influence of HO-1 on the cell cycle.

Tauber and colleagues recently performed a transcriptome analysis of potential target genes associated with HO-1 expression in human cancers. They first undertook genome-wide expression profiling of HO-1 expressing versus HO-1 silenced human choriocarcinoma cells to identify putative target genes of HO-1 protein. A subsequent comparative meta-profiling of a pre-existing expression database was performed to
include gene expression analysis of 190 individual tumours from 14 types of human cancer. This research led to the identification of 14 genes whose expression correlated strongly and universally with that of HO-1 protein from choriocarcinoma cells. The genes identified to be up-regulated downstream of HO-1 protein included moderators of cell adhesion, signalling and transport and other critical functions of malignant cells. The overall findings demonstrated a distinct molecular signature identifiable in malignant cells that was determined by HO-1 expression and promoted tumour cell growth and metastatic spread.

Recent published studies have examined the expression of HO-1 in human head and neck squamous cell carcinoma and its correlation with clinical and pathological features. A recent study by Gandini and co-workers investigated the expression of HO-1 using immunohistochemistry in tissue microarrays in a large collection of human primary head and neck squamous cell carcinomas with correlation to clinical parameters. Real-time quantitative PCR was performed on selected oral tissue tumour samples to further validate HO-1 expression. Results indicated a significantly increased HO-1 expression in tumour tissues as compared to healthy samples. Localisation of HO-1 protein to the cell nucleus was associated with malignant progression in human and murine models of squamous cell carcinoma.

A body of evidence suggests that HO-1 activity exerts potent and comprehensive pro-tumour effects in established tumours. Up-regulation of HO-1 improves the survival of various cancer cell lines exposed to stressful stimuli both in vitro and in vivo. Overexpression of HO-1 protein in murine and human melanoma models caused an increase in cell proliferation. Knockdown of HO-1 protein mediated via siRNA transfection was associated with a statistically significant inhibition of cell growth in human pancreatic cancer cell studies. The effects of altered HO-1 expression on cell proliferation appear to be markedly dependent upon the cell type. Down-regulation of HO-1 protein inhibits the growth of pancreatic carcinoma cell lines whereas up-regulation of HO-1 significantly increased the proliferation of human melanoma cells. Alternatively increased HO-1 activity was associated with significant anti-proliferative responses in human and rodent breast cancer cell lines.
Promotion of angiogenesis and tumour metastasis due to increased HO-1 activity has been demonstrated in various human and animal models. Up-regulation of vascular endothelial growth factor (VEGF) is promoted by HO-1 in many in vitro cultured cell types. Increased VEGF activity can mediate the pro-angiogenic activities of HO-1 in selected cancers and promote tumour growth and metastasis.

Pharmacological inhibition or genetically-mediated knockdown of enzyme activity resulted in reduced cell proliferation in studies of malignant mast cells, hepatocellular carcinoma, prostate carcinoma and urothelial cancer cell lines. Conversely, an antiproliferative effect of HO-1 activity has been demonstrated in experimental models of prostate cancer, lung adenocarcinoma and breast cancer. Treatment with the HO inhibitor tin protoporphyrin IX (SnPP IX) caused a reversal in the growth arrest observed in human lung adenocarcinoma cells that overexpressed HO-1 protein. A small but significant increase in the proliferation of rat and human breast cancer cell lines was observed after administration of SnPP IX whereas HO-1 induction with heme substrate or cobalt protoporphyrin IX resulted in a marked reduction of cell growth. Prostate carcinoma cells that overexpressed HO-1 protein either constitutively or in response to hemin treatment displayed a significant decrease in cell proliferation.

The majority of in vivo studies report a permissive role of HO-1 activity in tumour growth with increased cell proliferation evident in the larger volumes of nodules or increased numbers of cancer cells observed. Reduced expression of HO-1 enzyme caused inhibition of cancer cell growth in hepatocellular carcinoma, lung adenocarcinoma, pancreatic cancer, sarcoma, angioma and selected prostate cancer cell lines. Experiments performed in various tumour cell lines including colonic adenocarcinoma, gastric adenocarcinoma, malignant melanoma and thyroid carcinoma indicate that HO-1 plays an important cytoprotective and anti-apoptotic role in the response of cancer cells to various therapeutic strategies with an overall improvement in cancer cell survival.

Under certain conditions up-regulation of HO-1 protein can promote the accumulation of ROS within the mitochondria and other intracellular compartments. Induction of HO-1 enzyme does not consistently protect cancer cells from oxidative damage. Up-regulation
of HO-1 in breast carcinoma cells did not prevent an increase in apoptosis induced by chemotherapeutic agents. Over-expression of HO-1 protein in some experiments compounded mitochondrial oxidative damage and generated increased apoptosis in breast cancer cells and vascular smooth muscle cells.

Heme oxygenase activity can facilitate angiogenesis and thereby promote the growth of primary and secondary tumours. Tumour progression may be further supported by HO-1 activity via moderation of inflammatory cell function and suppression of the immune response. A significantly increased frequency of metastatic lung nodules was observed in mice with melanoma or pancreatic cancer cells engineered to over-express HO-1 protein as compared to their wild-type counterparts. Pharmacological inhibition of HO activity achieved complete prevention of lung metastasis. Over-expression of HO-1 caused a significant reduction in cell proliferation and migration observed in prostate cancer cells, with a corresponding increase in cell growth and invasion after small interfering RNA-mediated silencing of HO-1 expression.

Inhibition of HO enzymatic activity via knockdown of HO-1 mRNA in human prostate carcinoma cells decreased activation of the p38-mitogen-activated protein kinase / extracellular signal-regulated kinase pathway with reduced cellular oxidative stress observed. Cells displayed concurrent reductions in cell proliferation, invasion and cell viability in vitro. Equivalent in vivo mouse experiments produced marked inhibition of primary prostate tumour growth and fewer lymph node and lung metastases.

An increased level of HO-1 expression has been shown to be associated with a higher frequency of lymph node metastases in patients with oral squamous cell carcinoma due to chewing areca nut and betel leaves. A recent study reported alternative findings with reduced levels of HO-1 expression in squamous cell carcinoma of the tongue and a correlation of low HO-1 expression levels with increased frequency of cervical lymph node metastases.

The majority of investigations indicate a marked increase in HO-1 protein expression in a variety of tumours, particularly in response to anticancer therapy such as chemotherapy, radiotherapy or photodynamic therapy. The mechanism of HO-1 up-regulation may serve to protect cancer cells against therapeutic interventions. Increased HO-1 expression in
pancreatic cancer cell lines was associated with increased chemoresistance to gemcitabine\textsuperscript{407}. A significantly shorter recovery from photodynamic therapy was observed in a mouse model of adenocarcinoma with tumours that up-regulated HO-1 expression\textsuperscript{342}.

1.3.6 Heme oxygenase and anti-cancer therapy

Pharmacological inhibition of enzymatic HO activity has been investigated as a novel therapeutic strategy with the potential to increase tumour sensitivity to chemotherapy, radiotherapy or photodynamic therapy in a range of cancers including urothelial cancer, lung carcinoma, melanoma, chronic myeloid leukaemia, hepatoma, pancreatic cancer and colonic adenocarcinoma\textsuperscript{334 342 366 420 421 426 427}. Administration of the potent HO inhibitor zinc protoporphyrin IX has been able to reduce tumour growth in murine models of hepatoma, sarcoma, B-cell lymphoma and lung carcinoma\textsuperscript{337 356 359 361 427}. Furthermore, administration of pegylated zinc protoporphyrin IX to mice with chronic myeloid leukaemia enabled the cancer cells to overcome chemoresistance to imatinib therapy\textsuperscript{417}.

A major drawback to interpreting the results using pharmacological HO inhibitors and exploiting their therapeutic potential is the exertion of significant HO-independent effects associated with these molecules\textsuperscript{345 428}. Zinc protoporphyrin IX can be demonstrated to reduce tumour blood flow in rat models and cause an accumulation of ROS in sarcoma, colonic carcinoma and lung cancer models\textsuperscript{355-357 360 429-432}. Reported HO-1-independent effects mediated by protoporphyrin HO inhibitors include the reduction of cyclin-D1 expression in colonic adenocarcinoma with consequent inhibition of cell proliferation and increases in cellular apoptosis\textsuperscript{427 431}.

A more specific approach to modulation of the HO pathway involves the targeted knockdown of the HMOX1 gene achieved by siRNA transfection. This approach has been shown to cause reduced cell proliferation and inhibit tumour growth and angiogenesis in orthotopic hepatocellular tumours with induction of apoptosis demonstrable in cultured colonic adenocarcinoma and leukaemia cells\textsuperscript{352 356 422 433 434}. Knockdown of HO-1 mRNA in human prostate carcinoma cells reduced cell proliferation, invasion and cell viability \textit{in vitro} and growth inhibition of both primary and secondary prostate tumours \textit{in vivo}\textsuperscript{419}. Reduced HO activity mediated by siRNA HMOX1 knockdown increased apoptosis and
enabled the chemosensitisation of lung cancer cells toward cisplatin therapy\textsuperscript{405, 432}. Similarly, siRNA-mediated HO-1 gene silencing was able to potentiate the anticancer effectiveness of gemcitabine chemotherapy or \(\gamma\)-radiation against pancreatic carcinoma\textsuperscript{334}.

Pharmacological inhibition of enzymatic HO activity or siRNA-mediated HO-1 knockdown has the ability to increase the chemosensitivity of cells in pancreatic and lung carcinoma and acute myeloid leukaemia\textsuperscript{405, 407, 435}. Not all experiments produce consistent results. HO inhibition by zinc protoporphyrin IX was unable to potentiate the antitumour effects of 5-fluorouracil, cisplatin or doxorubicin chemotherapy in three different tumour models including orthotopic in vivo melanoma\textsuperscript{431}. The detailed mechanism of chemosensitisation associated with suppression of HO-1 activity is yet to be fully elucidated. Pharmacological inhibition of HO-1 by zinc protoporphyrin has been shown to produce significant increases in the formation of ROS\textsuperscript{334, 362, 431}.

Recent studies of possible chemoresistance include the exposure of human hepatocellular carcinoma (HepG2) cells to the environmental pollutant crotonaldehyde\textsuperscript{436}. Crotonaldehyde is a highly reactive aldehyde that occurs naturally in many foods and can be generated from lipid peroxidation\textsuperscript{437, 438}. High levels of crotonaldehyde have been measured in cigarette smoke\textsuperscript{439}.

Treating HepG2 cells with crotonaldehyde caused a significant increase in the nuclear translocation of Nuclear factor erythroid 2-related factor 2 (Nrf2) and induced expression of HO-1 protein via p38 MAPK-Nrf2 pathways. The overall observed effect was a reduction in apoptosis. Inhibition of HO activity by zinc protoporphyrin IX or HO-1 knockdown via siRNA transfection produced an increase in the G0/G1 phase of the cell cycle in crotonaldehyde-stimulated HepG2 cells\textsuperscript{436}. These studies provide a mechanism by which induction of HO-1 expression may promote tumour resistance to oxidative stress and a survival advantage for carcinoma cells when exposed to environmental pollutants.

Kongpetch and colleagues recently investigated the role of HO-1 enzyme in cytoprotection against gemcitabine and doxorubicin chemotherapeutic agents in human cholangiocarcinoma (CCA) cell lines\textsuperscript{440}. Cell lines were treated with gemcitabine (0.001 –
0.1 mM) in the presence of zinc protoporphyrin (0.01 and 0.1 mM) for 24 hours. A clear downward shift in the dose-response curves of both gemcitabine and doxorubicin was observed in the presence of pharmacological HO inhibition with zinc protoporphyrin (zinc PP). Cell lines became highly susceptible to cytotoxic effects of chemotherapy with significantly increased inhibition of cell growth and induction of apoptosis after co-incubation with either doxorubicin or gemcitabine and zinc PP. A slight cytotoxicity was inherent in the effects of zinc PP alone at the concentrations used in these experiments.

The generation of reactive oxygen species (ROS) was shown to be essential for the observed chemosensitisation effect as scavenging of ROS effectively prevented any sensitisation with zinc PP treatment. Inhibition of HO-1 activity may increase cellular oxidative stress and produce ROS derived from cell metabolism\textsuperscript{201,404}.

Co-incubation of cells with gemcitabine and zinc PP caused a significantly increased induction of p21Cip/WAF1 protein which is a p53-dependent downstream gene product and a potent cyclin-dependent kinase inhibitor up-regulated by mitochondrial dysfunction\textsuperscript{441}. Treating cells with gemcitabine or zinc PP alone did not produce any significant changes in the levels of p21 protein\textsuperscript{440}. A reduction in active HO-1 protein levels by specific siRNA-mediated knockdown of HO-1 mRNA exerted a similar sensitising effect with transfected cholangiocarcinoma cells rendered highly susceptible to gemcitabine cytotoxicity\textsuperscript{440}. Induction of HO-1 protein by tin (II) chloride (SnCl\textsubscript{2}) was associated with a 2-fold increase in cholangiocarcinoma cell viability and reduced apoptotic and necrotic cell death after gemcitabine or doxorubicin treatment. The drug resistant effects of HO-1 induction by SnCl\textsubscript{2} were observed regardless of the basal HO-1 expression levels in the cell lines studied\textsuperscript{440}.

These studies indicate that HO-1 plays a substantial role in the resistance of human cholangiocarcinoma cells to chemotherapeutic agents regardless of constitutive levels of HO-1 protein expression. Treatment with anticancer agents caused a marked up-regulation of HO-1 protein in cells with very low basal levels of HO-1 expression suggesting an adaptive cellular response to chemotherapy-induced stress.

Therapeutic inhibition of HO-1 enzyme is able to overcome intrinsic resistance to chemotherapy in cancer cells that constitutively over-express HO-1 and also the acquired
resistance to anticancer agents in tumours that rapidly up-regulate HO-1 protein in response to treatment.

Inhibition of HO activity either as an independent therapeutic approach or as a chemo or radio-sensitisation strategy should ideally be specifically targeted to the cancer cells given that HO activity can exert beneficial effects and protect healthy tissues against carcinogenesis\textsuperscript{350}.

1.3.7 Mechanisms of heme oxygenase activity in cancer

The precise cellular mechanisms responsible for producing the cytoprotective effects of HO-1 activity observed in tumour cells are yet to be elucidated. One potentially important pathway is the reduction of intracellular levels of free heme, a pro-oxidant and substantial mediator of signal transduction\textsuperscript{124}. Heme can be released from heme proteins and may directly catalyse free radical reactions together with the released Fe\textsuperscript{2+} via the Fenton reaction and can interact with nitric oxide to enhance heme uptake\textsuperscript{442}. Heme induces neutrophil activation and increases the expression of endothelial cell adhesion molecules causing the subsequent initiation of robust inflammatory reactions\textsuperscript{443-445}. Heme-binding proteins including albumin and haptoglobin block the cellular uptake of heme and prevent the resulting increase in cellular susceptibility to oxidative damage\textsuperscript{156,446,447}. Although free heme can be harmful to cells it can simultaneously directly regulate the activity of Nrf2 and Bach1 transcription factors\textsuperscript{448,449}. Heme rapidly induces the expression not only of HO-1 protein but also a selection of genes with cytoprotective and detoxifying activity\textsuperscript{450,451}.

There is evidence for the importance of the Nrf2 transcription factor in the cytoprotective activity of HO-1 enzyme\textsuperscript{433,452}. Up-regulation of HO-1 and the resultant cytoprotection was mediated by Nrf2 signalling in neuronal cells exposed to nitric oxide and in monocytes treated with epigallocatechin 3-gallate, a polyphenol compound with anti-carcinogenic properties found in green tea\textsuperscript{365,453,454}. Induction of HO-1 and prevention of oxidative damage in response to nitric oxide exposure was dependent upon Nrf2 signalling activity in colonic carcinoma cells\textsuperscript{451}. Nrf2 is an upstream transcription factor causing HO-1 induction and can also be up-regulated in response to increased HO-1 activity creating a positive feedback loop to promote HO-1-mediated cytoprotection\textsuperscript{455}.
The major source of endogenous carbon monoxide (CO) in mammalian cells is provided via the enzymatic degradation of heme. CO is recognised as a gaseous mediator that plays important roles in neurotransmission and vascular smooth muscle relaxation. CO derived from HO-1 activity produces vasodilatation via a cyclic-GMP-dependent pathway and via cyclic-GMP-independent activation of voltage-gated potassium channels\textsuperscript{456}. Inhibition of platelet aggregation, reduced leucocyte adhesion, and decreased endothelial cell apoptosis is mediated by CO via induction of soluble guanylyl cyclase (sGC)\textsuperscript{326 457}. Increased activation of both sGC and p38-MAP-kinase signalling pathways mediated by HO-1-derived CO can produce cytoprotective and anti-inflammatory responses\textsuperscript{268 358 458}. Increased CO levels are associated with a decreased production of pro-inflammatory cytokines such as tumour necrosis factor (TNF) and interleukin-1β (IL-1β) with the concomitant up-regulation of anti-inflammatory cytokine IL-10\textsuperscript{249}. CO has been demonstrated both to inhibit the release of mitochondrial cytochrome c (thereby preventing induction of apoptosis) and to reduce the expression of pro-apoptotic p53 protein\textsuperscript{357}.

Administration of exogenous CO has been shown to increase the viability of cultured cell lines including pancreatic β-cells, hepatocytes and endothelial cells and can also reduce doxorubicin-induced apoptosis in cardiomyocytes\textsuperscript{267 459-462}. The effects of exogenous CO may be specific to the cell type studied given that no cytoprotection was afforded by CO exposure in gastric cancer or colonic adenocarcinoma cells\textsuperscript{352 417}.

Bile pigment products of heme breakdown are acknowledged as potent antioxidants and inhibitors of the inflammatory complement cascade. The anti-apoptotic effects mediated by HO activity may be due to increased intracellular levels of biliverdin and bilirubin. Biliverdin and bilirubin have been shown to inhibit hepatocyte cell apoptosis both \textit{in vitro} and \textit{in vivo}\textsuperscript{463 464}. Unconjugated bilirubin is capable of scavenging the singlet oxygen molecule and can act as a reducing agent for certain peroxidases including horseradish peroxidase\textsuperscript{235}.

Ferrous iron (Fe\textsuperscript{2+}) is potentially harmful to cells by virtue of its propensity to generate toxic hydroxyl radicals via the Fenton reaction as described above. Hydroxyl radicals cause lipid peroxidation and direct damage to cellular proteins and DNA. Intracellular
Fe$^{2+}$ can be rapidly sequestered by ferritin or removed by ATP-dependent iron exporter pumps to prevent oxidative stress. Ferritin can contribute to the cytoprotective actions of heme oxygenase and shares common induction stimuli with HO-1 protein. Increased cellular levels of heavy-chain ferritin protein produced a marked resistance to cytotoxicity mediated by pro-oxidant hydrogen peroxide ($H_2O_2$) in breast and colon cancer cells. Increased HO-1 activity improved cell viability in malignant oral keratinocytes exposed to nitric oxide by up-regulation of iron regulatory proteins (IRP1 and IRP2), transferrin receptor (TfR), and ferritin protein.

1.3.8 Inflammation and cancer

Acute inflammation is an important physiological response to injury and infection that aims to rapidly restore cellular homeostasis and repair damaged tissue. The process of acute inflammation is characterised by vasodilatation and increased capillary permeability with infiltration of leucocytes into the site of infection to eliminate invading pathogens. Inadequate resolution of inflammation and uncontrolled inflammatory reactions can evoke produce a state of chronic inflammation with potentially pathological consequences. A variety of biochemical processes that are modified during chronic inflammation are implicated in carcinogenesis. There is increasing evidence of a central aetiological role for inflammation in the initiation, promotion and progression of cancer.

Inflammation can contribute to carcinogenesis via a number of mechanisms including: induction of chromosomal instability; stimulation of cell proliferation, metastasis and angiogenesis; alterations in epigenetic events with subsequent inappropriate gene expression; increased DNA damage; a shift in the cellular redox balance towards increased oxidative stress. A range of diverse pro-inflammatory mediators such as cytokines, chemokines, prostaglandins, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and nitric oxide (NO) are over-expressed or abnormally activated to play a fundamental role in the malignant conversion of cells in a chronic inflammatory environment. The primary molecular event connecting inflammation and cancer is the inappropriate transcription of genes encoding inflammatory mediators, survival factors, and angiogenic and metastatic proteins.
A variety of intracellular signalling pathways are inappropriately activated during chronic inflammation to cause disordered function of cell surface receptors, transcription factors, cytosol scaffold proteins and mitogen-activated protein (MAP) kinases. Activation of protein kinases causes propagation of disproportionate growth signals and abnormal expression of pro-inflammatory genes that allow the cells to acquire a malignant phenotype. The downstream activation of several transcription factors including nuclear-factor-κB, hypoxia inducible factor-1α (HIF-1α), signal transducer and activator of transcription (STAT) and activator protein-1 (AP-1) has been implicated in tumour growth, angiogenesis and metastasis\textsuperscript{468,472}. Inflammation can drive carcinogenesis intrinsically as well as via the extrinsic release of pro-inflammatory mediators within the tumour microenvironment. The transcription of genes involved in cell proliferation, resistance to apoptosis, angiogenesis and metastasis is thereby enhanced.

Exposure to environmental toxins or disordered cellular redox regulation causes oxidative or nitrosative stress resulting in local tissue inflammation. Oxidative stress can activate transcription factors to cause deregulated protein expression and lead to carcinogenesis\textsuperscript{477}. The generation of excessive ROS such as superoxide anion, hydroxyl radical and hydrogen peroxide causes DNA damage and can contribute to carcinogenesis by activating oncogenes and inactivating tumour suppressor genes\textsuperscript{478}. The sustained accumulation of reactive oxygen and nitrogen species enables covalent modifications of cellular proteins such as cysteine thiol modification and protein adduct formation that are associated with promotion of inflammation and an increased risk of malignant transformation. Increased oxidative stress leads to the accumulation of reactive aldehydes as a product of cellular lipid peroxidation. These reactive aldehydes can cause further damage via covalent modification of various proteins\textsuperscript{479,480}.

Nitric oxide plays a dominant role in carcinogenesis associated with oxidative or nitrosative stress by direct modification of DNA and inactivation of DNA repair enzymes\textsuperscript{481}. The nitric oxide radical captures superoxide anion to form a powerful oxidant peroxynitrite that is capable of causing direct DNA damage by forming 8-nitroguanine, a useful biomarker of cancers associated with inflammation\textsuperscript{482-484}.
The cellular adaptive response to excessive oxidative stress is the up-regulation of a panel of endogenous antioxidant enzymes including superoxide dismutase and heme oxygenase-1. The antioxidant-response element (ARE) is a consensus sequence located at the promoter regions of genes encoding various cytoprotective enzymes. The redox-sensitive transcription factor Nrf2 binds to the ARE region in response to mild oxidative stress to cause up-regulation of HO-1 protein\textsuperscript{485}. The protective role of Nrf2 against inflammation and oxidative stress may be exploited as a potential target for chemoprevention\textsuperscript{486 487}. Loss of Nrf2 function via gene knockdown or blockade of the Nrf2 activation pathway renders healthy cells vulnerable to inflammatory damage induced by oxidative stress and subsequent malignant transformation. Recent studies indicate however that the activation of Nrf2 can facilitate growth in established tumour cells and encourage chemoresistance\textsuperscript{488 489}. The increased activity of Nrf2-regulated antioxidant proteins such as HO-1 may provide an overall survival benefit to malignant tumour cells.

The link between inflammation and cancer is especially relevant in oesophagogastric cancer with both chronic gastric inflammation and chronic acid reflux damage to the oesophagus predisposing to malignant disease\textsuperscript{472 480}. The induction of genomic instability in cells subjected to chronic inflammation can be demonstrated in the progression of Barrett’s oesophagus to invasive adenocarcinoma\textsuperscript{490}. Analysis of tissue samples in patients with Barrett’s oesophagus and adenocarcinoma arising in a background of Barrett’s oesophagus demonstrated a 64% alteration in chromosome 11 and a 43% change in chromosome 12. These chromosomal instabilities were found in non-dysplastic Barrett’s epithelium that maintained a normal expression of tumour suppressor protein p53\textsuperscript{490}. Therefore the genomic instability associated with persistent tissue inflammation is induced before the inactivation of tumour suppressor genes in patients with Barrett’s metaplasia that progresses to dysplasia and invasive adenocarcinoma.
1.4 Study Aims and Objectives

1.4.1 Hypotheses of study

Oxidative stress plays an important role in the development and progression of oesophagogastric cancer. The heme oxygenase system has a critical role in the adaptive cellular responses to oxidative stress and chronic inflammation. Alterations in HO activity may have an important role in the cell biology of upper gastrointestinal tract cancer. Modifications in the heme oxygenase pathway may influence cellular signalling pathways with the ability to regulate cell proliferation and apoptotic activity. Changes in heme oxygenase activity and the production of its downstream products can affect the proliferation of oesophagogastric cancer cells.

1.4.2 Aims of study

1. To characterise constitutive and inducible HO protein expression in malignant and benign oesophagogastric cell lines.
2. To explore the mechanisms of HO activation and the influence of pro-inflammatory and antioxidant mediators on HO expression.
3. To investigate the role of oxidative stress in the regulation of epithelial cell cycle kinetics, apoptosis and proliferation.
4. To investigate the effects of modified HO protein expression and enzymatic activity on epithelial cell cycle kinetics, apoptosis and proliferation.
5. To examine the specific effects of downstream products of heme oxygenase activity and their potential regulatory roles in the HO pathway and influence on cancer cell proliferation.
CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Culture Experiments

2.1.1 Cell Culture Techniques

The role of the heme oxygenase system was investigated by experiments carried out on the following commercially obtained human cell lines: OE-33 oesophageal adenocarcinoma; HET-1A normal oesophageal squamous epithelium; AGS gastric adenocarcinoma; and HGC-27 metastatic gastric adenocarcinoma. OE-33, AGS and HGC-27 were purchased from the European Collection of Cell Cultures (ECACC) and HET-1A obtained from the American Type Culture Collection (ATCC).

The OE-33 cell line was established from a poorly-differentiated adenocarcinoma of the lower oesophagus of a 73 year old female patient. These cells are known to be tumourigenic in nude mice. The culture medium used was RPMI 1640 with 2 mM L-Glutamine and 10% foetal bovine serum as recommended, with added antibiotics 100 mgmL⁻¹ streptomycin and 100 IUmL⁻¹ penicillin. The AGS cell line is derived from an adenocarcinoma of the stomach of a 54 year old Caucasian female with no prior anticancer treatment. The culture medium used was Ham's nutrient mixture F12 with 2 mM L-Glutamine and 10% foetal bovine serum (FBS) with added penicillin and streptomycin at 1% concentration. The HGC-27 cell line originates from a metastatic lymph node deposit of a patient with undifferentiated mucin-producing gastric adenocarcinoma. The recommended culture medium is Dulbecco’s Modified Eagle’s Medium (DMEM) with 2 mM L-Glutamine and 10% foetal bovine serum. RPMI, Ham and DMEM culture mediums were purchased from Sigma-Aldrich as were all antibiotic solutions, glutamine and FBS.

The HET-1A cell line is derived from oesophageal autopsy tissue of a 25 year old male and has been shown to be non-tumourigenic in athymic, nude mice for more than 12 months. The growth of these cells is inhibited by foetal bovine serum, and the culture medium used was bronchial epithelial basal medium (BEBM) plus additives including growth supplements as provided (BEGM SingleQuots, Cambrex Bioscience, UK). The HET-1A cells were grown in flasks precoated with a mixture of 0.01 mgmL⁻¹ fibronectin, 0.03 mgmL⁻¹
bovine collagen type I (PureCol) and 0.01 mg/mL bovine serum albumin dissolved in culture medium.

Cells were grown in adherent cultures using tissue culture flasks with a surface area of 75 cm² and a hydrophobic filter cap (T75 Nunc Easyflasks, Thermo Fisher Scientific). All culture vessels were kept in a humidified incubator maintained at 5% CO₂ and 37°C. All manipulations of the cultured cells were carried out in a Class II biosafety hood using a sterile technique. All surfaces were cleaned with a 70% industrial ethanol solution. All solutions and growth media were warmed to 37°C prior to use. Growth medium was renewed every 48-72 hours for all cell lines, using a volume of 12-15 mL per flask. Cells were assessed by light microscopy and subcultured when 70-80% confluent to avoid possible senescence associated with a higher cell density.

For passaging cells, after discarding the spent medium the cell layer was briefly rinsed with warm phosphate-buffered saline to remove all traces of serum to avoid inhibition of trypsin enzyme. The monolayer was then treated with 3 mL of a mixture of 0.25% (weight/volume) trypsin and 0.1% EDTA (ethylenediaminetetraacetic acid) and left in the incubator for up to 10 minutes, until dispersion of the cell layer was observed using the inverted microscope. A further 3 mL of complete growth medium was then added to quench the trypsin reaction and the cells aspirated by gentle pipetting, transferring the suspension into a 15 mL conical centrifuge tube. The cell suspensions were centrifuged at 300 x g for 6 minutes, the resulting cell pellet resuspended in medium and appropriate aliquots of the new cell suspension added to the desired culture vessels, maintaining a subcultivation ratio of 1:3. For the HET-1A cells grown in serum-free medium, the trypsin-EDTA solution contained 0.5% polyvinylpyrrolidone (PVP) added. Once the cells were detached from the flask the trypsin was neutralised with 0.1% soybean trypsin inhibitor. The cells were resuspended and centrifuged as above and dispensed into coated flasks or plates.

Cells were stored as a 1 mL frozen suspension in cryovials at -70°C. Cells were preserved by resuspending the cell pellet in 90% complete growth medium with 10% dimethyl sulfoxide (DMSO) solvent. The HET-1A cells needed a separate freeze medium consisting of Leibovitz’s L-15 medium with 2 mM L-glutamine and 10 mM HEPES (4-(2-hydroxyethyl)-
piperazineethanesulfonic acid) buffer supplemented with 1% PVP, 10% foetal bovine serum and 7.5% DMSO. When taking cells out of storage, the frozen suspension was thawed quickly at 37°C and added to 5 mL of complete growth medium. This volume was centrifuged and the cell pellet resuspended in fresh medium to extract the DMSO solvent.

2.1.2 Cell seeding and cell counting technique

To achieve correct cell seeding, a flask of cells was passaged and the resulting cell pellet resuspended to a volume of 1 mL. A 10 μL volume of this well-mixed suspension was added to an equal volume of 0.4% trypan blue solution (Sigma-Aldrich) and made up to a 100 μL with medium, being allowed to stand for up to 5 min at room temperature. Trypan blue is a diazo dye that crosses the cell membrane of dead cells but is not taken up by viable cells with an intact membrane. After mixing thoroughly again with gentle pipetting to ensure a representative sample, 10 μL was placed on a haemocytometer and the slide examined under the microscope. The number of viable cells was counted as the average of cells in 4 large squares (area = 1 mm²) on the haemocytometer grid. The number of cells per mL can then be calculated with the following equation:

Cells per mL = [average cells in one large grid square] x 10⁴ x dilution factor

An appropriate dilution of the cell suspension was chosen with regard to the number of cells likely to be counted. Under-dilution of the sample would make the cells crowded and difficult to count, while a sample size that is too dilute does not contain enough cells to adequately represent the concentration in the original mixture. For seeding out cells from a 70-80% confluent T75 flask, the cell suspension of 1 mL will contain an adequate number of cells to allow a dilution factor of 10 (10 μL into 100 μL). However when counting cells per well for a 24-well plate, the number of cells was anticipated not to be sufficient for a volume of 1 mL. In this situation, after first discarding the well medium and rinsing briefly with warm PBS, 125 μL of trypsin-EDTA solution was added to the well. An equal volume of 125 μL was then added and the well gently scraped with a cell scraper. The sample volume of 250 μL was aspirated by pipette and transferred into an eppendorf tube. From this sample, 10 μL was placed on the haemocytometer and the cells counted as above. Because the original volume is 250 μL the cells per well is calculated as the average number of cells in one large grid square multiplied by 10⁴ and
divided by 4. Similarly, when counting cells per well for a 96-well plate, 31.25 μL of trypsin-EDTA is added to the well to be followed by 31.25 μL of trypan blue to give a total volume of 62.5 μL and the cells per well is calculated by the average number of cells in one large grid square multiplied by $10^4$ and divided by 16.

2.1.3 Measurement of cell growth: Cell counts and MTT assay technique

In addition to cell counts, cell growth was studied by the use of Thiazolyl Blue Tetrazolium Bromide (MTT) assay. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is a yellow solution that is reduced to water-insoluble purple formazan by mitochondrial dehydrogenases of living cells. The crystals are solubilised with acidified isopropanol and the intensity measured with standard colorimetric assay at a wavelength between 500 and 600 nm. The quantified absorbance should be directly related to the number of viable cells. MTT was prepared to a concentration of 0.25 mg per mL in complete medium and sterilized through a 0.2 micron pore size filter (Sartorius Minisart). For 96-well plates, a volume of 100 μL was added and for 24-well plates, 250 μL was added to the well once the well medium was discarded. The plate was then left for 2 hours to allow formation of purple formazan crystals. After a 2-hour incubation period the MTT reaction medium was discarded and the purplish-blue MTT formazan crystals dissolved by adding 0.04 molL⁻¹ HCl-Isopropanol. An equal volume of solubilisation buffer was added, mixed by careful pipetting and incubated for 30 min to dissolve the crystals. The solubilisation buffer consisted of 5 g Triton X-100 detergent and 430 μL hydrochloric acid made up to 50 mL with isopropanol. From each well, 200 μL of the dissolved coloured solution was transferred to a 96-well plate and the absorbance measured by spectrophotometer (Multiskan EX, Thermo labsystems) at 562 nm wavelength.

To evaluate the relationship between MTT optical density and number of viable cells, cell seeding experiments were performed with all cell lines seeded in 24-well and 96-well plates at a known number. Cells were seeded at 1000, 5000, 10000, 20000, 40000, 60000, 10000 and 150000 cells per well in a 24-well plate. Cell counts using trypan blue exclusion and MTT assay were performed at 24, 48 and 72 hours.

The number of viable cells counted from each well showed a strong correlation with the number of cells seeded in the well (Pearson correlation 0.992; $p < 0.001$). The number of
dead cells counted was far fewer and was not related to the number of cells seeded. The results are displayed in Appendix II. Absorbance measured by MTT assay demonstrated a strong positive correlation to the number of cells seeded per well (Pearson correlation 0.967; \( p < 0.001 \)). This relationship was evident for both AGS and HGC-27 cell lines seeded into 24-well plates and for AGS cells seeded into 96-well plates (Figures II.1b & II.2, Appendix II).

The results support the MTT and cell counting techniques as a valid measure of cell proliferation for cells seeded in a 24-well or a 96-well plate.

2.1.4 Obtaining growth curves for cell lines under different conditions

To investigate growth of the four available cell lines under various conditions, cells were seeded at the same number and grown using recommended culture media with or without serum. Cell counts and MTT assay were performed at 24, 48 and 72 hours to obtain growth curves for each cell line.

Growth curves generated by both cell counting and MTT assay demonstrate exponential growth for all cell lines under the specified conditions. Standard curves generated by MTT assay for all cell lines studies are displayed in Appendix III. There was significantly increased growth observed in malignant cell lines studied compared to benign epithelial oesophageal cell line HET-1A (Figures III.1 – 5, Appendix III).

2.2. Investigating Protein Expression with Western Blotting

Expression of heme oxygenase proteins in each cell line was assessed under basal and experimental conditions using protein electrophoresis and Western blotting techniques.

2.2.1 Protein Assay

The protein content in cell lysates was measured against a known standard concentration. Cell lysates were collected by adding 50 µL of lysis buffer to each well, collecting 3 wells together with a further 50 µL lysis buffer to wash the wells. The lysis buffer was made up as sodium dodecyl sulfate (SDS) dissolved in phosphate-buffered saline to make a 1% solution. To inhibit proteolytic activity in the cell lysates, one complete protease inhibitor tablet (Roche) was added per 10 mL of 1% SDS solution. Cell
lysates were stored at -20°C for future use. A solution of bovine serum albumin (BSA) was diluted with 1% SDS with protease inhibitor (lysis buffer) solution to make concentrations of 0.4 – 1.6 mgmL\(^{-1}\) for the protein standard. The first step was to decide what concentration of cell lysate to use to ensure that the sample fell within the range of the protein standard. Initial assay was performed on neat samples and those diluted with lysis buffer at a ratio of 1:1, 1:2 and 1:4. Protein assay was performed using a bicinchoninic acid (BCA)-based assay kit (Pierce BCA Protein Assay, Thermo Scientific). Samples were assayed in duplicate, with 10 µL of sample and 100 µL of working reagent per well, in a 96-well plate. Visual assessment of colour change was made with the initial samples to choose a concentration of lysate sample whose colour was within the range of the BSA standard. For the formal protein assay of the chosen concentration, the plate was read by spectrophotometer at 562 nm wavelength. The absorbance value was plotted against the BSA concentration to produce a standard curve using Microsoft Excel. A curvilinear regression was applied using a 2-parameter polynomial equation calculated by the spreadsheet program. This equation was then used to determine the protein concentration of the samples with known absorbance value, accounting for any dilution factor used. Once the µg per µL concentration is known for the sample, then an appropriate volume for 30, 20 or 10 µg of protein can be calculated.

2.2.2 Gel electrophoresis

Protein electrophoresis was performed using the NuPage Bis-Tris gel system. These are polyacrylamide gels that are buffered to a pH of 6.4, with a separating gel that operates at pH 7.0. The protein samples are loaded onto the less dense stacking matrix of the gel. With the use of SDS running buffers and a sample reducing agent, the proteins are unfolded and denatured with breaking of the disulfide cysteine bonds between polypeptide chains. The tertiary globular protein is broken down to its single-stranded primary structure, and coated in SDS detergent with an overall negative charge in proportion to its mass. A positive charge at the distal end of the gel pulls the protein down through the denser resolving matrix of the gel, in which the proteins are separated according to their molecular weight. The separated proteins are then transferred onto a nitrocellulose membrane for immunoassay. At a neutral pH the protein structure is more stable and proteins are kept denatured, whereas at a higher pH cysteine bonds can re-
form between protein chains. Furthermore, at a higher pH proteins are potentially subjected to chemical modifications such as deamination and alkylation that may reduce band sharpness and alter the efficiency of transfer onto the membrane. The included reducing agents move into the gel ahead of the protein stack to maintain a reducing environment.

During preparation of the samples to load onto the gel, the volume of lysate containing an equal amount of protein (at least 15 µg) was calculated for each experiment sample and made up to a total volume of 15.5 µL with 1% SDS solution. The maximum recommended load volume was 25 µL per well. A volume of 5.5 µL 1% SDS was added to 10 µL of full-range rainbow molecular weight marker (GE Healthcare) to run with each gel. Sample buffer (6.25 µL) and ultrapure water (0.75 µL) was added to each sample. The NuPage sample buffer uses lithium dodecyl sulfate (LDS) that stays in solution better than in the sodium form SDS. The hydrophobic dodecyl group interacts with hydrophobic amino acids in the core of proteins, disrupting their 3-D structure and transforming globular proteins into linear molecules now coated with negatively charged lithium groups. The sample buffer contains glycerol to increase the density of the sample to be loaded and hence maintain the sample at the bottom of the well, restricting overflow and uneven gel loading. To enable visualization of the migration of proteins Coomassie G250 and Phenol Red are included in the sample buffer as tracking dyes. These are small, anionic molecules that migrate faster than any component in the mixture to be separated and provide sharp viewing of the migration front to monitor the separation progress. Reducing agent was added to each sample (2.5 µL) and mixed by vortexing just prior to heating at 70°C for 10 minutes. The reducing agent contains 0.5M dithiothreitol (DTT) that reduces the disulfide bridges to break up quaternary protein structure and also prevents cysteine residue binding, working effectively under the denaturing conditions described. The samples were vortexed again after heating and centrifuged with pulse spin at 300 x g for 5 – 10 seconds prior to loading onto the gel.

The NuPage gels were assembled using the XCell SureLock mini-cell system (Invitrogen) according to manufacturer instructions. Normally 2 gel cassettes were run together. If only one gel was run, a buffer dam was placed between the buffer core and the tension wedge. The inner buffer chamber was filled with 200 mL of 1x NuPage MOPS (3-N-
morpholino-propane sulfonic acid) SDS running buffer with 500 µL antioxidant added to prevent re-oxidisation of the reduced proteins as the gel runs. The reducing agent DTT does not migrate with the sample through the gel in the neutral pH environment of the NuPage gels, tending instead to remain at the top of the gel. Although disulfide bonds are more stable at neutral pH and less likely to reoxidize than in a gel with a higher pH, antioxidant is required to avoid potential reoxidation resulting in slightly diffuse bands. NuPage antioxidant (a proprietary reagent) migrates with the proteins during electrophoresis maintaining their reduced state, and also protects sensitive amino acids such as tryptophan and methionine from oxidizing. After making sure there were no leaks from the buffer core, the outer chamber was filled with 600 mL of 1x MOPS running buffer. The prepared 25 µL volume samples were then loaded carefully into the wells using a fine pipette. The gels were then run at a constant voltage of 200 V for 1 hour, with an expected starting current of 100-115 mA per gel.

2.2.3 Western Blotting Gel Transfer

The separated proteins were transferred onto a Hybond ECL Nitrocellulose membrane (GE Healthcare) using the XCell II Blot module (Invitrogen) system. Transfer buffer was made up containing methanol 80 mL, 20x NuPage Transfer Buffer 20 mL to maintain the neutral pH environment, 400 µL of NuPage antioxidant to maintain the reduced protein state and ultrapure water to a total volume of 400 mL. Transfer buffer with 20% methanol was used for transferring two gels within a single blot module and 10% methanol for a single gel. The nitrocellulose membrane was soaked in the transfer buffer for several minutes prior to use. The gels were removed from the cassettes and placed between pre-soaked filter paper and the prepared membrane. The proteins within the gel have a negative electrical charge conferred by the SDS and are induced to travel towards the positively-charged electrode of the applied electrical field. The gel and nitrocellulose membrane are tightly sandwiched between blotting pads and filter paper with careful arrangement of the gel and membrane ensuring no air bubbles are trapped between them. The moving proteins are stopped by the nitrocellulose membrane and bound to its surface, thereby blotting the proteins from the gel.
The blot module was secured in the XCell SureLock system, making sure that the gel-membrane sandwich was covered in transfer buffer and filling the outer chamber with deionised water to act as a heat sink during the transfer run. The lid was secured and connected to electricity to run at a constant voltage of 30 V for 1 hour, with an expected starting current of 170 mA per gel. After the transfer run was complete, the stack was disassembled by reversing the assembly order and carefully peeling the membrane off the gel. The membrane was immediately placed in a blocking solution made up of phosphate buffered saline (PBS) with 0.1% added Tween detergent and 5% skimmed milk. The membrane was left in blocking solution for at least 1 hour at room temperature or overnight at 4°C with gentle agitation. The milk proteins bind to all the available sites on the nitrocellulose membrane where the target proteins from the gel transfer have not attached, thereby preventing non-specific binding of the probing antibodies to places other than the specific binding sites of the target protein. In addition, any unbound proteins are washed off into the blocking buffer. The nitrocellulose membrane has a high affinity for binding protein, so blocking is required to eliminate false positives in the final product of the blot and to produce clearer results.

Figure 2.2.1: Arrangement of the gel-membrane sandwich for transfer of 2 gels
2.2.4 Probing the membrane and generating a signal

The nitrocellulose membrane was probed for heme oxygenase proteins using specific monoclonal antibodies targeted against HO-1 (BD Biosciences) and HO-2 (Santa Cruz). Antibodies bound to protein were detected by a secondary anti-mouse antibody covalently conjugated to horseradish peroxidise (DAKO). The membrane was incubated with the antibodies in blocking buffer (PBS + 0.1% Tween with 5% low-fat milk) for 1 hour at room temperature. After incubation the membranes were washed with PBS + 0.1% Tween in cycles of 3x 5 mins and 1x 15 mins to wash away all unbound antibody. Primary monoclonal mouse anti-HO-1 antibody was diluted to a 1 in 500 concentration, the primary anti-HO-2 antibody to 1 in 100 and the secondary anti-mouse antibody used at 1 in 3000 dilution. Membranes were also probed for β-tubulin protein to check for equal protein loading across the wells using monoclonal anti-β-tubulin antibody produced in mouse (Sigma) at a 1 in 1000 dilution.

A signal was generated using the ECL Plus chemiluminescent reagents (GE Healthcare) with oxidation of the luminol substrate producing a sustained light emission detected on radiography film (Hyperfilm, GE Healthcare). Autoradiographs were developed through an MI-5 X-ray film processor (Jencons). HO-1 has a molecular weight of 32 kilo Daltons (kDa), HO-2 has a molecular weight of 36 kDa while that of β-tubulin is 55 kDa.

2.3 Oxidative Stress Experiments

Hydrogen peroxide (H₂O₂) is a powerful oxidant that denatures proteins by reacting with available thiol groups and methionine residues. The oxidative properties of H₂O₂ have enabled its use as an antiseptic and antimicrobial agent for many years. It is a relatively stable reactive oxygen species that is capable of diffusing across cellular membranes. By interaction with free ferrous iron (Fe²⁺), H₂O₂ is readily converted to the highly reactive hydroxyl free radical (OH⁻) capable of indiscriminate oxidative damage. There is recent evidence to suggest a physiological role of H₂O₂ at lower concentrations in cell signalling pathways involved with acute inflammation. In smooth muscle cells and vascular endothelial cells, hydrogen peroxide has been shown to activate intracellular NADPH oxidase resulting in the production of superoxide anion free radical and consequential oxidative damage. *In vitro* cellular studies examining the potential...
pathological effects of H₂O₂ commonly employ the oxidant in the concentration range of 20 – 250 µM.

*N*-acetylcysteine (NAC) is a well-known pharmacological agent with clinical indications including adjuvant mucolytic therapy for certain respiratory conditions, emergency treatment of paracetamol overdose, and to prevent the onset of acute renal failure with administration of intravenous radiographic contrast in patients with renal impairment. *N*-acetylcysteine is derived from the amino acid cysteine, and is a substrate for intracellular glutathione produced *in vivo*. Antioxidant properties are conferred by available thiol groups that break disulfide bonds linking protein chains and bind directly to free radicals.

The aim of the experiments was first of all to establish the basal expression of heme oxygenase enzyme isoforms in cultured cell lines. The influence of modulating oxidative stress on the proliferation of gastro-oesophageal cancer cells and their expression of heme oxygenase isoenzymes was investigated.

Oxidative stress was simulated by treating the cultured cells with hydrogen peroxide for 24 hours, at concentrations of 0.2 – 200 µM. Hydrogen peroxide was available as a solution at 10 Molar (M) concentration. This solution was diluted in Ham culture medium for a working stock at 2 mM concentration, and appropriate serial dilution was performed to give stocks of 2, 20, 100, 200, 500 µM and 1 and 2 mM at sufficient volumes.

The influence of antioxidant *N*-acetylcysteine on cell growth and heme oxygenase expression was studied by exposing cells to concentrations of 0.1 – 20 mM over 24 hours. NAC was obtained as a water-soluble white powder with molecular weight 163.2g. To make a stock solution at 200 mM concentration 0.163g was weighed and dissolved in 5 mL of medium. The stock was sterile filtered and protected from the light. Serial dilution of this solution gave further stocks at 1, 2.5, 5, 10, 50 and 100 mM.

Cell lines were seeded into 24-well plates such that the wells were 50% confluent at the time of adding reagents. The medium in each well was replaced with 450 µL of serum-complete medium warmed to 37°C. A volume of 50 µL stock reagent was added to the corresponding well. For the control wells, 50 µL of medium was added at the same time.
as adding the reagents. Cells were thereby treated with \( \text{H}_2\text{O}_2 \) at doses of 0.2, 2, 10, 20, 50, 100 and 200 µM and NAC at doses of 0.1, 0.25, 0.5, 1, 5, 10 and 20 mM including triplicate wells for each experiment dose. After 24 hours the reagent medium was washed out and replaced with 250 µL of MTT medium, performing MTT assay to assess cell growth.

Experiments were extended to 72 hours and to 168 hours (7 days) with AGS and OE-33 cell lines to further assess observed effects with selected reagent doses. Expression of heme oxygenase proteins HO-1 and HO-2 was determined by Western blotting and heme oxygenase activity assay performed on representative experiment samples. Apoptosis studies were performed to evaluate possible mechanisms of the observed reductions in cell growth using flow cytometry techniques and caspase activity assay.

2.4 Pharmacological modulation of Heme Oxygenase activity

Cell lines were treated with Hemin, an iron-containing porphyrin used as a substrate analogue to increase heme oxygenase activity\(^{125, 165}\). Cells were treated with Zinc Protoporphyrin-IX (ZnPP), a normal metabolite that is formed in trace amounts during heme biosynthesis which is a competitive inhibitor of all isoforms of the heme oxygenase enzyme\(^{498-500}\). Hemin powder was obtained from Sigma chemicals and zinc protoporphyrin purchased from Frontier Scientific. These compounds were found to have limited solubility in dimethyl sulfoxide (DMSO) but were readily soluble in sodium hydroxide (NaOH) at 1 mM concentration. To evaluate the effects of the chosen vehicle for hemin and zinc, an experimental plate was first set up to include NaOH at 1 mM concentration at time points of 24, 48 and 72 hours. Cells were then exposed to hemin at concentrations of 2 – 200 µM and treated with zinc PP at concentrations of 0.1 – 40 µM.

Hemin powder has a molecular weight of 651.94g. A stock solution of 2 mM concentration was made with hemin at 0.0013 gmL\(^{-1}\). The powder was first dissolved in a certain volume of 1M NaOH and further diluted with culture medium such that the hemin content was 0.0013 gmL\(^{-1}\) and the concentration of NaOH 10 mM. The stock was sterile filtered and protected from light. Serial dilution of the stock was performed to create stocks at 10x the desired concentration in the well. A volume of 50 µL stock reagent was
added to 450 µL in the corresponding well. Thus the maximum final concentration of NaOH in the wells was 1 mM.

Zinc powder has a molecular weight of 626.03g. A stock solution of 4 mM concentration was made with zinc at 0.0025 g mL\(^{-1}\). The initial stock was dissolved in NaOH and filtered as above, with subsequent serial dilution to obtain the appropriate stock concentrations for the experiment plate.

2.5 Chemotherapy studies

2.5.1 Cisplatin

The chemotherapeutic agent \textit{cis}-diamminedichloroplatinum(II), \textit{cis}-DDP, or cisplatin is one of the most effective chemotherapeutic agents used in the management of a variety of human cancers\(^{501}\). Cisplatin is a key component of treatment regimens for carcinoma of the oesophagus. Response rates of up to 40% can be achieved using single-agent cisplatin in patients with advanced disease while administration of cisplatin with continuous intravenous infusion of fluorouracil (5-FU) doubled the response rate reported in the EORTC trial (19% versus 35\%)\(^{502-504}\).

Cisplatin is also the mainstay of combination therapy regimens for gastric cancer. The cisplatin-based combination ECF (epirubicin, cisplatin and 5-FU) demonstrated a significant survival benefit at 5-years (36\% for perioperative chemotherapy versus 23\% in surgery alone)\(^{505}\).

2.5.2 Fluorouracil

Fluorouracil (5-FU) is an anti-metabolite chemotherapeutic agent that is a pyrimidine analogue. Fluorouracil remains one of the most potent chemotherapeutic agents for solid tumours even at limited response rates of between 10 – 30\%\(^{506,507}\).

2.5.3 Chemotherapy study protocol

Initial chemotherapy experiments were performed to establish the median lethal dose (LD\(_{50}\)) with cytotoxicity assays using Trypan blue exclusion and the equivalent half maximal inhibitory concentration (IC\(_{50}\)) with MTT assay to measure cell proliferation.
Dose response growth curves were obtained to obtain the IC\textsubscript{50} concentration of chemotherapeutic agent causing 50\% inhibition of cell growth. For the cytotoxicity assay cells were seeded at optimal density in serum-complete media into 12-well plates and returned to the incubator for 24 hours to establish monolayer cell adherence. Media supplemented with a range of concentrations of the chemotherapeutic agents cisplatin and 5-FU were then added to the plate and the plate returned to the incubator for either 48 hours or 72 hours. Cells were harvested from three control wells prior to adding the reagents and counted using Trypan blue exclusion to assess cell viability at time = 0. Further wells were similarly harvested and counted at appropriate time to obtain cell viability at 48 and 72 hours with various reagent concentrations. All subsequent cell experiments were based on the IC\textsubscript{10} and IC\textsubscript{50} concentrations of the chemotherapy agents representing the cytotoxic dose resulting in a 10\% and 50\% decrease in viable cell numbers respectively at 48 hours so that time was the only variable. Cell proliferation curves were obtained using equivalent ranges of reagent doses in 24-well plates and performing MTT assay after 24 hours for control wells (t = 0) and 48 and 72 hours for treated wells.

Cytotoxicity and cell growth assays were repeated to determine the influence of the heme oxygenase pathway and oxidative stress response. Cells were treated with a range of Cisplatin doses between 0.5 – 30 µM (0.15 µgml\textsuperscript{-1} – 9 µgml\textsuperscript{-1}) and 5-FU doses between 1 – 50 µM (0.13 µgml\textsuperscript{-1} – 6.5 µgml\textsuperscript{-1}) in the presence or absence of NAC 10 mM to determine the effect of the antioxidant on IC\textsubscript{10} and IC\textsubscript{50} chemotherapeutic concentrations.

Growth curves were extended to 144 hours to determine the latent toxicity of the chemotherapeutic agents with and without NAC exposure. Cells were plated in 24-well plates and treated for 48 hours with IC\textsubscript{10} and IC\textsubscript{50} doses of each chemotherapy agent alone and in combination with NAC. Media was then removed and the cells washed with warm PBS (37°C) to remove any traces of residual chemotherapy. Fresh serum-complete media was then added to each well and the plates returned to the incubator. MTT assays were performed at 24, 48, 72, 96, 120 and 144 hours to determine any recovery of cell growth after exposure to chemotherapy and evaluate the influence of heme oxygenase reagents. Spent media was changed to fresh serum-complete media after every 48 hours to encourage cell growth. Each experiment was repeated in triplicate with six replicate
wells for each experiment reagent and time point with the total time for each experiment extending to 192 hours (8 days).

Previous studies on AGS cell lines using the same chemotherapeutic agents provided a suitable range for establishing IC$_{10}$ and IC$_{50}$ doses. Published IC$_{10}$ and IC$_{50}$ doses for cisplatin are 0.156 and 5 – 10 µg/mL and those for 5-FU 0.156 and 10 – 20 µg/mL respectively$^{508,509}$.

2.5.4 Preparation of reagents

Cisplatin was supplied by Sigma Aldrich as cis-Diammineplatinum(II) dichloride crystalline powder with molecular weight 298.029g. A stock solution was prepared by dissolving cisplatin powder in 0.9% Normal Saline solution to a 10 mM concentration of 3 mg/mL. Stock solution was stored at 2°C protected from light to avoid precipitation. A range of concentrations was prepared by serial dilution so that the final concentration in experiment wells was between 0.15 – 15 µg/mL [0.5 – 50 µM] per well$^{509}$.

Cisplatin [FINAL]  
50 µM = 15 µg/mL  
30 µM = 9 µg/mL  
10 µM = 3 µg/mL  
5 µM = 1.5 µg/mL  
1 µM = 0.3 µg/mL  
0.5 µM = 0.15 µg/mL

Fluorouracil (5-FU) was supplied by Sigma Aldrich as a white powder with molecular weight 130g. Stock solution of 100 mM was prepared by dissolving supplied dry powder in DMSO to a concentration of 13 mg/mL. Stock solution was stored at 2°C protected from light with the solution expected to be stable for at least 4 months under these conditions. An initial range of concentrations was prepared by serial dilution so that the final concentration in experiment wells was between 0.13 – 78 µg/mL [1 – 600 µM] per well$^{509}$. The final DMSO concentration per well was always less than 0.1%.

5-FU [FINAL]  
600 µM = 78 µg/mL  
200 µM = 26 µg/mL  
100 µM = 13 µg/mL  
50 µM = 6.5 µg/mL  
25 µM = 3.25 µg/mL  
5 µM = 0.15 µg/mL  
1 µM = 0.13 µg/mL
2.6 Targeted knockdown of HO mRNA

2.6.1 Short Interfering RNA Technology

Small interfering or short interfering RNA (siRNA), are double-stranded RNA molecules of 20-25 nucleotides in length that interfere with the expression of a specific gene. Small interfering RNA strands have complementary antisense nucleotide sequences to the targeted cellular messenger RNA strand. When introduced into a cell or organism, the siRNA strand is recognized as exogenous genetic material and activates the RNA interference (RNAi) pathway. The RNA interference pathway is a naturally occurring mechanism of post-transcriptional gene silencing that inhibits the translation of specific gene expression. The Nobel Prize in Physiology or Medicine was won by Fire and Mello in 2006 for their discovery of RNA interference.\(^{510,511}\)

In this process the antisense strand of the siRNA duplex becomes integrated with several proteins to form a nuclease complex known as the RNA-induced silencing complex (RISC). The active components of an RNA-induced silencing complex are endonucleases called argonaute proteins. The siRNA duplex unwinds and then pairs by means of its antisense strand to target messenger RNAs (mRNAs) that carry a high degree of nucleotide sequence complementary to the siRNA. Endonucleases within the RISC then proceed to cleave the associated mRNA at sites not bound by the siRNA. The mRNA is broken down into smaller portions that can no longer be used as a template for translation into protein. This inactivated mRNA releases the RISC and is further degraded by cellular nuclease enzymes. The guiding antisense strand is protected from degradation and can direct the cleavage of multiple mRNA molecules. Inhibition of the translation process ultimately results in a loss of targeted protein expression.

RNAi has a unique degree of sequence specificity: introduction of a single nucleotide base mismatch in the siRNA structure nullifies the RNA interference effect.

Chemically synthesized siRNAs can be introduced into mammalian cell cytoplasm by distorting the integrity of the cell membrane. Lipofectamine transfection creates reparable pores in the cell membrane which facilitate gentle siRNA uptake. Transfection of an exogenous siRNA can be problematic because the gene knockdown effect is only
transient, particularly in rapidly dividing cells. Other problems include off-target effects that occur when the siRNA sense strand has a complementary base sequence to untargeted mRNA and becomes incorporated into a RISC complex and activates the RNAi pathway. SiRNAs can also be degraded over time with exposure to cellular nucleases. Furthermore, certain siRNAs can induce non-specific cellular stress response pathways including the interferon response that can alter the cellular phenotype.

2.6.2 siRNA transfection

The mode of delivering the siRNA into the cells involved using a positively-charged lipid transfection reagent, Lipofectamine (Invitrogen). The siRNA nucleic acid strand is encapsulated into liposomes with the resulting positive surface charge enabling interaction with the negatively charged cell membrane. The liposome-nucleic acid complex enters the cell by a process of endocytosis and diffuses through the cytoplasm. The Lipofectamine™ RNAiMAX reagent was chosen as it is specially designed and manufactured for delivery of siRNA double-stranded molecules. All experiments used Opti-MEM reduced serum medium to dilute siRNA duplexes and Lipofectamine prior to forming a transfection complex, in accordance with manufacturer recommendations. Antibiotics were not added to cell culture media during transfection as this may cause cell death. A forward transfection protocol was employed for all siRNA experiments, with cells being plated in the wells and the transfection mix prepared and added the next day.

2.6.3 siRNA and Negative Universal Control

To ensure that any observed results are due to effects mediated by the targeted RNAi molecules and not an artefact of the delivery method, a negative control needs to be included in the siRNA experiment.

For targeted knockdown of HO-2 gene expression, three pairs of Human Stealth™ Select HO-2 siRNA (HMOX2) were obtained from Invitrogen. Stealth™ RNAi uses proprietary chemical modifications to provide higher specificity and increased stability in cell culture, reducing off-target effects with a reduced potential to activate cellular stress response pathways. HMOX2 was obtained as RNA duplexes of 25 base pairs with low GC content. The recommended predesigned negative universal control (NUC) for experiments using
Stealth™ RNAi was obtained from Invitrogen, featuring the same base pair length and low GC content to match that of the experimental siRNA. The NUC sequence is not homologous with any known vertebrate gene, and does not induce non-specific cellular stress responses according to information from the supplier.

**2.6.4 RNA purification**

Purification of total RNA was performed using the RNeasy mini-kit supplied by QIAGEN. This uses a column-based solid phase extraction method to quickly purify nucleic acids, and relies on the fact that nucleic acid binds by adsorption to the solid phase (silica) depending on the pH and the salt content of the buffer. The selective binding properties of a silica-based membrane are combined with the speed of microspin centrifugation. A high-salt buffer system allows up to 100 µg of RNA longer than 200 bases to bind to the RNeasy silica membrane.

Initially, 10 µL of Beta-Mercaptoethanol (β-ME) was added to every 1 mL of RNeasy lysis buffer (RLT buffer). Medium was removed from the experiment well and a volume of 350 µL RLT buffer/β-ME was added to lyse the cell monolayer. This was mixed well by pipetting to homogenise the sample and transferred to an eppendorf. These samples were frozen at -20°C for future use. The RLT buffer contains guanidine thiocyanate that is highly denaturing, immediately inactivating RNases to ensure purification of intact RNA.

Defrosted samples were processed according to QIAGEN protocol to yield total RNA. Ethanol was added to the defrosted lysate sample to provide appropriate binding conditions, and the sample then applied to the RNeasy silica membrane in a spin column. Total RNA binds to the membrane and contaminants are efficiently washed away by treatment with wash buffer and repeated centrifugation. High-quality RNA was then eluted in 60-100 µL of RNAse-free water.

Isolated RNA was treated with Turbo DNase enzyme (Ambion Applied Biosystems) to digest contaminating DNA. Purified RNA was checked for DNA contamination using the polymerase chain reaction with GAPDH primers.
2.6.5 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a technique widely used in molecular biology to amplify specific regions of a DNA strand (the DNA target) by enzymatic replication in vitro. The heat-stable DNA polymerase, such as Taq polymerase, assembles a new DNA strand from nucleotide DNA building blocks, using single-stranded DNA as a template and DNA oligonucleotides (DNA primers) required for initiation of DNA synthesis. As PCR progresses, the DNA thus generated is itself used as template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified.

Components of the PCR reaction and reagents required include: the DNA template that contains the DNA region (target) to be amplified; two primers that have a complementary sequence to the DNA at the 5’ (five prime) or 3’ (three prime) ends of the DNA target region; Taq polymerase with an optimum temperature at approximately 70°C; Deoxynucleoside triphosphates (dNTPs), the building blocks from which the DNA polymerase synthesizes a new DNA strand; divalent magnesium cations (Mg$^{2+}$), monovalent potassium cations (K$^+$) and a buffer solution, all to provide the suitable chemical environment for optimum stability and activity of the DNA polymerase enzyme.

In order to assess the purified total RNA for possible DNA contamination, PCR for the GAPDH gene sequence was performed with the GAPDH sense primer 5’-TGAAGGTCGGTGCAACGGATTG-3’ and antisense primer 5’-CATGTAGGCCATGAGGTCCACCAC-3’. A negative control PCR reaction was set up to ensure no reagent contamination using 1 µL of RNase-free water as a substitute for 1 µL of sample RNA.

PCR was carried out using small reaction volumes in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction, with a heated lid to prevent condensation at the top of the reaction tube.

The PCR consisted of a series of 40 repeated temperature changes called cycles, and each cycle had 3 discrete temperature steps:
**Denaturing step:** heating the reaction to 94°C for 60 seconds causes denaturing of the DNA template and primers by disrupting the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA.

**Anneal primers:** the temperature is lowered to 55°C for one minute – this allows the forward and reverse primers to anneal (base pair) to their complementary sequences. Typically the annealing temperature is about 3-5 degrees Celsius below the melting temperature of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. Upstream and downstream primers are designed to bracket the DNA region to be amplified.

**Extend primers:** the temperature is raised to 72°C for one minute, allowing Taq polymerase to attach at each priming site and extend (synthesize) a new DNA strand. The DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction.

At its optimum temperature, the DNA polymerase is capable of polymerizing a thousand bases per minute. If there are no other limitations such as lack of substrates or reagents, at each extension step the amount of DNA target is doubled, leading to exponential amplification of the specific DNA fragment. After 2 complete cycles, 4 DNA copies are produced. When the strands are then denatured during the first step of the 3rd cycle, the DNA fragments of desired length are denoted and 2 copies made after primer binding sites are extended. The region of interest is then replicated geometrically such that by the end of 20 cycles the PCR products include 1,048,576 DNA copies and 1,048,536 target sequence copies. This is the exponential amplification stage of the PCR process. As the DNA polymerase loses activity and consumption of substrate and reagent (including dNTPs and primers) becomes a limiting factor, the enzymatic reaction slows. The efficiency of amplification is decreased because of contaminants (inhibitors), competitive reactions, inactivation of the polymerase and target re-annealing. As the number of cycles increases, the amplification efficiency decreases. Finally, a plateau phase is reached when there is no more accumulation of product due to exhaustion of reagents and enzyme.
The PCR method is extremely sensitive, requiring only a few DNA molecules in a single reaction for amplification across several orders of magnitude. Therefore, adequate measures to avoid contamination from any DNA present in the laboratory environment were required.

2.6.6 Agarose Gel Electrophoresis

Agarose gel electrophoresis was employed for size separation of the PCR products in order to assess whether the PCR generated the anticipated DNA fragment. Negatively charged nucleic acid molecules are moved through an agarose matrix with electrophoresis applied, and molecules are separated according to length with shorter molecules migrating further.

Gels were prepared by dissolving 1.8g agarose per 100 mL 1x Tris-Borate EDTA buffer (TBE Buffer). The solution was brought to the boil carefully in a microwave to dissolve the agarose, and kept at 65°C in an incubator. Then 50 µL of 1mgmL\(^{-1}\) ethidium bromide solution was added per 100 mL of 1.8% agarose solution, to give a final concentration of 5 µgmL\(^{-1}\). Ethidium bromide is an intercalating agent that interacts in between base pairs of double-stranded DNA, inducing local structural changes to the DNA molecule. These structural modifications result in altered DNA function, hindering transcription and DNA repair processes and thereby increasing the frequency of DNA mutations. By this mechanism, ethidium bromide is widely assumed to be carcinogenic. Ethidium bromide is useful as a nucleic acid stain because when exposed to ultraviolet light it fluoresces with an orange colour, intensifying 20-fold when intercalated into DNA. By running samples through a gel treated with ethidium bromide, any band containing more than 20 ng of DNA becomes distinctly visible under UV light.

The warm solution containing ethidium bromide was poured into a gel rack with a comb in one end and allowed to cool and solidify. PCR samples were loaded into the gel wells with a negatively charged loading buffer (Orange G) that also marked the progress of DNA fragments through the agarose matrix. A DNA ladder containing DNA fragments between 100 and 1,500 base pairs in length was loaded to run alongside the PCR products on the gel. This enabled the size of the PCR products to be determined by direct comparison with a molecular weight marker.
The gels were run with 1x TBE running buffer at a constant 100 V for 30 minutes. The gel was then transferred to the UV illuminator and the presence or absence of bright PCR product bands was recorded.

2.6.7 Reverse Transcriptase Polymerase Chain Reaction

The PCR technique described above is only applicable to DNA strands. However, PCR analysis of RNA molecules is made possible by first using reverse transcription to convert RNA to complementary DNA. The double-stranded DNA transcribed thus can be used in a standard polymerase chain reaction using primers targeted for analysis of the original single-stranded RNA. With this reverse transcriptase PCR (RT-PCR) method it is possible to isolate, amplify and identify a known sequence from total cellular RNA. For these experiments this technique was employed to assess the efficacy of siRNA-mediated knockdown of the HO-2 gene by qualitative analysis of the gene transcript in total cellular RNA samples.

Complementary DNA (cDNA) is synthesized from messenger RNA (mRNA) template in a reaction catalysed by reverse transcriptase enzyme. This enzyme operates on a single strand of mRNA, generating the cDNA sequence according to the pairing of RNA base pairs adenine (A), uracil (U), guanine (G) and cytosine (C), to their DNA complements thymine (T), adenine (A), cytosine (C) and guanine (G) respectively.

cDNA was synthesized from purified total RNA with the Improm-II reverse transcriptase system (Promega). The first step was a brief heat denaturation of the RNA template prior to reverse transcriptase, recommended to improve sensitivity. A poly-T oligonucleotide primer was combined with the RNA template and RNase-free water and incubated for 5 minutes at 72°C. This disrupts any secondary structures formed in the RNA and makes a more linear arrangement allowing for more effective binding by the primer. Cooling to 4°C for 5 minutes allows the poly-T oligo primer to hybridise onto the poly-A tail of the mRNA template by means of base pairing. Reverse transcriptase requires this double-stranded segment as a primer to start its operation. An alternative is to add random hexamer primers that contain every possible 6 base single strand of DNA and can thereby hybridise anywhere along the RNA template with complementary base pair sequence.
The next step was to add reverse transcriptase, along with deoxynucleoside triphosphates (dNTPs) substrate (A, T, G, C) and reaction buffer, magnesium chloride, RNase-free water and ribonuclease inhibitor to protect the RNA. This mixture was incubated at 25°C for 5 mins to ensure annealing of the oligo (dT) primer. This also enables the reverse transcriptase to extend the oligo (dT) primer and allows the primer to remain hybridised at higher temperatures. The mixture was then heated to 45°C for 60 minutes for the first-strand cDNA synthesis reaction, when the reverse transcriptase generates the cDNA sequence according to RNA base pairs using the dNTPs as substrate. A full length of cDNA is synthesized and includes all gene transcripts included in the available RNA template. This enables subsequent priming of different gene sequences from the same cDNA sample.

The final stage was inactivation of the reverse transcriptase by heating to 70°C for 15 minutes. The resulting cDNA product required no clean-up or dilution prior to being added to amplification reactions, and was suitable for storage at -20°C for future use.

The cDNA provided the necessary template for the heat stable Taq polymerase to amplify regions of interest in a standard PCR process.

2.6.8 Semi-quantitative RT-PCR

To investigate HO-2 gene knockdown in the RNA samples, standard PCR was performed using cDNA and specific HO-2 primers (sense primer 5'-CAAGGAAGGACCAAGGAA-3' and antisense primer 5'-CTCCTCCCAGTTTTTTACCAA-3'). The PCR reaction included 40 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. To assess the efficiency of amplification, PCR was also carried out with cDNA and an internal control GAPDH primer pair (sense primer 5'-TGAAGGTCCGAGTCAACGGATTGC-3' and antisense primer 5'-CATGTAGCCATGAGGTCCACC-3'). The GAPDH gene was expected to have constant expression levels throughout all the experimental conditions compared. Amplification of the GAPDH gene verifies acceptable quantity and quality of the target nucleic acid and reaction components. The pair of RT-PCR series was also used to semi-quantitiate the levels of HO-2 mRNA present against GAPDH mRNA levels, allowing comparison between experiment samples. However, differences in amplification
efficiencies between the GAPDH and HO-2 transcript due to variations in PCR product size or primer annealing efficiency may still have occurred.

Possible contamination with extraneous DNA was assessed with a negative control in every PCR reaction. This control reaction was performed alongside the experimental sample PCRs with the same reagents but without template cDNA added.

Assessment of RT-PCR-derived products was again performed using ethidium-treated agarose gel TBE electrophoresis with a DNA ladder for reference.

2.7 Real-Time Quantitative Polymerase Chain Reaction

2.7.1 Introduction

Real-time Polymerase Chain Reaction (PCR) is the ability to measure DNA amplification during PCR as it occurs. Data is collected throughout the PCR process as amplified product is measured at each PCR cycle rather than at the end of the PCR, changing the approach to PCR-based quantitation of DNA and RNA. During real-time PCR a fluorescent signal is generated that is directly proportional to the amount of accumulating PCR product. This signal is detected, calibrated and used to provide a measure of initial target gene abundance and calculate relative gene expression in experiment samples. Real-time PCR monitors the point in time during cycling when amplification of a target is first detected rather than the amount of target accumulated after a fixed number of cycles. The higher the starting copy number of the nucleic acid target, the sooner the fluorescent signal increases above a statistically significant threshold.

In traditional PCR, an endpoint assay measures the amount of accumulated PCR product after the reaction is complete, making it impossible to determine the starting concentration of nucleic acid and introducing variability in results due to changes in reaction kinetics.
2.7.2 Polymerase Chain Reaction

The polymerase chain reaction (PCR) method allows exponential amplification of short DNA sequences (up to 600 bases) within a longer double stranded DNA molecule.

PCR uses a pair of primers that are complementary to a defined target sequence on each of the two strands of the DNA molecule. These primers, each approximately 20 nucleotides in length, are designed to bracket the region that is targeted for amplification. The primers are extended by a DNA polymerase thereby making a copy of the designated sequence. This copy can then be used as a template for further copies using the same primers, leading to exponential amplification.

Double-stranded DNA must be denatured by heating to more than 90° Celsius to expose two single strands for each PCR cycle. The isolation of a DNA polymerase (Taq polymerase) from Thermus aquaticus, a bacterium that grows in hot pools, was significant progress for the PCR method. Researchers demonstrated that Taq DNA polymerase had 5’ to 3’ exonuclease activity at high temperatures, avoiding the need to add new polymerase for every cycle of amplification.

2.7.2.1 PCR Reaction Cycles

To quantify mRNA within a sample, the mRNA is first copied to cDNA by reverse transcriptase using an oligo dT primer and random hexamers. The reverse transcriptase has endoglycosidase H activity that digests the mRNA strand template prior to real-time PCR allowing the second strand of DNA to be formed. The PCR experiment set up mix includes Taq polymerase, specific primers for the gene of interest, deoxynucleotides and a suitable buffer. The steps involved in the reaction are as follows:

2.7.2.2 PCR Cycle 1

Denaturation: The sample is heated to 94-96°C for several minutes to separate double stranded DNA into single strands.

Anneal Primers: The sample temperature is lowered to 50-65°C for one to several minutes. This allows specific primers to base-pair (anneal) to their complementary sequences on each strand. Primers are designed to bracket the region that is targeted for
amplification. The primers sites may be up to 600 bases apart but there is often about 100 bases between upstream and downstream primer sites.

Extend primers: The temperature is raised to 72°C for one to several minutes. At this higher temperature the heat-stable Taq polymerase attaches to each primer site and adds nucleotides to synthesise a new DNA strand.

2.7.2.3 PCR Cycle 2

Denaturation: The sample is again heated to 94-96°C for several minutes to separate double stranded DNA into single strands.

Anneal Primers: The temperature is lowered to 50-65°C for one to several minutes to allow primers to anneal.

Extend primers: The sample is heated to 72°C again for one to several minutes. Taq polymerase binds to each primer and extends from the primer to the end of the new cDNA strand.

2.7.2.4 PCR Repeated cycles

The steps of denaturation, annealing and extension are repeated, doubling the number of target sequence copies at every cycle (2, 4, 8, 16, 32 etc.). The PCR reaction generates an exponential number of smaller double stranded cDNA molecules that are the size of the distance between the two primers. After 3 cycles this target sequence defined by the primers begins to accumulate so that by the 5th cycle 24 of 32 strands (75%) are the same size. After 30 cycles up to 1 billion copies of the target sequence or amplicon are produced from a single starting double-stranded DNA molecule. The strands of larger size only increase arithmetically and are soon a small proportion of the total number of molecules.

Analysis of PCR products on an agarose gel can be performed when product is abundant enough to be detected with the rather insensitive ethidium bromide stain. Ethidium bromide dye binds to double stranded DNA by interpolation (intercalation) between the base pairs. When irradiated in the UV part of the spectrum the dye is fluorescent. Detectable bands can provide a qualitative tool for detecting the presence or absence of a
particular DNA. The amount of end-product of PCR may not however be related to the amount of input DNA template as the exponential stage of amplification is over. The addition of the reverse transcriptase step makes standard PCR even less accurate to quantify relative mRNA levels in different samples.

2.7.3 Principles of Real-Time PCR

Real-time PCR technologies include the use of dual-labelled oligonucleotide fluorogenic probes such as TaqMan® assay probes. A labelled oligonucleotide probe specifically designed to hybridise within the target sequence is introduced into the PCR assay. The probe is labelled with a reporter fluorescent dye at its 5’ end and a quencher dye at its 3’ terminus, and anneals to PCR product strands to generate a substrate suitable for exonuclease activity. Cleavage of the target probe during PCR by the 5’ to 3’ exonuclease activity of Taq polymerase only occurs if the target sequence is being amplified, and the fluorescence signal emitted on cleavage of the probe can be used to detect amplification of the amplified product.

While the probe is still intact, the close proximity of the quencher dye significantly decreases the fluorescence emitted by the reporter dye by permitting fluorescence resonance energy transfer (FRET). Following denaturation, the primer anneals to the cDNA strand if the target sequence is present and the fluorogenic probe anneals downstream from one of the primer sites. During this initial annealing stage both primers and probe base-pair bind to the complementary DNA target sequence. Polymerisation of a new DNA strand is extended from the primers. When the polymerase reaches the downstream probe the 5’ to 3’ exonuclease activity degrades the probe, physically separating the fluorescent reporter dye from the quencher dye. The unquenched reporter dye fluorescence can be detected after excitation with a laser.

Cleavage removes the probe from the target cDNA strand, allowing primer extension to continue to the end of the template strand. Thus, inclusion of the probe does not inhibit the exponential accumulation of PCR product. Additional reporter dye molecules are cleaved from their respective probes with each PCR cycle. When the product targeted by the reporter probe increases at each PCR cycle, breakdown of the probe and consequent release of the reporter signal causes a proportional increase in fluorescence. The
increase in fluorescence intensity is directly proportional to the amount of amplicon produced during the exponential phase of PCR$^{513,514}$.

### 2.7.4 PCR Chemistry

The introduction of fluorogenic-labelled oligonucleotide probes that use the 5’ nuclease activity of Taq DNA polymerase into real-time PCR experiments enables the detection of only specific amplification products. This is because specific hybridization between probe and target is required to generate fluorescent signal. The assay utilises the 5’ nuclease activity of DNA polymerase to hydrolyse the hybridisation probe bound to its target amplicon. Probes can also be labelled with different, discernible reporter dyes that allow amplification and detection of two distinct sequences in one reaction tube. Furthermore, this development eliminates the post-PCR processing step previously required to analyse probe degradation by polymerase.

#### 2.7.4.1 TaqMan® Probes

TaqMan® technology (Applied Biosystems, California, USA) uses hydrolysis probe chemistry with assay efficiency chiefly dependent on 5’ to 3’ exonuclease activity of Taq polymerase enzyme. The oligonucleotide probe has a covalently bonded fluorescent reporter dye at the 5’ end and quencher dye at the 3’ end. Reporter dyes commonly in use include 6-carboxyfluorescein (FAM), tetrachloro-6-carboxyfluorescein (TET), hexachloro-6-carboxyfluorescein (HEX) and VIC. A commonly used quencher dye is 6-carboxytetramethylrhodamine (TAMRA). For real-time PCR experiments a forward and reverse primer are used in addition to the labelled oligonucleotide probe.

#### 2.7.4.2 Assay Design

TaqMan® assays require both the usual pair of PCR primers with a sequence-specific fluorescently labelled probe that is flanked by the primers. The primers and probe should be contained within a target amplicon of between 50 – 150 base pairs in length, preferably straddling an exon-exon junction. Amplicons that are small in length promote high-efficiency assays and are more tolerant of variable reaction conditions. The melting temperature $T_m$ of the two primers should be within 1°C of each other and between 58
and 60°C. The \( Tm \) of the TaqMan® probe should be 10°C greater than that of the primers to ensure that the probe is fully hybridised during primer extension.

Nucleic acid techniques are highly sensitive and susceptible to small changes in assay conditions, being thereby prone to variability in results. Assays with the design, synthesis and optimisation of concentrations of probe and primers and determined amplification efficiencies prior to use are commercially available. Using a standardised validated biological assay can help to control variations in results and reduce the potential for error.

Pre-optimised TaqMan® assays for human heme oxygenase 1 (Hs00157965_m1) and heme oxygenase 2 (Hs00157969_m1) were performed for the real-time PCR experiments. These assays are designed to detect all splice variants of the heme oxygenase gene. Assays used TaqMan® probes with FAM as the gene of interest reporter dye and TAMRA™ as the quencher dye.

2.7.5 PCR Kinetics

Under ideal experimental conditions, the amount of DNA product doubles during each cycle of the PCR reaction. After 2 cycles the amount of product is \( 2 \times 2 \) the initial amount, after 3 cycles \( 2 \times 2 \times 2 \) (\( 2^3 \)) and after 4 cycles \( 2^4 \) the starting amount. This can be defined by the equation \( N = N_0 2^n \) where \( N \) is the number of amplified molecules, \( N_0 \) is the initial number of molecules and \( n \) is the number of PCR cycles. The exponential nature of PCR amplification only applies to the early phases of the reaction. The reaction cannot go on indefinitely and as it progresses the reagents involved are depleted and become limiting. A plateau effect can be observed as the rate of product amplification slows and eventually stops. Factors that can contribute to the plateau effect include: accumulation of DNA product to a concentration at which re-association competes with primer annealing and extension; components necessary for the reaction become limiting; inhibitors of polymerase enzyme activity accumulate such as pyrophosphates; the molar ratio of polymerase to template falls below a critical value and enzyme activity is impaired.
As the efficiency of the PCR reaction becomes increasingly impaired, PCR product amplification is reduced. In real-time PCR analysis all phases of the PCR is revealed for every sample, enabling quantification of the product early during the exponential phase.

PCR kinetics can be displayed by plotting DNA product copies against cycle number. Using a regular scale gives an exponential graph:

**Figure 2.7.1: DNA copies per PCR cycle regular scale**

![DNA copies per PCR cycle regular scale](image)

There is a linear relationship between DNA copies and cycle number using a logarithmic scale:

**Figure 2.7.2: DNA copies per PCR cycle log scale**

![DNA copies per PCR cycle log scale](image)
The kinetic approach used in real-time PCR involves looking at the reaction in the early stages when amplification is exponential and linear on a logarithmic scale. PCR machines contain a highly sensitive camera that monitors the fluorescence in each well of a 96-well plate at frequent intervals during the reaction. The ABI Prism 7700 systems used for these experiments collects a fluorescent signal from each reaction every 7 seconds. Over the course of a 40-cycle PCR lasting 1 hour and 56 minutes a total of 994 measurements are made for each reaction. The kinetics of fluorescence accumulation is displayed in real time when the data are plotted against time. Detection of fluorescence signal is directly proportional to product amplification. The PCR reaction can be described in three phases:

**Exponential**

The early phase of PCR when all necessary reagents are fresh and available and the reaction is precise and very specific. When PCR efficiency is assumed to be 100%, amplicon is doubled at every cycle. During the exponential phase, the increase in signal detection corresponds directly to an increase in PCR product and is dependent on the amount of starting material.

**Linear (High Variability)**

Reagents are consumed as a result of amplification as the reaction progresses. PCR efficiency is impaired, reactions slow down and amplicon is no longer doubled with every cycle.

**Plateau (End-Point)**

The PCR reaction has stopped. DNA copies are no longer being made and the PCR products will begin to degrade if left long enough. Each experiment sample has potentially different reaction kinetics and will reach the plateau phase at a different point in time. Traditional agarose gel-based analysis of PCR products measures the amount of product at the plateau phase. This method will detect misleading variations in end-product amounts even when the starting amount of DNA template is identical between samples. In contrast, real-time PCR uses the exponential phase to provide accurate and precise data for quantitation of PCR product. This is in the early stages of the reaction, at
the very beginning of the upturn of the curve and not in the linear region of the curve on a standard scale.

**Figure 2.7.3: Phases of PCR**

![Phases of PCR](image)

### 2.7.6 The threshold cycle

A fundamental measurement in real-time PCR is the threshold cycle (C\textsubscript{T}). The C\textsubscript{T} is the cycle at which fluorescence generated by the accumulating PCR product exceeds a fixed threshold.

At the start of PCR reporter fluorescent signal is beneath a baseline detection level defined as the PCR cycles during which signal is accumulating but is below instrument detection levels. PCR cycles 3 to 15 are set as the default baseline and the arbitrary threshold level calculated as ten times the standard deviation of the average signal of the baseline fluorescent signal between cycles 3 to 15. A fluorescent signal detected above threshold can be considered a true signal used to define the sample threshold cycle (C\textsubscript{T}). The threshold cycle can be defined as the fractional PCR cycle number at which reporter fluorescent intensity during the reaction is recorded as statistically significant above background. The C\textsubscript{T} thereby indicates the number of cycles required to detect appearance of PCR product above the minimal detection threshold level. The higher the abundance of target cDNA in the starting sample, the sooner the threshold fluorescence level is reached and the lower the C\textsubscript{T}.516
The threshold line and $C_T$ values are always calculated during exponential phase cycles of PCR target amplification when none of the reaction components are limiting. The $C_T$ value is proportional to the log concentration of the template cDNA (and target RNA). The amount of template cDNA and target RNA in experiment samples can be accurately determined by comparing the $C_T$ values of these samples against a series of standards with log transformation and calibration of the $C_T$ value. The observed $C_T$ is not an absolute measure of target abundance as its value will vary depending on where the threshold line is set. The $C_T$ value is dependent on the starting template copy number, the efficiency of amplification, the PCR system and the type of chemistry used. Comparison of the absolute $C_T$ value is only valid when comparing experiments using exactly the same reaction conditions including assay design, reaction volumes, reagents, primers and probes and PCR instruments.

**The $C_T$ value**

$C_T$ (threshold cycle) is the intersection between an amplification curve and a threshold line (Figure 2.7.4). It is a relative measure of the concentration of target in the PCR reaction.

**Figure 2.7.4: The CT threshold cycle value**
2.7.7 PCR Efficiency

Target PCR product abundance recorded at any time during a reaction is influenced by the amount of initial starting template, the efficiency of PCR and by the number of PCR cycles performed.

PCR efficiency can be described by the equation $X_n = X_0 (1 + E)^n$ where $n$ is the number of cycles performed, $X_n$ is the amount of PCR product at cycle $n$, $X_0$ is the initial amount of target mRNA or DNA and $E$ is the amplification efficiency. As the nature of the PCR reaction is exponential, very small changes in amplification efficiency can yield dramatic differences in the amount of product $X_n$ even if the initial number of target molecules $X_0$ is the same.

PCR reaction efficiency can clearly affect the threshold cycle. Two identical samples amplified under low and high experimental efficiency conditions produce different $C_T$ values for the same target template abundance. Although the high efficiency condition results in a later $C_T$ at high concentrations, it has a better sensitivity at low target concentrations.

Experimental factors that may affect the efficiency of amplification include impurities in the sample, the nature and length of the sequence being amplified, the sequence of the forward and reverse primers and the performance of other master mix components used in the reaction that affect the solution pH and salt concentration. Efficiency between 90 and 110% is generally considered acceptable.

The purpose of the PCR is to copy a specific DNA fragment corresponding to the size predicted from the position of the primers on the cDNA (if looking at mRNA) or the genomic or plasmid DNA according to the nature of the target DNA. Primer-dimer artifacts arise when primers anneal to themselves and create additional small templates for PCR amplification. Mispriming describes the process whereby primers anneal to complementary or partially complementary sequences on DNA strands that are not part of the target sequence\textsuperscript{516}. 

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2.7.8 Quantitation Assay

A quantitation assay measures the amount of a nucleic acid target during each amplification cycle of the PCR. The nucleic acid target may be DNA, cDNA or RNA using reverse transcription polymerase chain reaction (RT-PCR). The aim of the assay is to calculate from the final PCR product abundance either the initial number of target molecules or the relative starting levels of target molecules among several samples.

Absolute quantitation assays calculate the quantity of target nucleic acid in experiment samples from a standard curve. Absolute quantitation is the appropriate method if researchers need to know the exact copy number of the target DNA or RNA in a given sample. A common example is the correlation of viral copy numbers in biological samples to monitor the progress of disease in Human Immunodeficiency Virus infection.

Relative quantitation assays calculate target RNA or DNA abundance in a given sample relative to another reference sample or control group. The calculation methods used to perform relative quantitation with data from the ABI PRISM®7700 Sequence Detection System are the standard curve method or the comparative \( C_T \) method.

2.7.8.1 Standard Curve Method

Standard curves are generated using accurate serial dilutions of stock DNA or RNA containing the appropriate target. Three independent serial dilutions of the nucleic acid standard are assayed in duplicate together with positive and negative control reactions. The dilutions are made over the range of target copy numbers that include the amount of target nucleic acid expected in the experimental samples.

Target abundance is expressed relative to an untreated control or reference calibrator sample. Quantity of target nucleic acid in experimental sample is determined from the standard curve and divided by the target quantity of the control, expressed as n-fold difference relative to the control. Relative target quantities can be determined across multiple plates by using identical stock DNA or RNA to prepare standard curves.

The \( C_T \) value is inversely proportional to the log of the initial copy number, so to generate a standard curve the \( C_T \) values (with 95% confidence intervals) are plotted against the
logarithm of the initial target DNA or RNA copy numbers. The copy numbers of experimental samples can be calculated after real-time amplification from the linear regression of the standard curve\textsuperscript{517}.

Quantitation can be normalised to an endogenous control using standard curves prepared for both the target nucleic acid and the endogenous reference. The quantity of target and endogenous control is calculated from the appropriate standard curve for each experiment sample, dividing the target by the reference amount to obtain a normalised value. Each of the normalised values can be expressed relative to a reference calibrator sample.

2.7.8.2 Comparative C\textsubscript{T} method

The comparative C\textsubscript{T} method was one of the first methods to be used to calculate real-time PCR results. It is an approximation method that permits comparison of expression of a gene of interest among different samples. The expression level of the gene of interest is normalised to an endogenous control gene to normalise for variation in the amount and quality of RNA between samples. It is similar to the standard curve method but uses the arithmetic formula $2^{-\Delta\Delta C_T}$ to achieve the same result for relative quantitation\textsuperscript{518}.

Expression of target mRNA abundance in experimental samples is normalised to an internal standard such as 18S ribosomal RNA and related to the expression level in a control sample (e.g. normal or untreated cells). The difference between the C\textsubscript{T} value of the target mRNA and that of the internal standard is represented as $C_T(\text{target mRNA}) - C_T(\text{internal standard}) = \Delta C_T$. The expression of the target gene in the test sample relative to the control sample is therefore given as $\Delta C_T(\text{test}) - \Delta C_T(\text{control}) = \Delta\Delta C_T$.

Where the efficiency of PCR is close to 100\% (i.e. close to a doubling of product per cycle) the relative abundance of the target RNA between the two samples becomes $2^{(\Delta C_T(\text{EXPERIMENT}) - \Delta C_T(\text{CONTROL}))}$ or $2^{\Delta\Delta C_T}$. This applies to all amplicons designed and optimised according to Applied Biosystems guidelines. The formula $2^{\Delta\Delta C_T}$ represents the normalised expression of the target gene in the unknown sample relative to the normalised expression of the calibrator sample. The advantage of the comparative C\textsubscript{T} method is that standard curves do not need to be constructed to achieve the same outcome.
Throughput is increased because wells no longer need to be used for the standard curve samples. Adverse effects of any dilution errors made in creating the standard curve samples are also eliminated.

The PCR efficiency has a major impact on the fluorescence profile and the accuracy of the calculated expression result when the $\Delta\Delta C_T$ method is used. For reliable comparison between samples there needs to be constant amplification efficiency in all samples being compared. The $2^{-\Delta\Delta C_T}$ equation assumes that target and standard efficiencies are both 100%.

The PCR efficiency must be demonstrated for both the target gene and the internal standard assay and is assessed by observing how $C_T$ varies with template dilution. If the graph plot of template versus $C_T$ is close to zero, it implies that the amplification efficiencies of the housekeeping and target genes are very similar. When efficiencies for each PCR are equal, the $\Delta C_T$ value will be the same no matter what the dilution (standard curves have similar slope).

Before using the comparative CT ($\Delta\Delta CT$) method for quantitation a validation experiment must be performed to demonstrate that efficiencies of target and endogenous control amplifications are approximately equal and that the assumptions of the method are reasonably correct. A sensitive method for assessing if two amplicons have the same efficiency is to look at how $\Delta C_T$ varies with template dilution. A graph is plotted of log input cDNA amount versus $\Delta C_T$ values [$\Delta C_T = C_T(\text{target mRNA}) - C_T(\text{internal standard})$]. The absolute value of the slope is calculated by linear regression. Applied Biosystems stipulate that only when this $\Delta C_T$ plot gradient is less than 0.1 can the comparative $C_T$ calculation be reliably used for the relative quantitation of target without running standard curves on the same plate. For situations in which the efficiencies of the two systems are not equal quantitation should be performed using the standard curve method. Alternatively the validation experiment can be repeated using new primers designed and synthesised for the less efficient system to try and optimise efficiency.$^{519,520}$
2.7.9 Endogenous Control

Minor differences in the abundance of template RNA, variable RNA quality or disparities in the efficiency of cDNA synthesis and PCR amplification all introduce specific errors into real-time quantitative PCR experiments. Amplification of an endogenous cellular control may be performed for the quantitation of gene expression in experiment samples to minimise these errors and correct for inter-sample variation. Commonly used controls include β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal RNA (rRNA). The endogenous standard sequence is simultaneously amplified with the target gene using a second pair of gene-specific primers. The ratios of the amount of PCR products generated by target and endogenous standard sequences in all experiment samples are then determined and compared. An active reference has its own specific pair of primers and probe to generate an active signal as a result of PCR amplification. Using a DNA or RNA that is present in each experiment sample as an active reference enables normalisation of target messenger RNA for differences in the amount of total RNA in each sample. An alternative is to use an exogenous control which is a characterised RNA or DNA added or “spiked” into each experiment sample at a known concentration. This can be used as an internal positive control to distinguish true target negatives from PCR inhibition. An exogenous reference can also be used to normalise for variations in sample extraction efficiency or complementary DNA (cDNA) synthesis by reverse transcriptase PCR.

The main advantage of using expression of an endogenous sequence as an internal standard is that the reference mRNA and the target mRNA are usually processed together for the entire duration of the experiment from RNA extraction through to PCR amplification. This minimises differences in RNA yield between samples. Using oligo(dT) primers and random hexamers converts the entire population of mRNA to cDNA in an attempt to normalise the overall efficiency of cDNA synthesis.

Regardless of the use of an active reference, a passive reference is always used to provide an internal reference to which the reporter dye signal can be normalised during data analysis. This correction is required to account for any non-PCR-related fluctuations in fluorescence signal caused by changes in concentration or volume.
Choosing a suitable internal control for normalisation of the target mRNA is of crucial importance. The principal challenge of normalisation is to identify one or more endogenous control gene which evaluates RNA quality and quantity and takes into account factors influencing the various steps of PCR. Characteristics required for a suitable control gene include: stable cellular expression in all experimental samples analysed with expression unaffected by reagent treatment in the experiment; equivalent stability to the target gene transcript so that any impaired amplification is mirrored by a corresponding reduction in target gene transcript quantity; PCR amplification that reflects variations in RNA quality, quantity or the efficiency of cDNA synthesis; no association with pseudogenes to avoid genomic DNA amplification and a level of expression that is neither too low (C<sub>T</sub> > 30) or too high (C<sub>T</sub> < 15) since the applied correction should be more accurate with a medium copy number. Most importantly, expression of the internal control must be unaffected by the target mRNA. Commonly used standards include the cytoskeletal protein β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme. Reported drawbacks of using GAPDH are that expression levels are altered by glucose, insulin, heat shock and cellular proliferation. Levels of β-actin may also be modified by experimental treatments. Alternative common control genes include MHC I (major histocompatibility complex I), cyclophilin and 28S or 18S ribosomal RNAs. For 18S ribosomal RNA all cells present in the sample under investigation will contribute to the resulting signal. Ribosomal RNA (rRNA) is expressed at a much higher level than mRNA and therefore may not always be a good representative of total mRNA abundance in a cell. One advantage of using rRNA is the relatively stable expression it provides under conditions affecting mRNA transcription.

Since it may be said that the perfect standard does not exist, some researchers advocate the use of a minimum of three and five independent housekeeping genes in a single experiment. The mean expression of these multiple genes can then be used for normalisation\textsuperscript{521-523}. 
2.7.10 Multiplex PCR

A Multiplex PCR method can be used in relative quantitation when one primer pair amplifies the target gene of interest sequence and another primer pair amplifies the endogenous control simultaneously in the same reaction tube. A multiplex reaction can be performed for both the standard curve and the comparative method by colour-coding the independent reactions using TaqMan probes labelled with different fluorescent dyes. There are multiple reporter dyes available for TaqMan® probes including 6-FAM, TET, and JOE that are distinguishable from one another due to their different emission wavelength maxima:

6-FAM, \( \lambda_{\text{max}} = 518 \text{ nm} \)  
TET, \( \lambda_{\text{max}} = 538 \text{ nm} \)  
JOE, \( \lambda_{\text{max}} = 554 \text{ nm} \)

In addition the quencher dye TAMRA (\( \lambda_{\text{max}} = 582 \text{ nm} \)), and the passive reference dye ROX (\( \lambda_{\text{max}} = 610 \text{ nm} \)) are currently recommended for use in the TaqMan system. The ABI PRISM 7700 Sequence Detection System software features a mathematical algorithm called multicomponenting that uses pure dye reference spectra to calculate the contribution of each dye to a complex reaction spectrum. This process can thereby detect independent amplification of the relevant target sequences within the same experiment. Multicomponenting does introduce a degree of error into the accuracy of fluorescent signal quantitation due to the experimental variation of measuring more than one spectrum. The magnitude of this error can be minimised by maximising the spectral resolution between the various dyes used in the reaction. This can be achieved using reporter dyes with a large difference in emission maximum – e.g. 6-FAM and JOE.
2.7.11 Limiting Primer Concept

Common reagents are shared by PCR amplification reactions of multiple target sequences. If one target segment has larger initial copy numbers this may use up the shared reagents to a degree that impairs amplification of the less abundant target gene. This will lead to a disproportionate amplicon and inaccurate quantitation. To avoid this competition in amplification reactions the concentration of primers used can be limited to control the size of fluorescent signal generated.

Limiting primer concentrations are defined by running a series of forward and reverse primer concentrations. The limiting concentration is defined as the concentration that forces the fluorescent signal generated by PCR to plateau at a lower copy number without affecting the cycle threshold value. Soon after obtaining accurate C_T values the primers become exhausted and the reaction is halted. In this way, amplification of the more abundant target species is stopped before it can limit the common reagents available for amplification of the minority species.

The relative abundance of target and endogenous target sequences to be amplified must be taken into consideration when applying the limiting primer concept. For these experiments ribosomal RNA (18S) was used as an endogenous reference. The concentration of rRNA in total RNA is always greater than the concentration of any target mRNA. Therefore, in a multiplex reaction amplifying both target mRNA and rRNA we need only limit the concentrations of the rRNA primers\(^{525}\).
2.7.12 Experimental protocol for mRNA quantitation using TaqMan® Hydrolysis probes

1. RNA isolation

RNA was prepared using the purpose-made RNeasy kit (Qiagen, Crawley, UK) for RNA extraction. RNA is thermolabile and susceptible to rapid degradation by endoribonucleases (RNases) released as the intracellular matrix breaks down. During RNA extraction samples are disrupted and homogenised in a buffer containing guanidinium salts which simultaneously lyses cells and inactivates endogenous RNases. Ethanol is added to the sample lysate. Passing this solution through a silica-based filter allows other cellular components to pass through while the RNA binds to the filter. The final step is to wash the filter to remove contaminants and the RNA is eluted.

2. Primer design and optimisation

A standardised validated biological TaqMan® assay was used for heme oxygenase (gene of interest) and internal control gene assays. The design, synthesis and pre-optimised probe and primer concentrations have passed Applied Biosystem bioinformatics design criteria. A validated assay was used to minimise the potential errors arising from experimental variation. The reporter dye FAM was used for the gene of interest and the dye VIC to report the endogenous control 18S rRNA with TAMRA used as a quencher dye in the PCR reaction.

3. RNA quantification

The most common method of measuring RNA concentration is ultraviolet (UV) absorption spectroscopy which is quick and relatively simple but comparatively insensitive and typically requires minimum nucleic acid concentrations of 1μg per mL in order to obtain reliable measurements. RiboGreen RNA quantification reagent is a superior alternative (Molecular Probes Europe BV, Leiden, The Netherlands Cat No. R11490). RiboGreen is an RNA-binding dye that exceeds the sensitivity of UV absorbance spectrophotometry by 1000-fold and ethidium-bromide-based assays by 200-fold.

Conventionally RiboGreen reagent is used with a fluorescence microplate reader, standard spectrofluorometer or filter fluorometer. The reagent may also be used on any
real-time PCR instrument by selecting the SYBR dye layer (SYBR Green I) and deselecting other dye options – the reporter dye is changed from FAM to SYBR and the quencher dye TAMRA is deselected, also deselecting ROX as a reference dye. RiboGreen assay is linear between 10 and 500ng per mL when performed on ABI Prism 7700 in a 200 μL per well reaction volume with RiboGreen reagent at a final dilution of 1 in 400. RiboGreen reagent is stored at -80°C with a working 1X solution stored at -20°C. The reagent is light-sensitive and the working solution is protected from light using foil wrapping as required.

RNA samples were measured in triplicate for accurate quantification. RiboGreen standards across the range of concentrations 0.013 – 0.526 ng per μL were used to construct a standard curve, with 100 μL reaction volume and 100 μL of Tris-EDTA buffer used for blank samples.

Ribonucleases (RNases) are very active and stable enzymes that do not generally need cofactors to function. RNase contamination of plasticware or glassware must be eliminated prior to use as RNases are difficult to inactivate and only tiny amounts are sufficient to destroy RNA. To create and maintain an RNase-free environment, disposable RNase-free polypropylene tubes were used throughout RNA experiments. Clean disposable gloves were worn during handling and preparation of all materials and solutions.

Diethylpyrocarbonate (DEPC) is a strong inhibitor of RNase enzymes causing inactivation of RNases in water by the covalent modification of histidine residues. DEPC-treated water was used in RNA handling and to create RNase-free solutions in order to minimise the inadvertent introduction of RNases into the RNA sample during or after the isolation procedure. Water was treated with 0.1 mL diethylpyrocarbonate added to 100 mL (0.1% v/v) and the mixture vortexed to bring the DEPC into solution. The solution was incubated for 12 hours at 37°C then autoclaved to 100°C for 15 minutes to inactivate any trace of DEPC. Inactivation of DEPC in this manner yields carbon dioxide, water and ethanol.

Purified RNA was stored at −20°C or −70°C in RNase-free water. Under these conditions, no degradation of RNA is detectable after 1 year.\textsuperscript{527}
4. Reverse Transcription

Reverse transcription (RT) of RNA is necessary because RNA cannot act as a template for PCR. Two-tube RT-PCR generates a stock of stable cDNA that is more suitable for long-term storage.

Using random hexamers and oligo dT as primers will allow amplification of the whole mRNA population. If only use oligo dT primers then ribosomal transcripts (e.g. 18S) will not be represented in the cDNA since the oligo dT lack a poly-A tail. Use of random hexamers is recommended.

Gene-specific primers analyse a single gene specified by the initial RT primer.

PCR mix is prepared at room temperature because the DNA polymerase is inactivated either by chemical modification or by an antibody.

Mastermix used – contains buffer, MgCl$_2$, dNTPs, enzyme

Optimal probe concentration 50 – 200nM & optimal primer concentration 100 – 900nM

Total volume 50 μL

Negative controls – water – allow identification of contamination of reaction mix. An RT sample is prepared in which reverse transcriptase has been omitted.

Positive PCR controls can be used as a calibrator e.g. a cell line dilution

5. Amplify cDNA

The cDNA was amplified using TaqMan® Assays and TaqMan® master mix. These purchased products (Applied Biosystems, Foster City, CA, USA) contain all of the enzymes, buffers, primers and probe necessary to perform TaqMan® real-time PCR for accurate nucleic acid quantitation.

The ABI Prism 7700 instrument (Perkin-Elmer–Applied Biosystems, Foster City, CA, USA) is capable of combining amplification, detection and quantification of real-time PCR products using fluorescent signal techniques. The instrument contains a built-in thermal cycler with 96-well positions able to detect fluorescence between 500nm and 660nm.
The cycle starts at 50°C for 2 minutes, heating to 95°C for 10 minutes to activate the Taq polymerase enzyme. The instrument then runs through 40 thermal cycles of 95°C for 15 seconds to denature DNA strands and allow unwinding followed by 60°C for 1 minute to allow primer and probe binding to the template strands.

Amplification is performed in closed thin-walled optical tubes of the 96-well microplate within the combined thermal cycler/detector. Laser light is distributed to each of the 96 sample wells via a multiplexed array of optical fibres. Each well is irradiated sequentially thereby inducing fluorescence. The resulting fluorescence emission data for each sample are collected once every few seconds via the optical fibres and directed to a spectrograph as PCR products are being generated. The spectrograph has a charge-coupled device (CCD) camera enabling spectral resolution of the fluorescent light. Spectral analysis allows the simultaneous use of more than one spectrally distinct fluorescent probe as described above. The initial starting copy number is determined by monitoring when PCR product is first detected at the threshold cycle. The higher the starting copy number of the target, the sooner a significant increase in fluorescence is observed and the lower the threshold cycle. These data are fed to a computer, which analyses and displays the results, eliminating the need for post-PCR processing.
2.7.13 RNA Quantification

Detection and quantification of small amounts of RNA in molecular biology procedures can be achieved via numerous methods. The most common traditional technique for measuring nucleic acid concentration is the determination of ultraviolet (UV) absorbance at 260 nm ($A_{260}$) and at 280 nm ($A_{280}$) using a spectrophotometer. The Beer-Lambert law predicts a linear change in absorbance with concentration with an $A_{260}$ of 1 unit corresponding to approximately 40 μg per mL of RNA in solution. Absorbance readings should be greater than 0.15 to ensure significance. The linear relationship between absorbance and concentration is based on an extinction coefficient calculated for RNA in water, so this method is only valid for RNA samples diluted in water.

RNA has its absorption maximum at 260 nm and while the OD at 260 nm is used to determine the RNA concentration in a solution, the ratio of the absorbance at 260 and 280 nm can be used to assess the RNA purity of an RNA preparation. The $A_{260}/A_{280}$ ratio ($A_{260}/A_{280}$) can indicate contamination with solutes that absorb in the UV including proteins. The pH of the sample also has a marked influence on the $A_{260}/A_{280}$ ratio, with lower pH producing a lower $A_{260}/A_{280}$ ratio and reduced sensitivity to protein contamination. Measuring the purity of RNA samples in water can introduce variation in results as water is not buffered and the pH is not constant. The most accurate value can be obtained by measuring the absorbance ratio in 10 mM Tris-Chloride buffer at pH 7.5, in which solution pure RNA has an $A_{260}/A_{280}$ ratio of 1.9 – 2.1. Many protocols accept a value of 1.8 – 2.1 to indicate a pure RNA sample, depending on how the measurement is performed and the source of potential contaminants.

For all the RNA samples eluted for real-time PCR analysis, an estimation of purity and RNA concentration was first performed using UV absorbance measurement with a spectrophotometer. The solution volume measured in 1 mL cuvettes was 150 μL with 10 μL of RNA sample diluted with 140 μL of RNase-free distilled water giving a dilution factor of 15. The absorbance was measured at 260 and 280 nm. The concentration of the RNA sample was calculated according to the following formula:

Concentration of RNA sample = 40 x $A_{260}$ x dilution factor = 40 x $A_{260}$ x 15 μg per mL OR ng per μL
2.7.13.1 RiboGreen RNA Quantification

The principal drawbacks of the UV absorbance-based technique include the relative insensitivity of the assay, the relatively large contribution of free nucleotides and proteins to the absorbance signal and interference by contaminants often present in nucleic acid preparations. The use of sensitive fluorescent nucleic acid stains solves many of these issues. To provide accurate RNA quantitation prior to reverse transcriptase PCR and real-time RNA analysis a sensitive RNA-binding stain was used called Quant-iT™ RiboGreen® RNA reagent (Invitrogen, catalogue number R11490). The Quant-iT™ RiboGreen® RNA reagent enables quantitation of as little as 1 ng per mL RNA using fluorescein excitation and emission wavelengths and was supplied with concentrated assay buffer and ribosomal RNA standards. The sensitivity of the RiboGreen assay exceeds that achieved with ultraviolet absorbance determination by 1,000-fold, with as little as 200 pg RNA detectable in a 200 μL sample volume. The RiboGreen reagent accurately measures the amount of intact RNA polymers in potentially degraded samples and unlike UV absorbance the reagent does not detect significant sample contamination by free nucleotides.

The RiboGreen reagent also binds to DNA, and sample contamination by DNA generates fluorescence using the assay. Although no currently available purification method can guarantee that RNA is completely free of DNA, DNase digestion of the purified RNA with RNase-free DNase was performed in an effort to ensure that the entire sample fluorescence generated was due to RiboGreen dye bound to RNA.

Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) was used to dilute the Quant-iT™ RiboGreen® reagent and for diluting the RNA samples. The 20X Tris-EDTA buffer included in the Quant-iT™ RiboGreen® RNA Assay Kit is nuclease-free and nucleic acid–free. A 1X Tris-EDTA working solution was prepared by diluting the concentrated buffer 20-fold with nuclease-free water.

For the experimental RNA quantification assay, RiboGreen reagent was diluted to 1:400 in Tris-EDTA buffer and added to DNase-treated RNA in Tris-EDTA buffer. The DNase-treated RNA was diluted 100-fold in Tris-EDTA buffer and 100 μL added to 100 μL of dilute RiboGreen reagent per well. This reaction was left at room temperature for 10 minutes in
a 96-well microplate and read on the ABI Prism 7700 plate reader. The SYBR green dye filter on the ABI Prism 7700 was selected for the unknown dye, deselecting TAMRA as the quencher dye and ROX as the reference dye. This is because the excitation and emission spectra for RiboGreen and SYBR green dyes are very similar: the SYBR green binds DNA while RiboGreen binds RNA.

2.7.13.2 RNA Standard Curves

For the construction of a standard curve 16S and 23S ribosomal purified RNA preparations were used. In preparation of the standard curve, the RNA solution used was treated in the same manner as the experimental samples and estimated to contain similar levels of RNA and other possible contaminants.

The ribosomal RNA standard was provided at 100 μg per mL in the RiboGreen® Assay kit and diluted 50-fold in Tris-EDTA buffer to make a working solution of 2 μg per mL. This 2 μg/mL solution was further diluted in Tris-EDTA buffer to give a range of final RNA concentrations used in the assay between 13 and 526 ng per mL. Tris-EDTA buffer was used for blank samples in 6 wells of the 96-well microplate. The reaction volume for all RiboGreen assays was 200 μL per well, with 100 μL diluted RNA sample added to 100 μL of RiboGreen reagent diluted 400-fold. The plate was sealed and left in the dark at room temperature for 10 minutes before reading on the ABI Prism 7700. The mean fluorescence value of the reagent blank was subtracted from the fluorescence value of each of the standard samples. These corrected data were used to generate a standard curve and formula of fluorescence versus RNA concentration.

All RNA samples eluted from siRNA experiments were initially quantified using UV absorbance as described above. This provided a quality control measure to ensure purity of RNA samples with A_{260}:A_{280} ratio between 1.9 and 2.1. Accurate RNA concentrations were then obtained using RiboGreen assay. A total of 40 ng RNA was used for cDNA synthesis for each sample. The cDNA samples were diluted to 1 ng/mL with pure water and a total of 10 ng used in each PCR amplification reaction.
2.8 Heme Oxygenase Enzymatic Activity Assay

The method used to measure the activity of heme oxygenase enzyme was performed as described by Motterlini et al.\textsuperscript{532,533} The assay measures the total heme oxygenase activity present in the cell suspension sample, i.e. the summation of HO-1 and HO-2 enzymatic activity. It is not designed to determine the activity of individual HO enzyme isoforms.

All experiments were completed with the kind supervision of Motterlini and colleagues at the Northwick Park Institute for Medical Research. The validated technique described originally by Tenhunen et al and modified by Balla and co-workers has been demonstrated to produce the same results as the more refined mRNA assay\textsuperscript{133,137,165}.

2.8.1 Cell sample preparation

Cells were cultured in 175 cm\textsuperscript{3} flasks (Nunc, Thermo Scientific, Denmark) to facilitate careful cell scraping during harvest. Cells were cultured to achieve the desired confluency depending on the reagents used and the cell line under investigation. A working volume of 48 mL was used for the flasks with a culture area of 175 cm\textsuperscript{2}. Oesophageal cell lines HET-1A and OE-33 were seeded at 3.2 x 10\textsuperscript{6} cells in 48 mL for reagent experiments and at 2.4 x 10\textsuperscript{6} cells in 48 mL for siRNA transfection experiments. Gastric adenocarcinoma cell lines AGS and HGC-27 were seeded at 2.4 x 10\textsuperscript{6} cells in 48 mL for reagent experiments and at 1.44 x 10\textsuperscript{6} cells in 48 mL for siRNA transfection experiments. This strategy was consistent with corresponding experiments using 6-well, 24-well or 96-well plates, seeding at equivalent cells per mL to achieve cells that were 30 – 50% confluent at the time of siRNA transfection and 50 – 75% confluent prior to addition of experimental reagents. Preparation of reagents and siRNA transfection protocols were carried out as previously described. Cells were exposed to experimental conditions for the required predetermined duration.

At the end of the experiment cells were washed twice with cold sterile phosphate-buffered saline (PBS) at pH 7.4. The adherent monolayer cells were then gently scraped in 10 mL of PBS using a cell scraper (Sarstedt, Germany) with a further 5 mL portion of cold PBS to wash any remaining cells anchored to the flask culture area. The aspirated 15 mL cell suspension was placed on ice in polypropylene Falcon tubes (BD Biosciences,
USA). The cell suspension was finally centrifuged at 1,000 x g for 10 minutes at 4°C. Remaining supernatant was discarded and the cell pellet re-suspended in 550 μL MgCl₂ (2 mM) phosphate (100 mM) buffer at pH 7.4 with adequate vortex. This cell suspension was suitable for short-term storage at -70°C until required for enzyme assay.

### 2.8.2 Heme Oxygenase Assay

Cell suspensions were subjected to three freeze / thaw cycles at -70°C for 10 minutes immediately followed by placement in a water bath at 37°C for 5 minutes to disrupt cell membranes before a final vortex on ice for 30 seconds to complete cell lysis. The resulting cellular suspension was then divided into 400 μL for enzyme activity assay with 100 μL transferred to an eppendorf tube and kept at 4°C for subsequent sample protein assay as detailed below.

A reaction mixture was prepared for each HO activity assay sample containing:

- 300 μL PBS with 2 mM MgCl₂
- 90 – 120 μL rat liver cytosol containing ≈3 mg per mL of protein prepared from supernatant fraction centrifuged at 105,000 x g as a source of biliverdin reductase (prepared according to Tenhunen, Marver, and Schmid¹⁶⁵)
- 25 μL hemin at 20 μM final concentration from a freshly prepared 2 mM hemin stock solution (see reagent preparation details previously described above)
- 50 μL glucose-6-phosphate at 2 mM final concentration
- 15 μL glucose-6-phosphate dehydrogenase at 0.5 IU per mL final concentration

The remaining 400 μL cell suspension for each experiment sample was added to the corresponding labelled tube containing the reaction mixture. A positive control was included using liver microsomal fraction from a rat treated with hemin for 24 hours. When all tubes were ready, 25 μL nicotinamide adenine dinucleotide phosphate (NADPH) at 0.8 mM final concentration was added to each tube to start the reaction. All tubes were vortexed well, wrapped in aluminium foil and incubated in a 37°C waterbath. The reaction was conducted for 1 h at 37°C in the dark as NADPH is light-sensitive. The
reaction was terminated by the addition of 1 mL of chloroform to achieve a 1:1 ratio of reaction solution to chloroform.

All tubes were then centrifuged at 1,000 x g for 5 minutes at room temperature. Three layers were then expected to appear to form a top aqueous layer, a middle solid layer of cell material and lipids and a clear organic layer at the bottom. All samples were vortexed briefly to disturb the bottom layer and then spun repeatedly at 1,000 x g until three layers were well defined. This ensured extraction of all the bilirubin into the chloroform.

The extracted bilirubin was calculated by measuring the difference in absorption between 464 and 530 nm of the lower chloroform layer isolated by careful pipette aspiration. The pipette tip was advanced through the overlying solid layer creating a smooth puncture to avoid any solid debris appearing in the aspirated chloroform layer. A quartz cuvette was used with pure chloroform as a blank sample using the extinction coefficient 40 mM$^{-1}$ cm$^{-1}$ for bilirubin.

Heme oxygenase activity was expressed as picomoles of bilirubin formed per milligram of cell protein per hour. The formula for calculation of heme oxygenase activity is as follows:

1. Bilirubin concentration in chloroform:

\[
\frac{OD_{464} - OD_{530}}{40} \text{ nm} \quad \text{[extinction coefficient } \epsilon = 40 \text{ mM}^{-1} \text{ cm}^{-1} \text{ for bilirubin]}\]

2. Bilirubin is extracted with 1 mL chloroform for each sample reaction. 1 mL chloroform contains:

\[
\frac{\Delta OD}{40 \times 10^3} \text{ mmoles of bilirubin formed over 1 hour by one cell suspension}
\]

3. Heme oxygenase activity as pmol bilirubin formed / mg cell protein / hour

\[
\frac{OD_{464} - OD_{530}}{40 \times (\text{protein content})} \times 10^6 \quad \frac{\text{pmol}}{\text{mg} / \text{hour}}
\]

The protein content of confluent cultured cells per experiment sample was measured by Bio-Rad DC protein assay method (Bio-Rad Laboratories Inc, Calif. USA) using bovine serum albumin as a standard. The protocols for preparation of rat liver cytosol as a
source of biliverdin reductase and rat liver microsomal fractions for a positive control both of which were required for the heme oxygenase enzyme activity assay were kindly performed by colleagues at the Northwick Park Institute for Medical Research.

2.8.3 Preparation of rat liver cytosol as a source of biliverdin reductase

Male Sprague-Dawley rats weighing between 200 – 250 grams were housed under laboratory conditions for at least 3 days prior to use. They were fed with commercial rat chow and water ad libitum. Animals were anaesthetised with intravenous ketamine and the liver perfused in situ with ice-cold 1.15 % (w/v) potassium chloride solution (KCl) via the portal vein. The liver was quickly harvested and kept on ice bathed in KCl solution. All remaining procedures were performed at 0 – 4°C. The liver was finely minced and homogenised in 2 – 3 volumes of cold 1.15 % KCl – 20 mM Tris Buffer (pH 7.4). Each homogenate was transferred to polycarbonate tubes and centrifuged at 5,000 x g for 20 minutes. The supernatant fraction was collected and centrifuged at 105,000 x g for 60 minutes. Following each centrifugation the turbid lipid layer was removed with a Pasteur pipette. After the final centrifugation the cytosol was removed taking care not to disturb the microsomal pellet. The liver cytosol was divided into aliquots and kept at -80°C until required for use as a source of biliverdin reductase.

2.8.4 Preparation of rat liver microsomal fractions for heme oxygenase positive control

Male Sprague-Dawley rats housed in experimental conditions as above were treated with hemin at a final concentration of 20 – 30 μM for 24 hours. At the end of the experimental protocol animals were euthanised by inhalation with isoflurane for 30 – 40 seconds or sacrificed by decapitation. The liver was perfused in situ with cold 0.9 % sodium chloride solution (NaCl) via the portal vein for 2 – 3 minutes until the liver parenchyma became blanched. The liver was then quickly harvested and homogenised in 5 volumes of 0.25 M sucrose with 0.05 M Tris-Hydrochloric Acid (HCl) buffer (pH 7.4). The liver tissue homogenates were centrifuged at 27,000 x g for 20 minutes. The remaining supernatant was then centrifuged at 105,000 x g for 90 minutes to yield the microsomal pellet. The microsomal fraction was suspended in 1mL 0.1 M PBS – 2mM MgCl₂ (pH 7.4) and kept at -80°C until required for use as a positive control for heme oxygenase activity assay.
2.8.5 Protein Assay

Several reported methods are available to analyse protein content within a particular sample, and each has advantages and limitations. The method of choice depends upon the analysis required – qualitative analysis to determine purity or quantitative analysis to provide protein concentrations. The most accurate method of determining protein concentration is probably acid hydrolysis followed by amino acid analysis that can calculate amino acid composition from primary analytical data and provides precise and sensitive quantitative and qualitative information. Spectrophotometric techniques are available to characterise protein samples and measure protein concentration by measuring the absorbance of the aromatic amino acids in a sample at different wavelengths. Fluorescence spectroscopy provides a highly sensitive and more qualitative evaluation of amino acid composition.

Colorimetric procedures can conveniently and reliably quantify the amount of protein contained within a sample solution. A chemical reagent is added to the sample solution producing a visible colour change that is quantitated with a spectrophotometer or microplate reader. The measured absorbance from the sample solution is compared against a standard curve of known concentrations of protein. This determines the total quantity of protein in an unknown sample via interpolation from the standard curve. The Bradford and the Lowry methods are the most frequently used colorimetric techniques. Both methods are sensitive to the amino acid composition of the protein, and absolute concentrations cannot be obtained. The technique of protein assay employed may depend on the type of protein, or the amount of protein likely to be available for assay. The choice of assay may also depend on experimental factors such as the chemical components contained in the buffer, the filters available for the laboratory spectrophotometer or the settings available on the microplate reader.

2.8.6 The Lowry Method for Protein Quantitation

The Lowry technique was reported in 1951 and the method was developed and adapted from earlier work of Wu advocating the use of the Folin phenol reagent for the determination of protein concentration. The method is based on both the Biuret reaction and its subsequent amplification by reaction with the Folin phenol reagent (Folin–
Ciocalteu reagent\textsuperscript{540}). It quantifies the colour obtained from the reaction of Folin-Ciocalteu phenol reagent with the tyrosyl residues of an unknown protein sample.

The Biuret reaction is based on the complex formation of cupric ions (Cu\textsuperscript{2+}) with the peptide bonds of proteins under alkaline conditions. In this reaction copper sulphate is added to a protein solution in strong alkaline solution containing sodium potassium tartrate. A purple-violet coloured chelate complex is formed between cupric ions and peptides containing three or more amino acid residues. The intensity of the colour produced is proportional to the number of peptide bonds participating in the reaction. The nature of the biuret reaction with unknown sample proteins is independent of the composition of the protein\textsuperscript{541-544}. The name of the reaction is derived from a similar reaction between copper and the organic compound biuret (NH\textsubscript{2}-CO-NH-CO-NH\textsubscript{2}) that is a product of excess urea and heat. Biuret forms a light blue tetradoentate chelate complex with cupric ions\textsuperscript{545}. Although protein composition is not a factor in the reaction, both protein purity and association state can influence the results obtained with the biuret reagent\textsuperscript{537 538 542 544}.

The Folin–Ciocalteau reaction is poorly understood. The principle depends on the reaction of the phenolic (R) group of tyrosine residues with colourless phosphomolybdotungstate ions in the presence of cupric ions. Folin-Ciocalteau reagent contains phosphomolybdotungstate complex which is a mixture of sodium tungstate, sodium molybdate and phosphate. The phenol groups within the tyrosyl of aromatic amino acids are oxidised in a reaction catalysed by alkaline copper sulphate. The phosphomolybdotungstate complex is reduced to a blue chromogen heteropolymolybdenum blue. The intensity of the blue-purple colour formed due to this oxidation-reduction reaction depends partly on the amount of aromatic amino acids tryptophan and tyrosine present in the sample. The intensity can be estimated spectrophotometrically by measuring the absorbance at 650 – 750 nm wavelength. Adding the use of Folin-Ciocalteau reagent greatly enhances the sensitivity of the Lowry assay. The method is reliably sensitive down to approximately 0.01 mg of protein per mL and is best used on solutions with expected concentrations in the range 0.01 – 1.0 mg per mL of protein.
Various substances can interfere with the Lowry reaction, including many nitrogen-containing buffers. There are two principle disadvantages of the Lowry protein assay method, namely that the amount of colour development is not strictly proportional to protein concentration and that this amount is variable depending on protein composition. The variation in colour development with different proteins reflects the pivotal contributions of specific amino acids tyrosine and tryptophan to the reaction.

Studies by Viner et al observed that oxidative inactivation of tyrosine and tryptophan decreased protein reactivity in the Lowry reaction. Protein variability can be minimised by performing the assay at an appropriate wavelength. Overall, the Lowry method sensitivity to amino acid composition is fairly constant from protein to protein. The method has been so widely used that Lowry protein estimations are an acceptable alternative to rigorous absolute determinations of protein content for various experimental approaches. Applicable circumstances include the measurement of mixed tissue protein, highly diluted protein samples or analysis of large numbers of similar protein samples such as antigen-antibody precipitates.

2.8.7 Protein Assay for HO activity assay

DC Protein Assay (Bio-Rad Laboratories Inc, Calif. USA)

The Bio-Rad DC (detergent compatible) protein assay is a colorimetric assay for protein concentration following solubilisation of any detergent contained in the samples to be analysed. If the test samples contain detergent, 20 μL of surfactant reagent is added to each ml of copper tartrate reagent needed for the assay. The DC protein assay technique was used to quantify protein in cell suspensions tested for heme oxygenase activity. The reaction is similar to the Lowry assay but has been modified to reach 95 % of its maximum colour development within 15 minutes of incubation and to have stable absorbance for at least 2 hours with a colour change of less than 5 % within 1 hour. It is based on the initial reaction between protein and copper at alkaline pH coupled with the subsequent reduction of Folin-Ciocalteu reagent by the copper-treated protein. Proteins reduce the Folin-Ciocalteu reagent by the loss of 1, 2, or 3 oxygen atoms yielding several possible reduced species that have a characteristic blue colour with a maximum absorbance at 750 nm and minimum absorbance at 405 nm. The intensity of the colour development
depends on the contribution of the amino acids tyrosine and tryptophan and to a lesser extent cysteine, cystine and histidine present in the sample proteins analysed.

The experimental protocol used 100 μL of protein sample with 100 μL of PBS as a blank sample. The Bio-Rad kit included an alkaline copper tartrate / carbonate (CTC) solution (reagent A) and a dilute Folin-Ciocalteu reagent at 20% (v/v) concentration (reagent B). For the protein assay, 500 μL of reagent A was added to the 100 μL test sample and vortexed to mix well. Then 4 mL of reagent B was added and vortexed followed by incubation at room temperature for 30 minutes. The absorbance was measured using standard disposable polystyrene cuvettes with the spectrophotometer at 750 nm within 1 hour. The spectrophotometer was equipped with a constant temperature cuvette chamber. Bovine serum albumin was used as a protein standard. The DC protein assay shows little difference in colour development between bovine serum albumin and bovine gamma globulin. A standard curve was prepared over a range of BSA concentrations from 0.125 – 1.5 mg/mL with a final volume 100μL. The BSA standard was prepared in the same buffer as the cell suspension test samples to minimise any interference effects. The dilutions for the standard curve were prepared according to the table below:

<table>
<thead>
<tr>
<th>Final concentration (mg/ml)</th>
<th>Volume of a 2mg/ml standard (μl)</th>
<th>Volume of water or dilution buffer (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (blank)</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.2</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>0.4</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>0.6</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>0.8</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>1.0</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>1.5</td>
<td>75</td>
<td>25</td>
</tr>
</tbody>
</table>

**Table 2.8.1: Standard dilutions for preparation of a calibration curve**

The standard curve fit is quadratic of the form \( y = ax^2 + bx + c \).
2.9 Flow cytometry

Measurement of DNA content for cell cycle analysis and characteristics of labelled probes for studies of apoptosis was performed using flow cytometry techniques.

2.9.1 Flow Cytometry Sample Preparation

When preparing a sample of cells for flow cytometry analysis the aims are to produce a suspension of single cells with minimal cell aggregation, minimal cell debris and the lowest possible number of dead cells. Cell clumps can cause disruption of fluid flow through the cytometer or block the flow channels within the instrument. Care must be taken during sample preparation especially when using cells in monolayer. Various enzyme techniques can alter cell-surface antigens and affect cell viability that introduces bias into the results obtained by cytometry analysis. In all flow cytometry experiments conducted, cells were filtered through a 70 μM nylon mesh immediately prior to analysis through the cytometers in order to prevent cell clumping.

2.9.2 Cyan ADP Flow Cytometer (Beckman Coulter)

The instrument used for all flow analysis was the CyAn ADP (three laser) 9-colour flow cytometer produced by Beckman Coulter. It has eleven parameters (forward scatter, side scatter and fluorescent channels FL-1 to FL-9) and is equipped with 488 nm, 405 nm, and 642 nm solid-state lasers. The CyAn ADP is capable of flow rates up to 150 µL per minute. The instrument includes sophisticated optics and utilises high performance electronics with pulse processing speed up to 70,000 events per second complementing a high acquisition and analysis capability of up to 1 x 10⁸ event data files. Summit software version 4.3 (Dako) was used for all data analysis applications.

2.9.3 Cell preparation and staining protocol

Measurement of DNA content for cell cycle analysis was achieved using propidium iodide (PI) staining of cell samples. Cells were prepared using ethanol to fix and permeabilise the plasma membrane to enable DNA staining of intact cells. Binding of propidium iodide to DNA is not covalent and is reversible, essentially following the law of mass action. Equilibrium exists in solution between available intracellular DNA binding sites and the
concentration of free fluorochrome. For diploid cells in G\(_1\) phase of the cell cycle there are 3 \(\times\) 10\(^9\) DNA base pairs per cell but some nucleosomal DNA within the cell is inaccessible to intercalating dyes and only a fraction of the potential binding sites (10-70\%) are actually available for staining\(^{551}\). If cell samples include an increased population of tetraploid cells arrested at the G\(_2\)/M phase then the higher DNA content of the sample causes a shift in equilibrium towards reduced concentration of free dye that consequently reduces the ability to stain DNA binding sites and altered fluorescence\(^{552}\).

Recommended standard conditions include using a fluorochrome ligand at 100 µM concentration to stain 1 x 10\(^6\) cells in a volume of 500 µL – 1 mL. Under these conditions there is an excess of available dye ligand per available DNA binding site meaning that an increase in cell number from 1 to 2 million (changing the concentration of free dye by 1\%) should not be reflected by any change in DNA staining greater than 1\%. Nevertheless it remains critically important to keep the ratio of dye:cells relatively constant especially when comparing DNA content histograms between cell samples of a specific experiment. For this reason every cell sample prepared was counted using a haemocytometer prior to staining with PI\(^{553}\)\(^{554}\).

Cell lines were seeded into experiment wells at an appropriate number to achieve 60 – 70\% density at the time of adding reagents 24 hours after seeding. For OE-33 and HET-1A cell lines cells were seeded into 6-well plates at 165,000 cells per well in 2.5 mL and into 24-well plates at 33,000 cells per well in 0.5 mL from a cell suspension of 1 x 10\(^6\) cells in 15 mL. For AGS and HGC-27 cell lines comparatively fewer cells were required to achieve the equivalent density after 24 hours. AGS and HGC-27 cells were seeded into 6-well plates at 125,000 cells per well in 2.5 mL and into 24-well plates at 25,000 cells per well in 0.5 mL from a cell suspension of 0.75 x 10\(^6\) cells in 15 mL.

Cell collection was performed at the appropriate time point with every step carried out on ice. Floating cells were first harvested into a 15 mL Falcon tube. Attached monolayer cells were then harvested by incubating with 4 mM EDTA PBS at 37°C for 5 minutes followed by aspiration into the corresponding Falcon tube. Cell suspensions were pelleted by centrifugation at 300 \(\times\) g for 6 minutes and re-suspended in 1 mL of cold PBS in the falcon tube to count the cells using a haemocytometer. Cell suspensions were then
aliquoted to use at approximately $1 \times 10^6$ cells per mL, washed with 10 mL PBS and resuspended in 250 µL cold PBS in the falcon tube. Cells were fixed and permeabilised by adding 1 mL of ice-cold 70% ethanol drop by drop with constant slow vortex to shake the tube. Cells were thoroughly re-suspended prior to adding the 70% ethanol to achieve a single cell suspension as fixation of cells that are in aggregates while suspended in PBS stabilises the aggregates which are then impossible to disaggregate. The fixed permeabilised cell suspension was incubated overnight at 4°C. Cells suspended in ethanol can be stored at 0–4°C for several months to a year.

The following day 10 mL of PBS was added to the ethanol-fixed cells, the suspensions centrifuged at 300 x g for 6 minutes and the ethanol carefully decanted. The resulting cell pellet was re-suspended in 5 mL of binding buffer and left for 1 minute prior to repeat centrifugation at 300 x g for 6 minutes. Finally the cell pellet was suspended in 500 µL of PI staining solution (50 µg·ml$^{-1}$ PI and 50 µg·ml$^{-1}$ RNase A in PBS). Stained cells were incubated at room temperature protected from light for 30 minutes prior to transferring the samples to the FACS laboratory for flow cytometry analysis. Cell cycle distribution was assessed counting 10,000 cells for each experiment sample.

### 2.9.4 Cell staining reagents

EDTA (Ethylenediaminetetraacetic acid) has a molecular weight of 380.2 so 0.38g powder was weighed and dissolved in 10 mL PBS to achieve a 100 mM stock solution. Stock solution was diluted further in PBS to produce a 4 mM solution to add to the cell monolayer for cell detachment.

Binding buffer was prepared as a 10x concentrated calcium chloride and sodium chloride buffer at pH 7.5 using 100 mM HEPES, 1.4 M NaCl and 25 mM CaCl$_2$. Sodium chloride (NaCl) was prepared to a 1.4 M solution by dissolving 8.18g in 100 mL ddH$_2$O (1M = 58.44g in 1000 mL). Calcium chloride was prepared to a 25 mM solution by dissolving 0.3675g in 100 mL ddH$_2$O (1 M = 147.0g in 1000 mL).

Ribonuclease A (RNase A) is an endoribonuclease that cleaves single stranded RNA. The enzyme attacks pyrimidine nucleotides at the 3'-phosphate group and cleaves the 5'-phosphate linkage to the adjacent nucleotide. RNase A works in the absence of cofactors.
and divalent cations and although active under a wide range of reaction conditions it can be inhibited by RNase inhibitor that occurs naturally in cell cytosol. At sodium chloride concentrations of 0.3 M or higher RNase A specifically cleaves single-stranded RNA.

For this application DNase-free ribonuclease A from bovine pancreas (Sigma-Aldrich R4642) was supplied as a solution in 50% glycerol containing 10 mM Tris-HCl buffer at pH 8.0 and diluted in PBS to a stock solution of 5mg per mL. The supplied preparation did not require the traditional boiling of RNase A solution to inactivate DNase and remained active for at least 2 years under recommended storage conditions at −20°C.

Propidium iodide (BD Biosciences) was provided in a PBS buffered pH 7.4 stock solution at a concentration of 1 mg per mL. A PI staining solution was prepared containing 50 μg per mL PI and 50 μg per mL RNase A in PBS. The PI solution contained 18.8 mL PBS, 1 mL PI stock at 1 mg per mL and 200 μL of RNase A stock at 5 mg per mL and was stable at 4°C for up to 3 months. Cell pellets of 1 x 10⁶ cells were suspended in 500 μL of staining solution containing 25 μg PI and 25 μg RNase A.

### 2.9.5 Cell Cycle Analysis

Cell growth and proliferation in eukaryotic cells is characterised by five distinct phases of development that constitute the cell cycle. Cell division represents a highly conserved and ordered process involving a tightly controlled series of molecular events. The sequence of events can be described as follows: an initial quiescent or resting state (G₀ phase) followed by cell growth with RNA and protein synthesis as the cell prepares for chromosomal replication (G₁ phase); DNA synthesis as chromosomes are replicated (S phase); preparations for cell division and repair of duplicated chromosomes (G₂ phase); mitosis and cytokinesis of parent cells (M phase) to give two daughter cells that immediately enter G₀ phase to continue the cycle. The three gap phases G₀, G₁ and G₂ and DNA replication during S phase are collectively referred to as interphase or between mitoses.

The DNA content within cells varies as the cells progress through the cell cycle. The DNA content of cells can be measured rapidly and conveniently by flow cytometry techniques. DNA content measurements are based on the properties of fluorescent nuclear dyes such
as propidium iodide that selectively bind DNA under appropriate staining conditions. Cells stained with nuclear dyes emit fluorescence after excitation with ultraviolet or 488 nm laser light in direct proportion to their DNA content with flow cytometers able to simultaneously measure cell volume parameters. Cells in the G₀/G₁ phases of the cell cycle are diploid, cells in the G₂/M phases are tetraploid and S phase cells are somewhere in between. Staining of the DNA of apoptotic cells is a simple method to quantify apoptosis within a sample. Apoptotic cells are sub-diploid (less than 2n) and the fragmented DNA strands result in a typical sub-G₀ peak on the histogram. The size of this peak can be used as an approximate indicator of the number of apoptotic cells.

2.9.6 Cell Cycle Data Analysis

Acquisition settings for measurement of DNA content using flow cytometry included limiting the event rate below a maximum of 200 counts per second and obtaining histogram plots of events versus PE Texas Red fluorescence. Initial histograms of pulse width versus forward scatter detected cell doublets. A polygon gate was drawn to exclude the doublets and applied to area versus linear plots and the gating process repeated for linear side scatter versus linear forward scatter histograms to correct for the size and granularity of cells.

At cell concentration below 1 x 10⁶ per mL the flow rate of the sample through the cytometers has to be increased which can degrade the value of the coefficient of variation. Alternatively if the concentration is too high there may be insufficient dye to stoichiometrically stain the DNA present in the sample.

The histogram plots obtained were checked systematically to satisfy the following criteria: the number of channels in the histogram should be at least 512; the flow rate should be kept low in order to obtain the best CV (coefficient of variation); the linearity of the plot should produce a tight and short distance G₁ peak with a CV of less than 8%; the ratio between G₁ and G₂ peaks should be between 1.95 – 2.05 and the cell debris measured should be up to a maximum of 30%. A polygon gate was drawn around cell debris on a single-parameter count rate versus PE Texas Red histogram and if greater than 30% then the threshold Texas Red fluorescence was altered accordingly.
2.10 Apoptosis studies: Plasma membrane changes

2.10.1 FITC-Annexin V and PI Staining for Apoptosis

Sequential changes occur in the plasma membrane during apoptosis. An early and unique event in the apoptotic sequence is the exposure of membrane phospholipids as the maintenance of plasma membrane asymmetry is impaired\(^{556, 557}\). Changes in the lipid composition of the plasma membrane and its permeability to small cations have been documented as relatively early signs of the apoptotic cascade\(^{558, 559}\). Membrane alterations usually follow the loss of mitochondrial membrane potential and caspase activation and appear before nuclear DNA fragmentation and DNA laddering\(^{560}\).

An asymmetrical phospholipid distribution is sustained by cell membrane proteins including aminophospholipid translocase that facilitate the translocation of lipid molecules from one leaflet to the other in a magnesium/ATP-dependent process\(^{561, 562}\). The activity of membrane proteins not only keeps phosphatidyl serine (PS) on the inner surface but also provides cells with the ability to translocate PS to the outer plasma membrane leaflet when required. Phosphatidyl serine generally constitutes less than 10% of the total membrane phospholipid composition. During apoptosis the function of aminophospholipid translocase is reduced and activity of the enzyme phospholipid scramblase is relatively increased. Translocation of phosphatidyl serine to the outer leaflet is thereby achieved with exposure of PS residues on the cell surface\(^{563, 564}\). The exposed PS on the cell surface has an important function as a marker for specific recognition by macrophages with PS receptors\(^{565, 566}\). The macrophages are recruited to perform phagocytosis of apoptotic cells and apoptotic bodies. Expression of PS residues at the outer plasma membrane leaflet has been demonstrated to occur early during apoptosis before loss of plasma membrane integrity\(^{559}\).

Flow cytometry techniques were used to detect binding of Annexin-V conjugated with FITC (fluorescein isothiocyanate) to exposed phosphatidyl serine (PS) residues in the plasma cell membrane as an indicator of early apoptosis. Annexin-V is a 35 – 36 kDa anticoagulant protein with a higher affinity for phosphatidyl serine (PS) than most other phospholipids. Annexin V is not able to bind to normal living cells since the molecule cannot penetrate the phospholipid bilayer of the plasma membrane. The FITC-Annexin V
conjugate reversibly binds to exposed PS residues in the presence of defined millimolar concentrations of salt and calcium ions (Ca$^{2+}$). Annexin V conjugated to fluorochromes with different absorption and emission wavelengths can be detected by flow cytometry and fluorescence microscopy$^{560,567}$. Cationic vital dyes such as propidium iodide (PI) or 7-amino-actinomycin (7-AAD) stain cellular DNA but are only able to infiltrate the cell after plasma membrane breakdown. Cells that are live or going through early apoptosis are still able to exclude the cationic dyes. As secondary necrosis occurs the plasma membrane loses the ability to exclude the dyes$^{568}$. Therefore annexin-V assays can be used in conjunction with plasma membrane permeability marker and analysed by flow cytometry.

2.10.2 Cell Seeding

Experiments for flow cytometry analysis of apoptosis were conducted using 6-well plates and 25 cm$^2$ growth area culture flasks (Nunc). The growth area of each well in a 6-well plate is 9.6 cm$^2$. For cell seeding purposes, AGS and HGC-27 cells were seeded at 125,000 cells per well of a 6-well plate in a well volume of 2.5 mL. OE-33 and HET-1A cells were seeded at 165,000 cells per well of a 6-well plate in 2.5 mL. For the T25 culture flasks AGS and HGC-27 cells were seeded at 350,000 cells per flask while OE-33 and HET-1A cells were seeded at 420,000 cells per flask, both in a volume of 7 mL per flask.

Individual flasks or triplicate wells were used as negative controls to set up flow compensation and dot plot quadrants for cytometry analysis. Controls included:

1. Unstained cells

2. Cells stained with FITC-Annexin V alone (no PI)

3. Cells stained with PI alone (no FITC-Annexin V)

4. Untreated experimental control cells stained with FITC-Annexin V conjugate and PI solution

Experimental reagents were added across various time points for a kinetic study aiming to demonstrate the progression of cells through viable cells to early apoptosis to secondary
necrosis. Time points chosen were 6 hours, 12 hours and 24 hours as standard with extension to 48 hours when indicated.

As a positive control cells were exposed to 1 µg per mL staurosporine for 3 hours.

2.10.3 Reagents

Binding Buffer

A buffer to promote annexin V binding to exposed PS residue sites was prepared to include 100mM HEPES buffer, 1.4 M sodium chloride (NaCl), 25 mM calcium chloride (CaCl$_2$) adjusted with 1M NaOH to a final pH 7.5. Sodium chloride solution at 1.4 M was prepared by dissolving 8.18g in 100 mL sterile water. Calcium chloride solution at 25 mM was prepared by dissolving 0.3675g in 100 mL sterile water. The binding buffer thus prepared was at 10X concentration to provide a final concentration (1X) of calcium ions at 2.5 mM required for binding of annexin V to phosphatidyl serine.

Annexin V and Propidium Iodide

Annexin V conjugated with FITC fluorochrome was purchased from Sigma-Aldrich (catalogue number A9210). The annexin V-FITC reagent contained approximately 50 µg per mL 50 mM Tris-HCl buffer at pH 7.5 and containing 100 mM NaCl. Propidium Iodide solution was also supplied by Sigma (catalogue number P2667) containing 100 µg per mL in 10 mM potassium phosphate buffer at pH 7.4 and containing 150 mM NaCl. Fluorescence-labelled annexin V needs to be stored refrigerated at 2–8°C and loses biological activity due to dimerisation if stored frozen.

Staurosporine

The p21 cell cycle inhibitor staurosporine (Sigma-Aldrich, catalogue number S5921) was dissolved in DMSO universal solvent to a concentration of 100 µg per mL. A working stock solution was prepared for storage at 1 mM and further diluted to 10 µM stock to give a final concentration of 1 µg per mL when added to experiment wells or culture flasks.
2.10.4 Cell collection

Cell morphology was documented for each experiment condition with attention to obvious rounding or detachment of cells to indicate apoptosis. The culture supernatant was collected into 15 mL Falcon tubes to include detached cells in the analysis. Cells were washed twice with sterile PBS. For experiments using 6-well plates adherent cells were detached by the addition of 2 mL 4 mM EDTA [0.15%] in PBS and incubation for 3-4 minutes. EDTA solution (EDTA MW = 380.2) was prepared by dissolving 0.380g in 10 mL PBS to obtain 100 mM stock solution that was diluted further with PBS to a 4 mM solution. Cells were then gently scraped and collected into corresponding Falcon tubes with addition of 2 mL serum complete medium to facilitate cell collection.

For experiments using T25 culture flasks a technique of labelling the cells prior to harvesting was used. The cell monolayers were washed twice with PBS before adding FITC-Annexin V and PI reagents in 1x binding buffer. Flasks were incubated in the dark at room temperature for 10 minutes. After labelling all flasks were washed twice in 1x binding buffer to remove excess staining reagent and detached using EDTA and cell scraping as described above.

Falcon tube cell suspensions were centrifuged at 400 x g for 6 minutes and cell pellets resuspended in 1x binding buffer at a concentration of approximately 1 x 10^6 cells per mL. Unstained cell suspensions from 6-well plates were labelled by the addition of 5 mL FITC-Annexin V conjugate and 10 mL Propidium Iodide solution to each cell suspension. Tubes were incubated at room temperature for 10 minutes and protected from light. Cells were then analysed by flow cytometry using 30,000 cells for each experiment sample.

2.10.5 Flow Cytometry Analysis

FITC-Annexin V was detected as a green fluorescence with a maximum emission of 528 nm. This was measured in the standard FITC Channel on a flow cytometer (FL1). Propidium iodide was detected as a red fluorescence with a maximum emission of 620 nm. This was measured on the PE Texas Red short channel on a flow cytometer (FL2 or FL3).
Initial analysis of unstained control cells was performed to establish the correct instrument settings. Pulse width and forward scatter was first analysed on a linear scale to discard cell doublets or cell clumps and the associated false positive results. Applying a gate below 64 pulse width on the histogram included only single file cell interrogations. The histogram of FITC versus Texas Red provided analysis of annexin V and PI staining and fluorescence emission. The FITC and Texas Red populations were brought on scale and instrument voltages adjusted to gate the population so that most were in the first log decade.

Control cells untreated by experiment reagents were then analysed to apply appropriate compensation with single staining of annexin V-FITC and PI and stained with both annexin V-FITC and PI. A positive control double-stained sample of cells treated with the apoptosis-inducing staurosporine was used to establish correct placement of quadrant regions. Cells that are early in the apoptotic process stain with the annexin V-FITC conjugate alone. Necrotic cells stain with both the propidium iodide solution and annexin V-FITC conjugate. It is not possible to have cells that are PI positive without the cells also being positive for annexin V binding as the PI staining reflects compromise of the plasma membrane. As the plasma membrane is disrupted, annexin V can also enter the cell and bind to PS residue sites on the internal cell membrane surface. If cells appear to be PI positive but annexin V negative then the FITC channel histogram gating is adjusted to include annexin V staining for all PI positive cells. Viable cells show no staining by either annexin V-FITC or PI solution (Figure 2.12.2).
Figure 2.10.1: Quadrants applied for analysis of Annexin V-FITC and Propidium Iodide fluorescent staining

The cells identified in each of the four quadrants can provide quantitative analysis of the cell populations studied in the experiment (Figure 2.12.3)

Figure 2.10.2: Quantitative analysis of apoptosis in cell samples by Annexin V-FITC and Propidium Iodide staining
2.11 Apoptosis studies: DNA Fragmentation Assay

Fragmentation of the genomic DNA is an irreversible event that commits the cell to death and is a biochemical hallmark of apoptosis. DNA degradation is able to occur via several pathways but in many systems it results from activation of a group of endogenous Ca$^{2+}$ and Mg$^{2+}$-dependent nuclear endonuclease enzymes termed DNases. Caspase-activated DNase (CAD) is the best characterised enzyme that is activated by cleavage of its inhibitor by initiation of caspase-3 and caspase-7$^{570}$. Caspase-activated DNase then translocates to the cell nucleus where its activity causes characteristic DNA fragmentation$^{571}$.

One of the hallmarks of late stage apoptosis is the fragmentation of nuclear chromatin resulting in an abundance of DNA strand breaks$^{572-574}$. Cleavage of nuclear DNA can generate double-stranded low molecular weight DNA fragments (mono- and oligonucleosomal fragments) as well as single strand breaks or nicks in high molecular weight DNA. DNA fragmentation can be detected at a single-cell level. Assays can demonstrate specific DNA breaks by labelling the ends of the nucleosomal fragments with subsequent colorimetric or fluorescent detection using flow cytometry or microscopy$^{575}$. A common method to identify apoptotic cells involves the in situ labelling of DNA strand breaks generated in the cell nucleus with either absorption dyes or fluorochromes$^{576-580}$. The free 3'-hydroxyl termini of DNA strand breaks can be labelled with fluorochromes by the attachment of modified nucleotides. A direct approach is the use of fluorochrome-labelled triphosphodeoxynucleotides (e.g. fluorescein-deoxyuridine triphosphate) in a reaction catalysed preferably by exogenous terminal deoxynucleotidyl transferase enzyme$^{579,581}$. Terminal Deoxynucleotidyl Transferase (TdT) enables detection of DNA strand breaks when used with a fluorescent marker. TdT enzyme has the ability to label blunt 3'-OH ends of double-stranded DNA breaks independent of a template. It preferentially catalyses the repetitive addition of deoxyribonucleotide triphosphates (dNTPs) to the exposed 3’-OH group of a DNA fragment. To label the end of the fragment fluorescently conjugated deoxyuridine triphosphate nucleotides (dUTPs) are used as a template for exogenous terminal deoxynucleotidyl transferase activity. An alternative labelling enzyme for DNA nick translation is DNA polymerase. The end-labelling technique is generally termed the TUNEL (TdT-mediated dUTP nick end labelling) assay$^{582}$. 

150
The TUNEL acronym is something of a misnomer since double strand DNA breaks are labelled as opposed to single strand nicks, and nucleotide analogues other than dUTPS are commonly used for this approach. Labelling DNA strand breaks with fluorochromes enables rapid analysis of cells by flow or laser scanning cytometry. Dual staining techniques allow the concurrent measurement of DNA content and analysis of the cell cycle phase position of apoptotic cells.

The TUNEL assay is the most widely used in situ method for detecting apoptotic programmed cell death. Originally described in 1992, TUNEL relies on the presence of breaks in DNA that can be identified by terminal deoxynucleotidyl transferase enzyme. The TdT enzyme promotes the template-independent addition of dUTPs to the exposed 3'-hydroxyl ends of double-stranded or single-stranded DNA breaks. The nucleotides incorporated by TdT enzyme activity form a polymeric tail composed of unlabelled nucleotides and chemically modified nucleotides or nucleotide analogues in a random sequence.

Chemical modifications of the nucleotide templates enable labelling of the oligomer molecule formed from their addition to DNA strand breaks. Labelling with fluorochromes enables direct detection of modified nucleotides such as fluorescein-dUTP. Indirect approaches use alternative haptens such as biotin (biotin-dUTP detected with streptavidin protein) or bromine (BrdUTP detected with fluorescein-labelled anti-BrdU monoclonal antibody). Cells are treated in the assay system so that the plasma membrane is permeable to all reagents and enzymes required to label the DNA fragments. Fluorometric detection is applicable with fluorochrome-labelled DNA fragments incorporating fluorescein-dUTP. Addition of biotinylated nucleotides to exposed 3’-OH sites is detectable using streptavidin-conjugated horseradish peroxidase.

2.11.1 Apo-BrdU in situ DNA Fragmentation Assay

A suitable substrate for terminal deoxynucleotidyl transferase enzyme is the deoxythymidine analogue 5-bromo-2'-deoxyuridine 5'-triphosphate (BrdUTP). This is a brominated nucleotide analogue that labels the DNA strand breaks by incorporation into the DNA oligomer. The 3’-OH termini of the fragmented DNA serve as primers for the reaction catalysed by exogenous TdT. Exposed primer sites are only present at a
detectable level in cells undergoing apoptosis\textsuperscript{585}. Incorporated BrdUTP can be identified by anti-BrdU monoclonal antibody labelled with fluorescein isothiocyanate (FITC). The FITC-conjugated anti-BrdU reagent is commonly used in studies of cell proliferation to detect BrdU incorporated during DNA replication\textsuperscript{586, 587}. Bromo-deoxyuridine replaces thymidine in dividing DNA and can be detected by antibodies to BrdU to measure new DNA synthesis. Of all the available deoxynucleotides BrdUTP is considered to be the most advantageous to label DNA strand breaks with a highly sensitive, simple and low cost assay. The BrdUTP nucleotide is more readily incorporated into DNA strand breaks than alternative ligands of larger size such as fluorescein or biotin\textsuperscript{585}. The greater incorporation generates a brighter signal when BrdUTP sites are identified by FITC-conjugated anti-BrdU antibodies.

Cell fixation and membrane permeabilisation are essential steps for successful labelling of DNA strand breaks. Cells are treated briefly with paraformaldehyde that cross-links small low molecular weight DNA fragments to other cell constituents. This fixation prevents the extraction of fragmented nuclear DNA that would otherwise occur during repeated centrifugation and cell sample rinsing. After fixation cells are permeabilised by suspension in ethanol.

The relatively complex procedure of DNA strand break labelling involves multiple reagents and is technically demanding. A negative result may reflect technical or methodological problems such as loss of TdT enzyme activity or degradation of BrdUTP substrate rather than the absence of apoptotic DNA strand breaks. The inclusion of positive and negative cell controls is required for every assay.

Extensive DNA degradation is considered to be a hallmark event of late apoptosis with the presence of DNA strand breaks highly specific for an apoptotic process. The abundance of strand breaks in apoptotic cells generates intense labelling in the TUNEL assay enough to ensure their positive identification. False-negative results from the TUNEL procedure may arise in late apoptosis when prior shedding of apoptotic bodies leaves cells with reduced DNA content or when such extensive DNA fragmentation occurs that small DNA fragments cannot be retained in the cell after fixation with paraformaldehyde. False-negative results may also result from atypical apoptosis when internucleosomal DNA
degradation does not occur keeping the number of DNA strand breaks in apoptotic cells below a significantly detectable threshold level by the TUNEL method.

Distinctive features of apoptosis include specific DNA fragmentation and chromatin condensation with preservation of the plasma cell membrane integrity that is lost in the process of necrosis. In some circumstances necrosis causes extensive DNA fragmentation and these cells can be indistinguishable from apoptotic cells using TUNEL assay despite having morphological hallmarks of necrosis\textsuperscript{588-590}.

Additional false-positive TUNEL results arise from some developmental processes such as spermatid maturation that involve chromatin condensation and a degree of transient DNA strand fragmentation\textsuperscript{591}.

It is generally recommended to validate TUNEL staining results using additional alternative criteria for identification of apoptosis such as analysis of morphological features or changes in the plasma cell membrane.

2.11.2 Apo-BrdU in situ DNA Fragmentation Assay Protocol

A DNA fragmentation assay was performed using the Apo-BrdU assay kit purchased from MBL International (Watertown, MA, USA). The assay kit includes brominated deoxyuridine triphosphate nucleotide (BrdUTP) that is incorporated into the 3’-hydroxyl termini of double and single-stranded DNA. These sites are identified with flow cytometry by staining the cells with fluorescein-labelled anti-BrdU monoclonal antibody. Components of the Apo-BrdU assay kit included:

One 120 mL bottle of wash buffer and one 120 mL bottle of rinse buffer (both containing 0.05% sodium azide); one 0.6 mL vial of reaction buffer containing cacodylic acid (dimethylarsenic); one 0.3 mL vial of FITC-labelled Anti-BrdU mouse monoclonal antibody (1 µg per test); one 30 mL bottle of Propidium Iodide/RNase A staining solution (PI 5 µg mL\textsuperscript{-1}, RNase 200 µg mL\textsuperscript{-1}). The kit also provided one 45 µL bottle of TdT (terminal deoxynucleotidyl transferase) enzyme (200 µg mL\textsuperscript{-1} in 50% glycerol solution) and one 0.48 mL bottle of BrdUTP nucleotide (54.7 µg mL\textsuperscript{-1}) both of which require storage at \(-20^\circ\text{C}\).
After observing cell morphology with light microscopy experiment cells were collected with the supernatant and centrifuged to a pellet of $1 - 5 \times 10^6$ cells. Cell pellets were resuspended in PBS and fixed by adding 1% (w/v) paraformaldehyde and placing on ice for at least 15 minutes. To prepare a 1% paraformaldehyde solution 1g paraformaldehyde was weighed in a fume hood and 100 mL PBS added. The paraformaldehyde was dissolved by heating in a water bath at 65°C for 2 hours. This solution is stable for at least 2 weeks stored at 4°C.

Fixed cells were then centrifuged at 300 x $g$ for 5 minutes and washed twice with PBS to remove excess paraformaldehyde solution. Cell pellets were re-suspended in PBS and added to 5 mL of ice-cold 70% (v/v) ethanol solution for membrane permeabilisation and stored at -20°C overnight or up to several days prior to analysis. Fixed and permeabilised cells were resuspended and centrifuged at 300 x $g$ for 5 minutes with the ethanol removed from the pellet by careful aspiration. Pellets were resuspended in wash buffer at approximately $1 \times 10^6$ cells per mL and centrifuged as above. The washing step was repeated once. The cell pellet was then resuspended in 50 μL of the DNA labelling solution. The DNA labelling solution was prepared for 10 assays with the following reagents supplied in the assay kit: TdT enzymes 7.5 μL and BrdUTP nucleotides 80 μL (both stored frozen) and TdT reaction buffer 100 μL (stored chilled at 4°C). The labelling solution was made up to 510 μL with 322.5 μL double-distilled H$_2$O. Cells were incubated in the DNA labelling solution at 37°C for 60 minutes. Each falcon tube was shaken every 15 minutes to ensure the cells were well suspended in solution.

The cell solutions were rinsed with the addition of 1 mL rinse buffer provided in the kit followed by centrifuge at 300 x $g$ for 5 minutes and repeating the process once. Fluorescein-labelled antibody was then added by resuspending the cell pellet in 100 μL of antibody solution. The antibody solution was prepared for 10 assays using 50 μL of the Anti-BrdU-FITC antibody provided and making up to 1 mL with 950 μL of rinse buffer (both stored at 4°C). Cells were incubated with the antibody solution at room temperature in the dark for 30 minutes. Finally 0.5 mL of propidium iodide and RNase staining buffer was added to the cells and suspensions incubated for a further 30 minutes in the dark at room temperature. Cells were analysed by flow cytometry within 3 hours of staining to count 20,000 cells for each experiment sample.
2.12 Apoptosis studies: Measurement of changes in $\Delta \psi_m$

Collapse of the electrochemical gradient across the mitochondrial membrane as measured by the change in the mitochondrial transmembrane potential ($\Delta \psi_m$) has been observed to occur during the early phases of apoptosis. Mitochondrial alterations often occur long before any identifiable morphological hallmarks of apoptosis\textsuperscript{592-594}.

Dissipation of mitochondrial transmembrane potential is frequently associated with the insertion and oligomerisation of pro-apoptotic proteins including Bax, Bak and Bad that are believed to create pores in the outer mitochondrial membrane. The activation and assembly of pro-apoptotic Bcl-2 family proteins in the mitochondria creates pores in the membrane and enables the release into the cell cytoplasm of various apoptosis-modulating small proteins normally enclosed in the intermembrane space of the organelle\textsuperscript{595-601}. These proteins include cytochrome c that is essential for activation of pro-caspase-9. Pores created in the mitochondrial membrane causes a coincidental loss of transmembrane potential. Although dissipation of $\Delta \psi_m$ has been thought to be a prerequisite for release of cytochrome c and therefore for caspase activation, there are reported examples where loss of $\Delta \psi_m$ precedes, accompanies or follows permeabilisation of the outer mitochondrial membrane\textsuperscript{594 602}.

Loss of mitochondrial inner transmembrane potential may not represent an irreversible point of no return for cell commitment to apoptosis. Fluorometric or flow cytometric detection of $\Delta \psi_m$ loss can be a sensitive marker of events in early apoptosis\textsuperscript{603 604}. Detection procedures are based on lipophilic cationic probes that are readily taken up by viable cells and accumulate in mitochondria according to the Nernst equation. Lipophilic cationic probe dyes accumulate within the mitochondria of healthy eukaryotic cells because the electrochemical gradient across the mitochondrial membranes is larger than the $\Delta \psi$ across the cytoplasmic plasma cell membranes\textsuperscript{605 606}.

To signal the loss of the mitochondrial membrane potential in experiment cells the Apo-Logix JC-1 Assay Kit was purchased (Bachem, San Carlos, CA, USA). The kit uses a unique cationic fluorochrome $5,5',6,6'-$tetrachloro-1,1', 3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1)\textsuperscript{607 608}. In healthy cells, energised mitochondria with an intact membrane establish and maintain a negative charge in the mitochondrial matrix that facilitates
uptake of the lipophilic JC-1 dye bearing a delocalised positive charge. The dye accumulates in the mitochondrial matrix and forms aggregates once a critical threshold concentration is exceeded. These J-aggregates emit a bright orange/red fluorescence. In apoptotic cells, collapse of the mitochondrial membrane potential leads to dissociation of the J-aggregates and transition to monomeric form. The JC-1 cannot accumulate within the mitochondrial matrix and remains in the cytoplasm in its monomeric form that exhibits green fluorescence.

With flow cytometry analysis the green monomeric form of JC-1 has absorption and emission maxima of 510 and 527 nm when excited with a 488 nm wavelength laser. The red JC-1 aggregates formed at higher concentration have absorption and emission maxima of 585 and 590 nm. Therefore, cells with a high mitochondrial \( \Delta \psi_m \) display orange-red fluorescence while cells with low mitochondrial \( \Delta \psi_m \) emit green fluorescence. The extent of JC-1 dye uptake and formation of aggregates is proportional to \( \Delta \psi_m \) status and can be directly measured by the intensity of cellular fluorescence. Apoptotic cells show primarily green fluorescence and can be easily differentiated from healthy cells that show red and green fluorescence.

### 2.12.1 Apo-Logix JC-1 Assay Kit Protocol

Assay kit components included lyophilised JC-1 reagent and 10X assay buffer both stored at 2 – 8°C. Assay buffer was diluted to 1X with double distilled water (1 mL of 10X assay buffer added to 9 mL dd H\(_2\)O). The lyophilised vial of JC-1 reagent was resuspended with 500 µL DMSO to obtain a 100X stock solution. Reagent crystals were completely dissolved in the solvent by gentle vortex and inverting the vial at room temperature. The reconstituted JC-1 reagent was aliquoted in small amounts sufficient for one session of experimental work and stored at -20°C in amber vials protected from light and moisture. The shelf life of resuspended JC-1 is six months if stored frozen at or below minus 20°C.

Immediately prior to use one vial of 100X JC-1 stock was defrosted in a water bath and diluted to 1X using warm serum-free culture medium or 1X assay buffer. Using small aliquots of frozen 10X JC-1 stock avoided the need for multiple freeze-thaw cycles of stock solution. If any undissolved particles remained in the diluted JC-1 solution, this was
centrifuged at 13,000 x g for 1 minute with careful transfer of the supernatant into a fresh tube by pipette aspiration excluding the pelleted debris.

Experiment cells were harvested and collected together with their supernatant. Cells were analysed at concentrations not exceeding 1 x 10^6 cells per mL. Cell suspensions were centrifuged at 300 x g for 5 minutes at room temperature and the supernatant removed by careful aspiration. Cell pellets were resuspended in 0.5 mL 1X JC-1 reagent solution as prepared above. The cell suspension was incubated at 37°C in a 5% CO₂ incubator for 15 minutes. Cell suspensions were again centrifuged at 300 x g for 5 minutes at room temperature, the supernatant removed and the pellet resuspended with 2 mL 1X assay buffer before centrifugation at 300 x g. The wash step with 2 mL assay buffer was repeated one time. The resulting cell pellets were resuspended in 0.5 mL fresh serum-free culture medium or 1X assay buffer ready for flow cytometry analysis.

Cells were analysed immediately by flow cytometry and fluorometry. Red JC-1 aggregates in the mitochondria of healthy cells were detected in the FL-2 fluorescence channel and green monomers in apoptotic cells were detected in the FITC (FL-1) fluorescence channel. The untreated control sample was used first to generate log FITC (x-axis) versus log PE Texas Red (y-axis) dual parameter dot plots with gating regions dependent on the peak population of control cells. Induced samples were then analysed with gated dot plots with a cell population appearing in the gated region due to a loss of red fluorescence corresponding to the loss of mitochondrial membrane potential in treated cells. The dot plot of red versus green fluorescence with red - % green compensation resolved live cells with intact ΔΨₘ from apoptotic and dead cells with lost mitochondrial membrane potential.

Additional analysis was performed on a FLUOstar Optima plate reader (BMG Labtech, Aylesbury, UK) with the filters set to 485 nm excitation and 520 nm emission (green) and 544 nm excitation and 590 nm emission (red). Data were presented as the ratio of red to green signals generated (590 nm/520 nm). Viable cells were expected to generate a higher 590 nm/520 nm ratio than cell samples undergoing apoptosis.
2.13 Apoptosis studies: Caspase Activity

A family of cysteine proteases that cleave a variety of intracellular target proteins are fundamental participants in apoptotic pathways. The enzyme family is termed caspases (from cysteiny1 aspartate-specific proteases). Biological caspase substrates include poly-(ADP ribose) polymerase (PARP), protein kinase C (PKC) delta, DNA-dependent protein kinase (DNA-PK), topoisomerases and sterol regulatory element binding proteins (SREBP)\textsuperscript{611-614}. In mammalian cells there are probably at least 14 protein caspases that form a closely related family of cysteine proteases. Caspases are the central mediators of the proteolytic cascade leading to elimination of cells selected for apoptosis. Caspase function is tightly regulated both transcriptionally and by endogenous anti-apoptotic polypeptides that can block caspase activation and prevent the proteolytic cascade\textsuperscript{615}. While specific functions can be identified for individual caspases some degree of overlap exists between them with occasional redundancy of function observed\textsuperscript{616}.

Currently there are 8 caspases identified as participants in the apoptotic pathway: caspase-2, caspase-3, caspase-12 and caspases-6 to -10 inclusive\textsuperscript{617 618}. Caspase enzymes also have crucial roles in cell signalling pathways and demonstrate specialised functions consistent with their role in apoptosis such as inflammatory responses and cytokine processing. Mammalian cysteine proteases can be divided into initiator and effector caspases and these are especially useful targets for the detection of cellular apoptosis.

Under normal physiological conditions caspase enzymes are constitutively expressed in all cells and exist in the cytoplasm as zymogen precursors with very low intrinsic activity. Zymogens are activated by cleavage to form a single heterotetramer with two active enzymatic sites in a head-to-tail configuration\textsuperscript{619}.

Activation of caspases can result from a variety of stimuli including exposure to radiation or chemotherapeutic agents, withdrawal of growth factors or initiation of the intrinsic apoptotic pathway. The active heterotetramers drive apoptotic events in a concerted effort with a proteolytic cascade causing self-amplification and cleavage of vital intracellular substrates. Cell homeostatic and repair enzymes are disabled and the cell structure is systematically disassembled. Apoptosis is the end result\textsuperscript{616 618 619}.
Assays designed to directly measure the activity of endogenous caspases can provide valuable information about the mechanism of death in dying cells. Fluorescent and luminescent substrates for specific caspases have enabled homogeneous assays to detect their activity to be developed. Specific antibodies that recognize the products of caspase cleavage or the activated caspase enzyme rather than its zymogen can also be used to detect cellular apoptosis. Fluorescently conjugated caspase inhibitors can specifically label individual active caspases within cells. This is achieved through binding to a caspase recognition element made up of a four amino-acid peptide. The most common recognition element contains the valine – alanine – aspartic acid residue sequence (VAD) that facilitates binding to activated caspases.

An alternative approach for measuring caspase activity involves the use of caspase substrates that generate fluorescent products upon cleavage by active caspases. The Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega, Madison, WI, USA) enables fast and sensitive measurement of caspase-3 and caspase-7 activity. These caspase family members have central effector roles in the apoptotic process in mammalian cells. Numerous homologues of CED-3, a proapoptotic gene of *C. elegans* and caspase family member have been identified in mammalian cells. Caspase-3-like proteases display specificity for cleavage at the C-terminal side of the aspartate residue of the sequence DEVD (Aspartate – Glutamine – Valine – Aspartate). The caspase-3-like proteases are inhibited by the tetrapeptide inhibitor Ac-DEVD-CHO.

The purchased Apo-ONE® Homogeneous Caspase-3/7 Assay Kit provides all the necessary reagents for caspase-3/7 activity assays. These include a profluorescent substrate together with a dual-purpose buffer optimised for both cell lysis and caspase activity. The proprietary buffer rapidly lyses and permeabilises cultured mammalian cells and supports optimal caspase-3/7 enzymatic activity. The assay uses a caspase-3/7 substrate that exists as a profluorescent substrate prior to the assay. The profluorescent substrate is rhodamine 110, bis-(N-CBZL-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide; Z-DEVD-R110). After the buffer and substrate are mixed and added to experiment cell samples, sequential cleavage and removal of the DEVD peptide sequence by caspase-3/7 activity leaves the rhodamine 110 group. The rhodamine 110 emits an intense green fluorescence with excitation and emission maxima of 499 and 521 nm wavelengths. The
amount of fluorescent product generated is proportional to the amount of caspase-3/7 cleavage activity present in the sample analysed by a fluorescent plate reader.

The assay using the Z-DEVD-R110 substrate is sensitive over short incubation times and can be scaled to suit the required sample number provided that a 1:1 ratio of assay reagent volume to sample volume is maintained. Multiple assays on the same experiment plate can be performed to provide valuable internal control data. Specific inhibition of caspase-3/7 activity may be achieved using the addition of the caspase inhibitor Ac-DEVD-CHO (Promega).

2.13.1 Apo-ONE® Homogeneous Caspase-3/7 Assay Protocol

The components of the assay kit included 100 μL Caspase Substrate Z-DEVD-R110 (at 100X concentration) and 10 mL Apo-ONE® Homogeneous Caspase-3/7 Buffer (1X). The complete system supplied contained sufficient reagents to make 10 mL of Apo-ONE® Caspase-3/7 Reagent suitable for 100 assays of 100 μL per well in a 96-well plate. 96-well opaque black plates (Nunc International Brand) suitable for cell culture and fluorescent plate reader analysis were used for experiment purposes. A fluorescent plate reader with FLUOstar Optima software was used for analysis with a plate shaker function to mix the reagents in each experiment well.

The buffer and concentrated 100X substrate were thawed to room temperature and mixed by vortexing. The substrate was diluted 1:100 by addition of 100 μL of 100X substrate to 9,900 μL buffer. The assay reagent thus prepared was stored protected from light at 4°C for up to 24 hours prior to use.

The following controls were included in triplicate:

Blank wells containing complete cell culture medium without cells + Apo-ONE Caspase-3/7 reagent; Control wells containing untreated cell culture + Apo-ONE Caspase-3/7 reagent; Negative control wells containing vehicle-treated cell culture + Apo-ONE Caspase-3/7 reagent. The vehicle was the appropriate solvent or carrier used to deliver the reagent of interest. Blank controls provided a measure of background fluorescence associated with culture samples and the assay reagent and were subtracted from
experimental values. Untreated control cell sample reactions determined the basal caspase activity of the experimental cell culture system.

100 µL of Apo-ONE® Caspase-3/7 Reagent was added to each well of the black 96-well plate containing 100 µL of blank medium, controls or experiment cell samples. Gentle mixing of reagent and samples was performed using a plate shaker at 400 RPM for 30 seconds. Pipetting was not used to avoid creating bubbles that interfere with fluorescence readings or the cross-contamination of samples. 96-well plates were incubated at 37°C protected from light for 1 hour prior to measuring fluorescence of each well using a plate reader. Data were collected on the plate reader over 4 hours. A time course of experiments was performed to assess caspase activity associated with experimental reagents after 6 hours, 12 hours, 24 hours and 48 hours.

The FLUOstar Optima plate reader (BMG Labtech, Aylesbury, UK) was used to measure fluorescence generated by caspase-3/7 activity. The microplate reader is capable of measuring time-resolved fluorescence intensity using a high-energy xenon flash lamp with an excitation and emission spectral range of 240 – 740 nm wavelengths. The manufacturer’s protocol for the Apo-ONE caspase assay was developed using a spectrofluorometer configured to detect caspase-3/7 activity at an excitation wavelength range of 485 ± 20 nm and an emission wavelength range of 530 ± 25 nm. Researchers have reported the optimal excitation wavelength for detection to be 499 nm with emission maximum at a wavelength of 521 nm\(^{624}\).

A caspase protocol was set up using the instrument software. This included microplate readings over 250 cycles with a reading time of 60 seconds and 20 lamp flashes per cycle at 37°C temperature. Excitation filters were set at 485 nm and emission filter at 520 nm. Maximum absorbance was measured at 65000 units of absorbance. A gain adjustment well was selected prior to each test run choosing a well of greatest intensity and adjusting reading such that 90% was the required value for endpoint readings and 30% of maximum activity was the required value in test run kinetic readings. The full spectrum of kinetic absorbance data was collected to generate signal curves over 250 cycles. A line graph from the raw data was obtained and the X-Y scatter analysed to identify a linear portion of the graph indicating a steep part of the signal curve for all signals generated.
This identified a particular time point or cycle number for all values. These values were then chosen for absorbance measurement and normalised to MTT assay to obtain the relative caspase activity per 1000 cells. Cell proliferation MTT assay was performed concurrently using clear 96-well plates under the exact experimental conditions as the 96-well opaque black plates used for caspase activity assay. Cell numbers per well were calculated from an MTT standard curve assay as previously described. The measured absorbance value for each well was divided by the cell number per well and multiplied by 1000 to give caspase fluorescence per 1000 cells.

2.14 Methods for Detecting Apoptosis

Events in apoptosis provide the opportunity to examine the process with a quantitative approach. Structural and biochemical features used to identify apoptosis include the loss of plasma membrane phospholipid asymmetry, nuclear DNA fragmentation and the mitochondrial permeability transition pore. The complex signalling cascade of apoptosis and its multiple regulation points allows evaluation of the activity of the various proteins involved.

The array of apoptotic quantifiable markers for apoptosis may vary according to the cell type, the level of stress or stimulus applied to the cell and the unique cellular microenvironment that modulates the cellular response. For example, markers including oligonucleosomal DNA fragments may be undetectable in cells challenged with variant stimuli such as inflammatory cytokines or growth factor deprivation. It is therefore recommended to always study several parameters at a time to provide a multidimensional aspect of apoptotic progress.

2.15 Timing of Apoptosis Assays

Apoptosis is a dynamic process with variable induction and execution kinetics. There is a short time-window during which apoptotic cells may display their characteristic features. The induction and progress of apoptosis can vary widely depending on the type of cell investigated. Different cell lines treated with an identical apoptotic stimulus may undergo apoptosis across an interval of between 2 hours and more than 24 hours. The interval between induction and execution of apoptosis also varies depending on the initial
stimulus applied\textsuperscript{626}. Cells of haemopoietic lineage tend to have a much shorter apoptotic induction time when compared to cells of solid tumour lineage. The length of the apoptotic process from initiation to complete cell disintegration also depends on the cell type investigated. Apoptotic cells can remain detectable in cell culture systems for an extended time period due to the lack of clearance of apoptotic bodies by phagocytosis that occurs \textit{in vivo}.

When identification of apoptotic cells relies on a specific marker that is detectable at variable time intervals, clear knowledge of the relevant time-windows for appearance of the marker is required. To determine if apoptosis is the primary mechanism of cell death, the duration of exposure to the cytotoxic reagent, the concentration of the test reagent and the choice of assay endpoint are critical for the experimental system. Different methods to measure apoptosis can produce different results depending on the timing of the assay and the process of apoptosis\textsuperscript{627}.

Loss of the mitochondrial transmembrane potential has been observed to be an initial transient event followed by later permanent collapse during the apoptotic cascade as depolarization of the mitochondrial membrane is followed by release of cytochrome c and caspase activation\textsuperscript{602}.

In the early stages of apoptosis terminal deoxynucleotidyl transferase can be positive for DNA breaks (TUNEL assay) but negative for the fractional DNA content (sub-G1 peak) as DNA fragments are still maintained in the nucleus. DNA laddering will not be observed on agarose gel electrophoresis until DNA fragments become isolated later in the process\textsuperscript{628}.

Extensive DNA fragmentation is considered to be a specific marker of apoptosis. The intensity of TUNEL assay labelling due to the large number of DNA strand breaks in apoptotic cells enables their discrimination from primary necrotic cells. Apoptotic or apoptotic-like cell death processes may however occur without extensive internucleosomal DNA degradation\textsuperscript{629}. If this occurs the intensity of cell labelling with TUNEL assay will be inadequate to positively identify apoptotic cells.

The plasma cell membrane can expose phosphatidyl serine residues that stain Annexin-V positive in early phases of apoptosis. Changes in plasma membrane integrity with the
appearance of blebs and the morphological hallmarks of apoptotic bodies can be identified at a later stage in apoptosis. It may often be difficult to distinguish late apoptotic cells (secondary necrotic cells) from the primary necrotic cells that have undergone accidental cell death. In both instances the cells cannot exclude cationic dyes such as propidium iodide or Trypan blue as the integrity of plasma membrane is lost.

2.16 Carbon Monoxide Releasing Molecules

For all experiments investigating the influence of CO on the cell lines studied, CORM-3 tricarbonylchoro(glycinato)-ruthenium(II) was used exclusively with the kind collaboration of Dr Roberto Motterlini and colleagues at the Northwick Park Institute for Medical Research. The CORM-3 was obtained as a material transfer agreement between Imperial College and Hemocorm limited company, the manufacturer and owner of intellectual property rights of the CORM compounds.

2.16.1 CORMS Preparation and Administration Protocol

CORM-3 was supplied as an off-white powder stable stored at -20°C. The compound is mostly present as an hydrochloric acid adduct with an effective molecular weight of 310 and chemical formula Ru(CO)$_3$Cl(NH$_2$CH$_2$CO$_2$). The powder forms a low-pH solution in water that remains stable for several hours. The half-life of CORM-3 is approximately 24 hours when the powder is dissolved in double-distilled water (ddH$_2$O). Dissolving 310g in 1000 mL or 0.31g in 1 mL gives a solution at 1M concentration. A workable stock solution at 10 mM concentration was obtained by dissolving 3 mL ddH$_2$O to 0.0093g powder. This stock solution was aliquoted into small volumes and frozen at -70°C for use within one month thereby avoiding multiple freeze-thaw cycles and minimising half-life impact. The stock was defrosted quickly for each experiment and a serial dilution performed immediately prior to adding the final solution to experiment wells. One mL of the 10 mM stock was added to 9 mL of serum-free medium to give a 1 mM stock solution. A volume of 600 µL 1 mM stock was then added to 2400 µL serum-free medium to give a final stock solution of 200 µM concentration. The appropriate volume of 200 µM solution was added to experiment wells to give the final dose of CORM-3. A negative control was prepared by dissolving an equivalent quantity of CORM-3 powder in equivalent volumes of sterile phosphate-buffered saline and leaving the solution at room temperature for 48
hours. The solution was bubbled with nitrogen gas in a fume hood using a Pasteur pipette after 48 hours and was then serially diluted as described above. This gave an inactive form of the CORM-3 termed iCORM-3. Universal precautions were followed for preparation of all CORM-3 reagents as the recommendation is to assume the compound to be toxic.

2.17 Bilirubin studies

2.17.1 Bilirubin reagent preparation

Bilirubin was purchased from Porphyrin Products (Frontier Scientific, Logan, UT, USA) and supplied as a 99% pure bilirubin-α crystalline powder with molecular weight 584.7 g. Initial experiments were performed with mixed-isomer bilirubin obtained from Sigma Aldrich but subsequent doubts emerged regarding the trace impurities in β- and γ-isomers of this product. Therefore all results were rejected and experiments repeated using 99% pure free unconjugated α-isomer bilirubin available from Porphyrin Products. Bilirubin solution was prepared by dissolving powder in 0.01 M sodium hydroxide alkali (NaOH) to a concentration of 1 mg per mL. Once dissolved the solution was diluted with an appropriate volume of sodium phosphate buffer and adjusted with 1 M hydrochloric acid (HCl) to pH 7.4 – 7.6. The diluted solution was sterile filtered through a 0.2 µm pore size filter (Sartorius) to obtain a working stock solution at 500 µM concentration. The 1 mgmL⁻¹ solution (1.71 mM) was stored at -20°C and defrosted to make up a 500 µM stock solution fresh for each experiment. All bilirubin solutions were stored protected from light and all experiments carried out avoiding direct light.

Sodium phosphate buffer was prepared using 50 mM Na₂HPO₄²⁻ (3.45 g dissolved in 500 mL ddH₂O) and 50 mM Na H₂PO₄²⁻ (3.549 g dissolved in 500 mL ddH₂O). Each solution was prepared separately, sterile filtered and combined to prepare the buffer stored at 2°C.

2.17.2 Bilirubin Experiment Protocol

Cells were seeded at optimum density into 24-well plates such that cells were 40 – 50% confluent at the time of adding reagents. After 24 hours incubation media was changed and supplemented with bilirubin reagent at the following concentrations: 0.1 µM, 1 µM, 5
µM, 10 µM, 20 µM and 50 µM (0.06 µg/ml – 30 µg/ml). These concentrations were chosen to provide include a spread across the physiological range. Experiment plates also included a control set of wells to which only medium was added and a vehicle set of wells to which the reagent vehicle solution was added. The vehicle solution was prepared with equivalent 0.01 M NaOH and sodium phosphate buffer solution and pH adjusted with HCl to pH 7.4 – 7.6.

Effects of prolonged exposures to bilirubin over 24 – 72 hours were compared to the effects of 24 hour treatment with bilirubin. The reagent medium was replaced with serum-complete medium after 24 hours and modified MTT assay performed at 24, 48 and 72 hours as described below.

2.17.3 Cell proliferation assay

Cell counts and cell viability MTT assays were performed at 24, 48 and 72 hours to assess the influence of 24-hour and extended bilirubin treatment. Cell counts were performed using Trypan blue exclusion using a standard haemocytometer having disaggregated cell clumps by passing the cell suspension through a 21G needle. Cell proliferation was determined with an indirect 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) to assay mitochondrial activity.

Previous work by Ngai and colleagues has identified that application of the MTT assay technique to studies using bilirubin can present the problem of interference with colorimetric readings. The data obtained by the formazan colour index can be affected by the yellow bilirubin crystal deposits in microplate wells. The reported study demonstrated a significant misinterpretation of MTT readings with the apparent results 10 – 24% higher than the actual concentrations due to bilirubin deposition. Dissolving the MTT formazan crystals in 0.04 mol L⁻¹ hydrochloride-isopropanol does not disturb bilirubin precipitates as bilirubin is not readily soluble in acidic solvents: less than 5% of the total bilirubin deposits were extracted into the supernatant. The MTT-formazan solution was transferred to a clean 96-well microplate for direct reading of the optical density (OD) with a spectrometer at 562 nm wavelength as previously described. The OD due to formazan produced in control cells was taken as 100% viability and all other measurements were expressed as a percentage (%) of the control cell value. This
modified MTT assay was adopted for all experiments using the MTT assay as it does not change either the peak or the absorption spectrum of the assay and produces highly reproducible and consistent results.631

Studies of apoptosis were performed using flow cytometry techniques described above to evaluate possible mechanisms for observed proliferation effects after bilirubin treatment. Further experiments were performed to investigate the impact of bilirubin treatment on reversal or prevention of the effects of heme oxygenase competitive inhibition or gene knockdown. Initial experiments identified bilirubin doses of 1, 5 and 10 µM for 24 hours did not cause any alteration in cell viability or mitochondrial activity assessed by MTT assay. This dose range and exposure time was therefore chosen to determine bilirubin effects after competitive HO enzyme inhibition with zinc protoporphyrin and specific siRNA-mediated knockdown of HO-2 protein. Zinc protoporphyrin reagent (ZnPP) and siRNA and negative control transfection reagents were prepared as previously described. Cells were exposed to 10 µM ZnPP for 48 hours, the reagent media discarded and replaced with fresh media supplemented with 1, 5 and 10 µM bilirubin. Similarly cells transfected with 10 nM siRNA vectors using 6 pmole of oligonucleotide were incubated for 48 hours as described above prior to treatment with bilirubin. Cell counts, cell viability assays, RT-PCR analysis of mRNA and Western blot analysis of protein expression were performed 24 hours after bilirubin addition.

2.18 Statistics protocol

All experiment results from cell proliferation studies are expressed as percentage of control for each individual experiment. Statistical analysis used the non-parametric Kruskal-Wallis Independent samples test to look for significant differences between results of study experiments or the Mann-Whitney U test to compare 2 independent samples. Analysis was based on data from experiments performed in triplicate or higher as indicated in the results chapter. When histograms of continuous variable data demonstrated a normal distribution, one-way ANOVA or the Students t test for 2 independent samples were used to compare means.
CHAPTER 3: Downstream Effectors of Heme Oxygenase Activity

3.1 The Role of Carbon Monoxide

3.1.1 Carbon monoxide biochemistry

Carbon monoxide (CO) traditionally has a bad reputation as an odourless, colourless air pollutant and toxic gaseous molecule due to its strong affinity for hemoglobin that causes a significant reduction in the oxygen-carrying capacity of hemoglobin and results in tissue hypoxia. Carbon monoxide also binds to myoglobin and mitochondrial cytochrome oxidases to cause impairment of oxygen utilisation in these molecules. The physiological effects of carbon monoxide are however not all negative. Recent studies demonstrate that CO has considerable influence on intracellular signalling processes that culminate in anti-inflammatory, anti-proliferative, and anti-apoptotic effects. Heme oxygenase enzyme activity produces endogenous levels of CO in the body. Studies document the antioxidant and cytoprotective properties associated with activity of the inducible HO-1 isoform. Endogenous CO may be generated as an effector of HO-1 activity and may provide the means for a physiological protective response to stress. In experimental models looking at HO-1 in the response to stress, administration of CO at appropriate doses results in a comparable protective response even when heme oxygenase activity is totally abolished.

There are two major sources of CO in mammalian biology: production as a by-product of heme oxygenase enzyme activity and a heme oxygenase-independent source as a result of severe stress that is not achieved under normal physiological conditions. Severe stress causes oxidation of organic molecules, phenols, and flavonoids and the peroxidation of lipids that generates CO. The major source of CO in mammal is via the degradation of heme by heme oxygenase with consequent production of CO, iron and biliverdin. Mammalian tissues have the ability to generate CO locally as cells express both inducible (HO-1) and constitutive (HO-2) heme oxygenase isoforms. Heme oxygenase enzymes are found in most human tissues with high abundance in the spleen (HO-1), liver (HO-2 and HO-1), brain (HO-2), vascular endothelial cells and smooth muscle tissues (HO-1 and HO-2). Rapid increases in CO levels that occur in vivo are solely due to either the
induction of HO-1 or constitutive HO-2 activity\textsuperscript{636}. Recent evidence supports the concept that carbon monoxide functions as an important cellular signalling molecule\textsuperscript{236,637}.

Endogenous CO production can be detected in exhaled breath. Adults exhale approximately 6 cm\textsuperscript{3} of CO per day. The activity of heme oxygenase accounts for about 86\% of endogenous CO production in humans. The majority originates from the degradation of heme in the spleen and liver where senescent red cells are processed. A variety of sources such as lipid peroxidation, bacteria and xenobiotics generate the remaining 14\% of CO produced\textsuperscript{638}. Without external sources of CO, approximately 0.6\% of hemoglobin is bound to CO as carboxyhemoglobin as a reservoir of body carbon monoxide\textsuperscript{639}. CO is a relatively stable and unreactive molecule \textit{in vivo}. The amount of CO converted to CO\textsubscript{2} in mammals is very unlikely to be significant. CO binds to heme and heme proteins including cytochromes in a reversible reaction. The half-life of CO bound to myoglobin is approximately one minute before the carboxymyoglobin dissociates\textsuperscript{640}. There are two isoforms of heme oxygenase enzyme characterised: the inducible HO-1 that is up-regulated in response to a wide variety of insults that cause a threat to cell homeostasis and survival\textsuperscript{641-643} and the constitutive HO-2 isoform present in organs and tissues including brain, liver and endothelium that has regulatory functions associated with neurotransmission and changes in vascular tone\textsuperscript{159,641}. Various disease states cause a marked increase in CO production including asthma, bronchiectasis, cystic fibrosis and diabetes\textsuperscript{644-648}.

\textbf{3.1.2 Carbon Monoxide Toxicity}

Carbon monoxide toxicity results from a combination of tissue hypoxia and direct CO-mediated damage at a cellular level that is independent of HbCO-induced hypoxia. Several pathophysiological mechanisms for CO toxicity have been suggested including lipid peroxidation and oxidative stress and ischaemia-reperfusion injury\textsuperscript{649,650}. Intracellular targets of CO activity such as cytochrome c oxidase contribute to the adverse and ultimately lethal effects of CO at HbCO levels above 20\%. Binding of CO to cellular cytochromes has been demonstrated to contribute to mitochondrial CO toxicity. A sustained and significant inhibition of cytochrome c oxidase activity (complex IV of the mitochondrial respiratory chain) has been observed in acute CO poisoning of human
lymphocytes. Alonso and colleagues reported a statistically significant and progressive inhibition of cytochrome c oxidase activity parallel to increased concentrations of CO. Other enzyme complex activities did not show any significant variation in relation to the presence of CO.

Whereas CO is directly toxic to human mitochondria, carboxyhemoglobin is not. The toxic effects of HbCO are due to the consequent tissue hypoxia. Recent studies observe inhibition of cytochrome c oxidase activity that is dependent on oxygen concentration as CO directly competes with oxygen binding of the mitochondrial cytochromes. Tissue hypoxia caused by high affinity binding of CO to hemoglobin and myoglobin inhibits cytochrome c oxidase synergistically with CO as the reduced oxygen level enables competitive CO binding. The inhibition of cytochrome c oxidase may play a role in the development of acute CO poisoning symptoms and in disease effects associated with smoking. The significance of cytochrome c oxidase inhibition by endogenously produced CO is debatable. CO binds with such high affinity to hemoglobin and myoglobin that free levels of cellular CO are likely to be very low.

3.1.3 Endogenous CO production and prognostic value

Carbon monoxide levels measured in different animal models of oxidative stress and tissue injury have been demonstrated to correlate with both the degree of stress and with increased tissue survival. Limited human trials report a higher production of CO in critically ill patients when compared to healthy controls. Endogenous CO production is higher in septic patients than in controls with an elevated level of CO measured at 24 hours in patients with sepsis that survived the episode as opposed to non-survivors.

Increased serum leucocyte counts in intensive care have been found to have a significant correlation with HbCO levels as a marker of total body CO content. The increased white blood cell count was associated with increased severity of illness. In paediatric studies significantly higher levels of exhaled CO have been observed in full term septic infants admitted to neonatal intensive care compared to healthy neonates. The possibility of CO as a mediator in the pathogenesis of neonatal sepsis is supported by a described correlation between cyclic guanosine monophosphate (cGMP) and HbCO levels.
in preterm infants with respiratory distress syndrome. An increase in cGMP mediated by CO may contribute to systemic vasodilatation and hypotension in preterm neonates.

Studies of mechanically ventilated intensive care adults found higher levels of endogenous CO production in patients with cardiac disease and those undergoing dialysis as compared to other critically ill patients. The levels of CO correlated with serum bilirubin and with serum creatinine reflecting the severe renal failure prevalent in these patients. Recent studies have reported a strong trend of higher levels of exhaled CO in critically ill patients that survived the illness without a clear correlation demonstrated between exhaled CO and illness severity or degree of inflammation. Arterial HbCO levels measured at low or high extremes are associated with increased mortality in intensive care patients suggesting an optimal range of increased CO production.

There are numerous questions surrounding the clinical significance of carbon monoxide levels. CO levels may provide a marker of disease severity or progression and may have a direct role in disease pathogenesis. Increased endogenous CO production may reflect this role or indicate enhanced heme oxygenase activity in response to stressful conditions. Protective effects of heme oxygenase induction may become detrimental at excessive induction levels. Measurement of CO levels during the early stages of acute pathological conditions might be crucial in understanding and predicting survival outcomes in intensive care.

CO levels may provide a useful prognostic marker particularly in inflammatory disorders. Exhaled CO levels are elevated in patients with seasonal allergic rhinitis and can reflect the severity of pulmonary inflammation in asthmatic patients. Levels of exhaled CO are also higher measured in preterm infants with bronchopulmonary dysplasia and in patients with cystic fibrosis. In ulcerative colitis patients with active disease have an increased level of CO detectable in the colon. Patients with cirrhotic liver disease have elevated arterial HbCO levels although no correlation with disease severity has been reported.
3.1.4 CO at lower doses

Initial studies on lung injury in animal models found evidence to support a valuable action of CO in the stress response. These findings were corroborated in later studies examining more tissues including heart, liver, kidney, intestine and the reticulo-endothelial system. Endogenous production of CO and inhalation of exogenous CO gas can produce beneficial cytoprotective outcomes provided that the oxygen-carrying capacity of hemoglobin is not severely compromised. Studies suggest a threshold level of HbCO < 20% below which favourable responses are observed against inflammation, cell proliferation, apoptosis, organ injury, vasoconstriction and both systemic and pulmonary hypertension. Available evidence supports the potential use of small amounts of CO gas as a therapeutic agent to treat conditions of cellular stress.

Studies on cellular and animal models indicate a complicated CO-dependent cell signalling network that varies in response to a specific stimulus and the type of tissue involved.

The mechanisms and signalling pathways involved in CO-mediated activity are yet to be fully understood. Carbon monoxide is likely to target guanylate cyclase activation to mediate vascular tone and blood pressure regulation. Various signal transduction pathways are implicated in the anti-apoptotic and anti-inflammatory function of CO. These include the mitogen-activated protein kinase cascade (MAPK), particularly the p38 protein MAP kinases, phosphatidylinositol 3-kinases (PI3-K) and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) signalling pathways. The multiplicity of CO effects and variability of CO action make its specific targets difficult to interpret. The proposal of CO as a future therapeutic option is made more feasible with the advent of controlled methods for delivery of CO to the tissues using CO-releasing molecules (CORMs).

3.1.5 Carbon Monoxide and Nitric Oxide

Nitric oxide (NO) is generated by nitric-oxide synthase (NOS) enzyme that contains the heme moiety. Specific effects mediated by NO can be duplicated by CO including the regulation of action of certain neurotransmitters and muscle relaxants. Both CO and NO are active in the vascular endothelium with NO established as a powerful vasodilator.
While similarities exist between CO and NO gaseous monoxides there are substantial differences in the molecules regarding their modes of action. Physiological stimuli tightly regulate NO synthesis by the constitutive NOS isoforms via intracellular release of Ca\(^{2+}\). The half-life of NO is strictly limited due to its unpaired electron (NO·) causing rapid free radical reactions with reactive oxygen species, sulfhydryl groups or metal ions within the cell. A short-lived burst of NO production results from stimulation of the constitutively expressed NOS isoforms NOS1 and NOS3. This transient rise in NO leads to a rapid and brief increase in local cyclic GMP levels reflecting a 100 to 400-fold activation of soluble guanylate cyclase (sGC). A significant increase in sGC activity is due to NO binding with the prosthetic heme moiety of sGC leading to formation of a nitrosyl heme complex\(^{673,674}\).

Carbon monoxide is likewise considered a vasorelaxant via stimulation of cGMP but the relative potency of CO much smaller compared with that of NO. Carbon monoxide is not a free radical and its production by heme oxygenase activity is not so tightly regulated. When CO binds to the iron of the prosthetic heme group of sGC a heme complex is formed with only a five-fold increase in activity of the sGC enzyme\(^ {675}\). However, the potency of the CO-mediated increase in cGMP may be significantly increased by other mechanisms including the sensitization of sGC to CO in biological systems\(^ {676,677}\).

Recent evidence shows that HO-derived CO can regulate NO production by inhibition of NOS enzymes\(^ {678}\). The impact of the HO pathway in the control of cGMP levels can be thereby substantially augmented with recent studies demonstrating the contribution of CO product to hypertension and endothelial dysfunction in Dahl salt-sensitive rats\(^ {679}\).

Heme prosthetic moieties are commonly present in enzymes that catalyse reactions of single electron transfer. CO can bind to heme and may disrupt the activity of a variety of heme protein enzymes other than sGC. Published evidence supports a panel of susceptible enzymes including xanthine oxidase, NADPH oxidase, and prostaglandin-H-synthase all of which underlines the significance of the HO pathway in modulation of the oxidative stress response\(^ {680-683}\).
3.1.6 Carbon Monoxide Effects

Up regulation of heme oxygenase and measurement of HO-1 protein levels does not directly equate to measurement of HO activity. HO-1 gene expression is readily induced by a variety of stimuli. Studies by Kruger and colleagues reported that increased HO-1 protein in Zucker rats did not result in increased total HO activity. Antioxidants and peroxynitrite scavengers with inducers of HO-1 restored the activity of preexisting inactive HO-1 to active HO-1 with a resulting increase in the levels of CO and bilirubin, restored endothelial NOS, decreased blood pressure, and normalised kidney function. Therefore, the measurement of HO-1 protein or a description of an increase in HO-1 protein expression does not mean an increase in HO activity and consequent increased production of bilirubin and CO. The measurement of total HO enzymatic activity is essential.

Carbon monoxide and bilirubin derived from HO activity lead to relaxation of vascular smooth muscle mediated via both cGMP-dependent and cGMP-independent pathways such as stimulation of specific potassium channels and an increase in adiponectin levels. The vasodilator effect produced by CO on the endothelium may be due to a CO-mediated reduction in the cytochrome P450-dependent synthesis of vasoconstrictors. Cytochrome P450 metabolises arachidonic acid to generate the very potent vasoconstrictor 20-Hydroxyeicosatetraenoic acid (20-HETE). Activation of calcium-dependent potassium channels is an important mechanism for vasodilatation independent of cGMP.

Direct effects of CO can result from binding of high levels of exogenously administered CO to heme proteins. Excessive CO competes with NO for prosthetic heme binding sites causing a transient increase in unbound NO that will lead to the production of peroxynitrite anion (ONOO−) and oxidative tissue damage in the presence of reactive oxygen species. Binding of excess CO to heme proteins disrupts mitochondrial transport and leads to release of superoxide anions (·O2−) that react with NO− to form the powerful oxidant peroxynitrite and cause tissue damage. Administration of CO in the form of CO donors must therefore be carefully controlled. CO-releasing compounds have been shown to be effective in delivering CO and to cause induction of HO-1
protein$^{695}$. Treatment with CO can reverse or reduce the adverse effects of treatment with inhibitors of heme oxygenase enzyme, suggesting that endogenous CO contributes to the protective effects of HO activity. CORMs are able to deliver CO effectively thereby preventing renal injury and attenuating the endothelial cell death associated with diabetes in vivo$^{695,696}$.

Treating endothelial cells with CO-releasing molecules restores glutathione (GSH) levels that are diminished when cells are treated with short interfering RNA or HO inhibitors$^{655}$. Carbon monoxide up regulates the expression of the rate-limiting enzyme in GSH biosynthesis glutamate cysteine ligase via activation of the phosphatidylinositol 3-kinase pathway and HO-1 mediated Nrf2 transcription factor signalling$^{655}$. Cell treatment with a CO donor up regulated expression of the catalytic subunit of glutamate cysteine ligase associated with protective effects of CO against NO-induced cell death$^{697}$.

Carbon monoxide is a neurotransmitter in the autonomic peripheral nerves of the gastrointestinal and urogenital tract. In many synapses CO appears to act synergistically with nitric oxide. One or the other gas may be dominant at specific sites that may differ in different species. A physiological function of CO as a brain neurotransmitter has been proposed$^{236}$. Functions attributed to CO in the central nervous system include neuroendocrine functions, circadian rhythm, cerebellar development, learning and memory, temperature control and pain perception. CO may be the principal physiological regulator of cGMP levels in the brain via activation of guanylyl cyclase. The HO-2 isoenzyme is found closely associated with guanylyl cyclase, ALA synthase, cytochrome P450 reductase, and NOS in brain tissue$^{637}$. Although not yet clearly defined, the role of HO-2 in the brain could be the production of CO to function in cell signalling pathways.

Evidence for the physiological functions of endogenous CO is largely indirect and often inconclusive. In studies of HO knockout mice HO-1 deficiency causes adverse effects primarily from heme toxicity and iron recycling deficiency. HO-1 expression is induced as an adaptive response to a wide variety of stresses that cause acute or chronic inflammatory responses. The HO-2 deficient mouse phenotype demonstrates impaired intestinal mobility, pain perception and subtle behavioural defects all of which have been attributed to deficient CO production.
3.1.7 Carbon monoxide targets

The cellular fate of CO formed during heme degradation by HO enzyme is not completely understood. CO preferentially binds to and interacts with heme proteins or transition metal-containing proteins. As reported for nitric oxide, CO can bind to oxyhemoglobin as well as to other heme proteins to affect their heme prosthetic moieties and alter enzymatic activity\textsuperscript{159, 698}. CO has been demonstrated to inhibit apoptosis of endothelial and epithelial cells and to reduce proliferation of smooth muscle cells, fibroblasts and T lymphocytes. CO also inhibits the production of cytokines implicated in a variety of disease pathologies including tumour necrosis factor (TNF), interleukins IL-1 and IL-6, and endothelin. CO activity is mediated by its molecular interaction with proteins such as soluble guanylyl cyclase, high conductance calcium sensitive K+ channels and mitogen-activated protein kinases (MAPK).

CO effects may involve three broad mechanisms: activation of soluble guanylyl cyclase (sGC) with resultant increase in cyclic guanine monophosphate (cGMP) production, modulation of the MAPK pathway and binding of CO to a range of intracellular heme proteins with formation of metal-CO complexes and inhibition or activation of enzymes containing heme that generates varied biological effects.

3.1.8 cGMP dependent pathways

CO binding to the heme moiety of soluble guanylate cyclase activates the enzyme and increases cGMP levels to relax vascular smooth muscle and cause vasodilatation. Soluble guanylyl cyclase is classically considered as the target to mediate CO cellular actions, but there is far less CO stimulation of sGC compared to NO, with CO only about 1/80th as effective as NO in activation of sGC. Very high levels of CO are normally required to activate soluble guanylate cyclase. Furthermore, CO can be demonstrated to effect vasodilatation despite concurrent administration of blockers of the cGMP pathway. These observations suggest the involvement of cellular mechanisms independent of sGC activation\textsuperscript{699, 700}. Interaction of CO with the cGMP pathway may be particularly relevant in neuronal tissues. The HO-2 enzyme isoform co-localises with soluble guanylate cyclase primarily in neural tissues including the brain. At these sites there is therefore a close association between a potential source of endogenous CO and a potential target of CO
activity. Heme oxygenase inhibition by false substrates or knockdown of the HO-2 gene can cause adverse effects to central and peripheral nervous system functions\textsuperscript{236 674 701 702}.

3.1.9 cGMP independent pathways

3.1.9.1 Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels

Published reports suggest that vasodilatation produced by CO is mediated via activation of calcium-dependent potassium channels. Blockade of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels inhibits vasodilatation associated with CO and inhibition of HO activity compromises K\textsuperscript{+} channel activity in smooth muscle cells\textsuperscript{691 703}. Activation of vascular 238pS and 105pS Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels render smooth muscle cells less responsive to the actions of vasoconstrictors\textsuperscript{704 705}. In the normal physiology of smooth muscle cells, K\textsuperscript{+} channel activity is regulated by a local transient increase of Ca\textsuperscript{2+} concentration in the micromolar range. Activation and opening of large conductance potassium channels enables passive flow of K\textsuperscript{+} ions down the electrochemical gradient causing an efflux of K\textsuperscript{+} from the cell and plasma cell membrane hyperpolarisation. Cell excitability is decreased and voltage-dependent Ca\textsuperscript{2+} channel activity is reduced resulting in smooth muscle cell relaxation\textsuperscript{706}. Endogenously generated or exogenously administered CO increases the amplitude and frequency of K\textsuperscript{+} channel activity dependent on micromolar changes in Ca\textsuperscript{2+} concentration. This may be an essential mechanism for CO-mediated vasodilatation\textsuperscript{707}. The Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel has a tetrameric structure containing α-subunits that are critically involved in its activation process. Each α-subunit contains a heme-binding pocket and binding of heme to the channel inhibits its activity\textsuperscript{708}. CO activates the K\textsuperscript{+} channel by binding to protein-bound prosthetic heme and altering the interaction of heme with the intrinsic active protein of the α-subunit resulting in an increase in sensitivity to Ca\textsuperscript{2+} concentration\textsuperscript{688 709 710}.

Oxygen levels can regulate calcium-sensitive potassium channel activity via a similar mechanism of heme binding. Carbon monoxide derived from HO-2 activity has been demonstrated to be an essential component of this regulatory control of respiration by oxygen sensing. Carotid body cells demonstrate inhibition of Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channel activity induced by hypoxia that is dependent on HO-2 expression and augmented by increased HO-2 activity\textsuperscript{215}. 
3.1.9.2 Mitogen-activated protein kinases

Carbon monoxide has been demonstrated to regulate mitogen activated protein kinases (MAPK) related pathways with specific activation of p38 protein and down-regulation of extracellular signal-regulated kinase 1/2 (ERK1/2). MAPK-regulated CO effects include cytoprotective, anti-inflammatory and anti-proliferative activity via cell cycle arrest.

Production of inflammatory cytokines is suppressed by CO activity involving the c-Jun N-terminal kinase (JNK) pathway. Physiological concentrations of CO inhibit the production of inflammatory cytokines such as interleukin-1 (IL-1), macrophage migration inhibitory factor (MIF) and tumour necrosis factor (TNF-α) from activated macrophages. The secretion of interleukin-2 (IL-2) from activated T-lymphocytes is also suppressed by CO via selective activation of the MAPK pathway.

The protective effect of HO-1 up regulation has been attributed to an increase in cGMP levels induced by CO and CO-mediated activation of the p38 MAPK pathway. Recent studies in the human lung epithelial cell line A549 demonstrate inhibition of the expression and activity of the matrix metalloproteinases MMP-1 and MMP-2 by carbon monoxide effects regulated by the MAPK system. These findings imply a central role for CO in the pathophysiology of emphysema and other alveolar diseases involving a protease/antiprotease imbalance.

It has been demonstrated that different cell types and stimuli can produce variable effects of CO on MAPK signaling cascades. CO inhibits the proliferation of pancreatic stellate cells by activating p38 MAPK whereas CO produces a similar reduction in proliferation of human airway smooth muscle cells by inhibiting ERK activation. The mechanisms by which CO modulate MAPK pathways are not fully elucidated. CO slows the rate of electron transport in the mitochondria enabling electrons to accumulate at complex III. This promotes the production of superoxide anion (O$_2^-$) and its conversion to hydrogen peroxide (H$_2$O$_2$) by manganese superoxide dismutase. Hydrogen peroxide derived from mitochondria can activate the MAPK and Akt protein kinase signalling pathways.
3.1.9.3 Heme protein binding

Carbon monoxide binds to the heme moiety of other hemoproteins thereby affecting their enzymatic activity. Alternative targets of CO include nitric oxide synthase\textsuperscript{292 717 718}, cytochrome oxidase and mitochondrial complexes\textsuperscript{461 719-722} and NADPH oxidase\textsuperscript{722}. There is published evidence to support a role for CO in stimulation of cyclooxygenase (COX) activity\textsuperscript{723}. Treating rat hypothalamic explants and primary cell cultures of rat hypothalamic astrocytes with the heme oxygenase substrate hemin increased the production of prostaglandin E2 via activation of COX. Treatment with the HO inhibitor tin mesoporphyrin-IX reduced E2 production and adding hemoglobin as a CO scavenger abolished the stimulatory effect on COX activity. Furthermore, exposure of rat hypothalamus samples to a CO-saturated medium caused significant increases in prostaglandin E2 release\textsuperscript{723-725}.

Additional evidence indicates targeting of various heme proteins such as cytochrome P450 and biologically relevant protein ligand systems containing transition metals\textsuperscript{713 726}. CO can bind to iron, nickel and copper in bacteria, and copper in cytochrome c oxidase\textsuperscript{727}. Binding of CO to cytochrome P450 and cytochrome c oxidase alters their enzymatic activity and provides a potential mechanism for CO effects. Protection against reperfusion injury mediated by CO may be implemented via competitive binding of CO to cytochromes that prevents their activation by oxygen.

The potential for iron binding is particularly interesting given that HO activity produces biliverdin, CO and ferrous iron (Fe\textsuperscript{2+}). The ferrous iron is not oxidised to ferric iron (Fe\textsuperscript{3+}) in the heme oxygenase system. Ferrous iron reacts with cysteine to give Fe(cysteinate)\textsubscript{2} which readily absorbs CO to produce Fe(cysteinate)\textsubscript{2}(CO)\textsubscript{2}\textsuperscript{728}. Fe\textsuperscript{2+} binds CO much more readily than Fe\textsuperscript{3+} via these reactions.
3.1.10 Adverse CO Effects

Pharmacological levels of CO have cytoprotective properties when added to cell cultures and in vivo models. Appropriate CO levels can inhibit apoptosis, inflammation, platelet aggregation and proliferation of vascular smooth muscle. Carbon monoxide may also exert deleterious effects in certain organs, particularly by stimulating pro-inflammatory responses in the brain. CO activates cyclooxygenase enzyme in rat ex vivo hypothalamus and primary cultures of rat hypothalamic astrocyte cells resulting in the increased formation of pro-inflammatory prostaglandins. CO may cause oxidative tissue damage by increasing the formation of mitochondrial reactive oxygen species through CO binding to cytochromes residing in complex IV. Intracellular levels of antioxidants such as glutathione can be reduced by this mechanism. Inhibition of cytochrome oxidase by endogenously produced CO is thought to be physiologically insignificant. CO binds so tightly to hemoglobin and myoglobin that levels of available free CO are likely to be very low.

3.2 Clinical Aspects of Carbon Monoxide

3.2.1 CO in inflammation

The anti-inflammatory activity of CO could have the greatest potential for future therapeutic applications. Inflammation is the underlying cause of chronic diseases including diabetes, cardiovascular disease, cancer and obesity. Acute inflammation corresponds to the constitutive systemic response to acute conditions such as microbial infection.

Chronic inflammation is a pathological condition characterised by ongoing active inflammation, tissue loss and tissue repair processes. Chronic inflammatory tissue typically includes infiltration of activated macrophages and monocytes that generate inflammatory mediators including nitric oxide. Carbon monoxide has been demonstrated to inhibit NO production in macrophages and reduce inflammation. Nitric oxide synthase contains a heme prosthetic group that provides a binding site for CO. CO binding impairs the activity of NO synthase preventing the enzymatic oxidation of arginine to citrulline and NO.
The anti-inflammatory response mediated by HO-1 activity and its products CO and bilirubin also impairs expression of intercellular adhesion molecule-1 (ICAM-1). Production of pro-inflammatory cytokines such as TNF-α is reduced while important anti-inflammatory molecules such as interleukin-10 (IL-10) are upregulated\textsuperscript{730}. Evidence from several cell culture experiment models indicates that CO directly inhibits the production of pro-inflammatory cytokines TNF-α and IL-1β induced by lipopolysaccharide endotoxin (LPS) while stimulating the release of IL-10\textsuperscript{249, 297, 642}. Similar CO effects are documented from studies in animal models with induction of inflammatory responses caused by LPS, ischaemia–reperfusion injury and organ transplantation often involving various cytokines such as IL-2 and IL-6\textsuperscript{249, 642, 731-733}. Mice exposed to CO at 250 ppm for 1 hour before administration of LPS exhibited an inflammatory response with significantly lower levels of TNF-α and IL-1β and higher levels of IL-10 as compared to control mice subjects.

Cytoprotective effects of pharmacological or inhalation CO therapy in animal models of acute lung injury and sepsis are mediated by multiple cellular and molecular mechanisms including vasodilatation, maintenance of mitochondrial integrity and regulation of the redox state by controlling the production of reaction oxygen species (ROS). Levels of HO-1 and CO regulate cellular autophagy by means specific to the cell type involved.

CO has demonstrated a therapeutic benefit in experimental animal models of ventilator-induced lung injury, oxidative and acid-induced lung injury, challenge with LPS endotoxin and sepsis induced by caecal ligation and puncture\textsuperscript{734, 736}. A recent study describes the effects of timing of CO administration with respect to lung protection in animal subjects undergoing mechanical ventilation. Pre-treatment with CO had no measurable impact on ventilator-induced lung injury. Delaying administration of CO until 3 – 5 hours after the start of mechanical ventilation reduced the observed lung damage, cellular infiltrate and accumulation of neutrophils. Treatment with CO for 6 hours protected against ventilator-induced lung injury (VILI)\textsuperscript{736}. A similar study of VILI in a mouse model demonstrated protective effects of CO on mechanically-ventilated lung. CO treatment prevented the early up regulation of early growth response-1 (Egr-1) associated with mechanical ventilation with decreased neutrophil infiltration and prevention of lung injury. CO effects were mediated by peroxisome proliferator-activated receptor-γ and dependent on inhibition of Egr-1\textsuperscript{737}. 
The effects of CO inhalation on systemic inflammation have been studied during experimental endotoxaemia in humans. In a double-blinded randomized controlled trial with a two-way crossover trial design experimental endotoxaemia was induced in healthy volunteers by injection of 2 ng per kg LPS endotoxin. Inhalation of 500 ppm CO sufficient to increase HbCO levels was compared to inhalation of synthetic air as a placebo for 1 hour. No adverse effects of CO inhalation were observed despite an increase in HbCO levels from 1.2% to 7% in treatment subjects. CO inhalation had no effect on the inflammatory response as measured by systemic production of cytokines TNF-α, IL-1β, IL-1α, IL-6 and IL-8. A recent clinical feasibility study reported the administration of inhaled CO to human subjects with chronic obstructive pulmonary disease (COPD). Ex-smokers with stable COPD were subjected to CO inhalation at 100–125 ppm for 2 hours per day for 4 consecutive days. Arterial carboxy-Hb levels increased to 4.5%. CO inhalation demonstrated a trend towards reduced sputum eosinophils and an improved response to inhaled methacholine challenge.

The efficacy and potential of CO therapy in humans with inflammatory conditions remains relatively uninvestigated, and further controlled clinical studies in human subjects are required. A recently completed trial supported by the U.S. National Institutes of Health Clinical Center aims to examine how CO inhalation affects local pulmonary inflammatory responses in healthy volunteers subjected to bronchoscopic instillation of bacterial endotoxin. Clinical trials in the recruiting phase include studies of CO therapy for severe pulmonary hypertension and idiopathic pulmonary fibrosis and a trial of CO inhalation to reduce the duration of post-operative intestinal ileus.

3.2.2 Potential of CO as a therapeutic agent

Experimental models of acute lung injury including mechanisms of ischaemia, hyperoxia and acute respiratory distress syndrome (ARDS) have demonstrated increased expression of stress-induced HO-1 measured in broncho-alveolar epithelial cell and macrophage cell specimens. Administration of exogenous CO has shown promising therapeutic potential in animal models of acute oxidative lung injury, ventilator-induced lung injury, pulmonary fibrosis and lung transplantation. These observations suggest a contribution of the HO-1 pathway in protecting pulmonary function during pathological...
conditions. Comparatively high levels of exhaled CO have been observed in rodent models of acute lung injury induced by extracorporeal circulation and in experimental ARDS\textsuperscript{744 745}. These findings indicate that sustained levels of substrate are available to maintain increased heme oxygenase activity in pulmonary tissue.

*In vitro* studies performed with lung epithelial cells and fibroblasts confirm that up regulation of HO-1 conferred resistance to ischaemic lung injury, oxidative damage and apoptosis mediated by TNF-\textalpha\textsuperscript{279 746 747}. Cytoprotective and anti-oxidative effects in the respiratory and cardiovascular system can be observed with direct exposure to exogenous CO gas as well as other effectors of heme oxygenase activity biliverdin and bilirubin\textsuperscript{748}. Whereas the biliverdin and bilirubin products have direct capability to scavenge oxidants and prevent damage, the CO product has additional unique activity in cell signalling pathways.

Beneficial properties of CO have also been documented for *in vivo* experimental models. The majority of experiments involve administering CO gas at 200 to 400 ppm in the air supplied for inhalation. The first study to include CO inhalation at low concentrations as a therapeutic strategy observed a protective effect of CO gas against hyperoxic lung injury and proposed anti-inflammatory and anti-apoptotic CO activity as the likely mechanism responsible\textsuperscript{269}. Carbon monoxide inhibits the development of post-operative intestinal ileus in a mouse model and is involved in the control of gastrointestinal contractile activity\textsuperscript{749 750}. CO administration also reduces the inflammation associated with allergen-induced asthma in mice\textsuperscript{734}.

In rodent models CO has been demonstrated to protect against inflammation associated with orthotopic lung transplantation and oxidative lung injury. CO checks the progression of atherosclerotic lesions associated with chronic graft rejection and with intimal vessel wall injury following balloon angioplasty\textsuperscript{297 751 752}. Experiment rats exposed to 400 ppm of CO gas for 2 days following mouse-to-rat cardiac transplantation had graft survival evident at 50 days post-transplant. Control rats exposed to air alone had no suppression of graft rejection and the organs survived only 5 to 7 days after transplantation\textsuperscript{634}.

Further success with rodent models has been observed with CO treatment able to reverse established pulmonary hypertension\textsuperscript{271}. Cytoprotective effects on the respiratory system
produced by CO gas exposure seem to be mediated by mitogen-activated protein kinase pathways (p38, MKK3) and caspase-3 and cyclic-GMP-dependent mechanisms. CO inhalation treatment may reduce the deposition of fibrin in the microvasculature thereby preventing a major pathophysiological contribution to ischaemic lung injury.

CO exposure has similar actions in protecting against ventilator-induced lung injury by reducing TNF-α production and neutrophil recruitment to the affected alveoli. Evidence for the potential therapeutic benefits of CO inhalation in animal models of lung disease is not universal. Clayton and colleagues report no significant benefit of CO treatment on acute lung injury due to hyperoxia and observed neurotoxicity at relatively low levels of CO exposure (200 and 500 ppm).

The role of endogenous CO as a vasodilator in the cardiovascular system is associated with guanylate cyclase (sGC) activation and consequent increases of cGMP levels as well as stimulation of various potassium channels. In a porcine model CO exposure improved cardiac output and protected the heart during reperfusion after cardiopulmonary bypass. Although nitric oxide is the more important physiological vasodilator the role of endogenous CO may become more relevant when NO signalling pathways are disrupted. The CO-mediated responses of the vasculature may be tissue-dependent. Although CO treatment provokes vasodilatation in most vessels investigated including the aorta and cerebral vessels it has also been observed to cause vasoconstriction rather than dilatation in gracilis muscle arterioles.

3.2.3 Heme oxygenase-1 (HO-1) and stress induction

Induction of heme oxygenase-1 (HO-1) enzyme can produce beneficial effects mediated via an increased production of endogenous CO. Increased expression of HO-1 protein occurs in cells and tissues exposed to agents or conditions that cause an increase in oxidative stress. Enhanced HO-1 expression may contribute to the therapeutic activity of drugs including aspirin and statins in the cardiovascular system. Further experiments are required to establish clear evidence for this relationship.

A number of compounds that occur naturally in fruits and vegetables are capable of potent HO-1 induction, including curcumin found in turmeric, L-sulforaphane present in broccoli, resveratrol in grape skin and organosulfur compounds derived from red...
wine and garlic\textsuperscript{765}. Although again direct experimental evidence is lacking, a high intake of certain fruits or vegetables may exert beneficial action on the cardiovascular system via increased HO-1 activity and consequent increased endogenous CO production.

3.2.4 Clinical development of CO therapy

Initial attempts to use CO inhalation for therapy were performed for sickle cell anaemia and reported by Beutler in 1975\textsuperscript{766}. Carbon monoxide administration at a concentration of 1000 – 2000 ppm significantly prolonged the life span of chromium-labelled red blood cells in two patients with sickle cell disease. Decreased sickling of red cells results from binding of CO to hemoglobin and an increased affinity of hemoglobin for O\textsubscript{2} shifting the O\textsubscript{2} dissociation curve to the left.

A recent pilot study examined the effects of CO inhalation on the inflammatory response to experimental endotoxaemia \textit{in vivo} using a randomised, double-blinded, placebo-controlled crossover trial in healthy volunteers\textsuperscript{738}. Healthy male smokers aged 18 to 40 years randomly inhaled either synthetic air as placebo or 500 ppm CO for 1 hour with a washout period of 6 weeks in between both treatments. Carboxyhemoglobin levels were assessed as a safety parameter with an HbCO level greater than 10% defined as a stop criterion. Immediately after inhalation all volunteers received an intravenous bolus of reference endotoxin \textit{E. coli} lipopolysaccharide (LPS) at a dose of 2 ng per kg. CO inhalation increased carboxyhemoglobin levels from 1.2% to peak values of 7.0%. Infusion of LPS induced transient increases in cytokines (TNF, IL-6, IL-8, IL-10), c-reactive protein and prothrombin fragments in serum, as well as IL-1\textalpha{} and IL-1\textbeta{} mRNA levels in blood cells. Administration of inhaled CO had no significant effect on any LPS-induced response observed. Increases in blood pressure, heart rate and body temperature after LPS treatment were not prevented by CO inhalation. Contrasting results were reported in rodent models using CO inhalation at 250 ppm for 1 hour\textsuperscript{738}.

This trial was somewhat controversial as some observers considered the study to violate acceptable standards of ethical research. There remains a lack of consensus regarding the safety threshold of CO exposure in a trial context. Opponents of CO inhalation therapy studies maintain that low dose CO inhalation (e.g. 100 ppm CO for 1.5 to 2.5 hours) has been proven to have adverse neuropsychological effects. This opinion does
not consider the lack of such effects directly caused by CO in other studies and in smokers exposed to higher CO doses. Circumstantial evidence exists that CO has beneficial effects in humans as CO inhalation in smokers may partially protect women from pre-eclampsia\textsuperscript{767} and may provide partial protection against cell proliferation of the intimal vessel wall following balloon angioplasty\textsuperscript{768}.

Several CO inhalation trials are currently in progress with the aim of assessing the therapeutic viability of CO inhalation\textsuperscript{769-772}. The U.S. National Institutes of Health programme includes various phase I clinical trials that are either recently completed or currently recruiting to investigate trials of CO inhalation therapy in severe pulmonary hypertension, idiopathic pulmonary fibrosis and prevention of post-operative intestinal ileus\textsuperscript{741,773}.

The therapeutic use of CO gas presents several practical and clinical problems. It has to be administered through the lungs as a mixture with air at a typical dose of 200 ppm. The whole body is therefore exposed to it and the level of blood carboxyhemoglobin has to be carefully monitored. A preferable approach would be to selectively administer the CO to disease tissue targets that may be achieved using a metal carbonyl solution to deliver a tightly controlled therapeutic local dose.

There is recent reported interest in the targeted induction of mitochondrial biogenesis as a potential therapy for multiple organ failure that can be mediated by CO activity. A murine model of \textit{Staphylococcus aureus} sepsis was investigated using inhaled carbon monoxide at 250 ppm once daily for 1 hour. CO treatment activated mitochondrial biogenesis via induction of HO-1 enzyme and increased mitochondrial HO activity through Nuclear-related factor-2 transcriptional and Akt kinase activity. Survival of mice with \textit{S. aureus} sepsis was significantly enhanced with CO inhalation compared to control subjects with demonstrable resolution of hepatic mitochondrial damage\textsuperscript{774}. 
3.3 Carbon Monoxide Releasing Molecules

3.3.1 Development of CORMs

Increased expression of heme oxygenase-1 (HO-1) enzyme has been demonstrated to provide beneficial effects in a wide variety of pathological conditions. These findings have fostered interest in the therapeutic potential of heme oxygenase inducers, heme oxygenase gene transduction and cell permeable heme oxygenase-peptide conjugates. An alternative strategy is the direct application of carbon monoxide in its gaseous form, the end product of heme oxygenase activity. Experimental animal disease models have shown beneficial effects using inhalation of gaseous CO or oral administration of methylene chloride, a compound that generates CO through its metabolism in the liver.

Under pathological conditions endogenous CO production is delayed or reduced so that its potential for beneficial effect is not realised. Administration of exogenous CO may have therapeutic potential in clinical applications but the systemic delivery of inhaled gaseous CO is not practical outside a hospital setting. It requires ventilators, face masks, normobaric chambers or portable inhalers. Systemic inhalation delivers CO indiscriminately to tissues using the same pathway that eliminates endogenously produced CO under normal physiological conditions. Delivery of CO to therapeutic targets involves the binding and dissociation of CO with hemoglobin. The hemoglobin molecule has a very high affinity for binding CO so that a relatively high loading of hemoglobin with CO is required to deliver a CO dose to target tissues within the therapeutic range. This dose range is likely to be associated with the adverse effects of mild CO poisoning. An ideal solution would be the primary delivery of therapeutic CO to diseased tissue targets before the gas diffused into the blood to be eliminated by respiration\textsuperscript{732}.

The limitations of CO inhalation therapy may be overcome by the use of a promising alternative pharmacological method: the development of transition metal-based carbon-monoxide-releasing molecules (CORMs). These compounds have been designed with the aim of providing safe and controlled administration of gaseous CO to a specific tissue target.
3.3.2 CORM design

A substantial panel of organic compounds and metalloorganic complexes that generate CO under different conditions have been designed and synthesised by chemists. The nomenclature CO-releasing molecules was introduced by CORM pioneers Roberto Motterlini and colleagues at the Northwick Park Institute for Medical Research and the University of Sheffield. CORMs are essentially prodrugs that deliver active carbon monoxide without adverse effects on healthy tissues. Selective delivery of CO to diseased tissue targets may be further enhanced by incorporating CORMs into macromolecular carriers such as cyclodextrins and liposomes with attached specific targeting devices. The clinical applications of CORMs may be expanded by conjugating them with drugs such as aspirin to complement the therapeutic effects and potentially counteract the adverse effects of the drug.

The two major classes of CORMs are organic molecules and metalloorganic complexes. These are similar to organic nitrates and the metalloorganic compound nitroprusside that have longstanding clinical applications in the delivery of nitric oxide. There is a complex interaction between gaseous mediators NO and CO in human tissues with many similar effects on shared target proteins. Carbon monoxide may have several advantages over NO as a therapeutic principle due to much lower toxicity at comparable doses, its more restricted target range and greater stability. The goal of CORM candidate development is to establish drugs that are stable in healthy tissues but released in diseased tissue targets. Studies are ongoing in tissue culture and animal models to determine the kinetics of CO release under different conditions. Optimisation of CORM designs and specific properties of bioavailability, tissue distribution and pharmacokinetics are yet to be established.

3.3.3 Mechanism of CO release

Carbon monoxide is known to bind strongly to transition metals in organic solvents to form stable carbonyl complexes. CORMs are designed as a metal carbonyl compound or equivalent salt and at least one appropriate carrier with pharmacologically acceptable properties. Carbon monoxide becomes available for physiological effect from the metal carbonyl with a reversible process of CO binding via one or more of the
following mechanisms: dissociation of the metal carbonyl produces CO in dissolved form; the metal carbonyl releases CO on contact with a solvent, tissue, organ or cell; the metal carbonyl releases CO on irradiation.

3.3.4 Types of CO-releasing molecules

The first CORMs identified were transition metal carbonyl compounds Mn$_2$CO$_{10}$ (manganesedecacarbonyl) and Ru(CO)$_3$Cl$_2$ dimer [tricarbonyldichlororutheium-(II)-dimer] that subsequently became commercially available and termed CORM-1 (Mn$_2$CO$_{10}$) and CORM-2 [Ru(CO)$_3$Cl$_2$-dimer]. When stimulated under appropriate physiological conditions these compounds liberated CO and promoted typical CO-mediated pharmacological effects such as vasodilatation and hypotension in vivo$^{693}$. CORM-1 is insoluble in water and releases CO by photolysis when stimulated by light, producing a marked reduction of coronary vasoconstriction in isolated rat hearts$^{693}$ and increasing blood carboxyhemoglobin levels, renal blood flow, glomerular filtration rate, and urinary cGMP excretion$^{778}$. Both CORM-1 and CORM-2 are lipid-soluble and soluble in organic solvents such as dimethylsulfoxide (DMSO) commonly used as a vehicle for biological experiments. The pharmacological action of Mn$_2$CO$_{10}$ and Ru(CO)$_3$Cl$_2$ dimer has been confirmed to be directly linked to the CO liberated, and the CO-releasing properties of both chemicals have been well characterised$^{693}$.

In an effort to improve the compatibility of these compounds with biological systems water-soluble carbonyl complexes were designed and developed. Another ruthenium-based compound tricarbonylchloro(glycinato)-ruthenium (II) (CORM-3) dissolves in water and rapidly releases CO in physiological fluids$^{672, 779}$. The chemistry of transition metal carbonyls is varied and highly versatile with CORM development not restricted to particular compounds. Several subgroups of metal carbonyl complexes have been synthesised and assessed as CORM candidates including compounds containing manganese, iron, cobalt, molybdenum and ruthenium without restriction to transition metals$^{780}$.

There is substantial experimental evidence in animal disease models demonstrating the beneficial effects of CO administered in gaseous form or derived from CORMs. Therapeutic benefit has been shown in acute lung injury, liver and kidney injury, acute
and chronic rejection of a transplanted organ grafts and in cardiovascular disease, sepsis, and shock. A major difficulty of CORMs in medical applications is the rapid diffusion of the small molecular weight drugs after administration with release of their CO prior to reaching the diseased target tissue. Future work is directed toward developing a safe and efficient delivery system encompassing the design and synthesis of CORMs with molecular characteristics that provide appropriate absorption, distribution, metabolism, and excretion properties yet maintain an effective therapeutic action and low toxicity profile.

### 3.3.5 Water-soluble CORMs and alternative delivery mechanisms

A central challenge is the development of water-soluble compounds that release CO relatively fast with half-lives of less than two hours and are non-toxic to healthy cells and tissues. Slower release of CO will reduce the effective CO concentration as available CO is diluted in the systemic circulation and binds to hemoglobin. The CORM compound concentration required to produce therapeutic doses of CO may become prohibitively high from an adverse effect standpoint.

The first prototype of a water-soluble transition metal carbonyl was synthesised by coordination of the amino acid glycine to a ruthenium metal centre $[\text{Ru}(\text{CO})_3\text{Cl}]/[\text{glycinate}]$\textsuperscript{782}. This CORM-3 compound is relatively stable in aqueous solutions but rapidly releases CO in the presence of myoglobin, other biological stimuli that interact directly with the ruthenium metal and after contact with cellular components. The half-life ($t_{1/2}$) of CO release from CORM-3 in biological fluids conditions is typically less than 1 minute whereas the half-life is 24 hours when the molecule is dissolved in distilled water\textsuperscript{782}.

The specific CO-releasing properties of CORM-3 in biological fluid can be explained by what is believed to be the coordination of a strong ligand such as a thiol group. Experiments conducted \textit{in vitro} have demonstrated the prompt loss of CO from an aqueous solution of $[\text{Ru}(\text{CO})_3\text{Cl}]/[\text{glycinate}]$ only after addition of cysteine or pyridine. Both the chloride and glycinate ligands of the CORM-3 molecule are labile and readily replaced by stronger ligands\textsuperscript{782,783}. This binding chemistry limits a very slow release of CO
from aqueous solutions of [Ru(CO)$_3$Cl(glycinate)] until the compound meets suitable strong ligands present in biological fluids.

CORM-3 has been proved to effect vasodilatory and antihypertensive activity in biological experiments$^{782,783}$. CORM-3 protects an isolated rat heart from reperfusion injury following 30 minutes of ischaemia and markedly improves the survival time of mice following heart transplantation$^{782}$. CORM-3 administration produces demonstrable vasodilatation in an isolated aortic ring pre-treated with the vasoconstrictor phenylephrine$^{758}$. There is evidence the treatment with [Ru(CO)$_3$Cl(glycinate)] affords renal protection against cisplatin-induced nephrotoxicity and improved renal and cardiac graft function following cold ischaemia when used during organ preservation$^{720,732,784,785}$. Recent experimental in vitro studies of vascular and neural inflammation suggest important anti-inflammatory properties of CORM-3 activity with evidence of inhibition of platelet aggregation$^{786-788}$. CORM-3 is undoubtedly the most-studied of all the CORMs developed to date. Several subsequent generations of CORMs have been synthesized and studied and are the subject of unpublished patent applications. A key challenge that so far remains incompletely solved is the controlled delivery of therapeutic amounts of CO to target tissues without adverse effects of the carrier molecule or generation of excessive carboxyhemoglobin levels. A consistent strategy to achieve this aim is the use of stable molecules that release CO only after activation by a specific stimulus$^{779,789,790}$. Suitable mechanisms are the pH-dependent release of CO from boranocarbonate compound (CORM-A1) and its aminoderivatives or the release of CO from transition metal carbonyl complexes triggered by UV-radiation$^{694,791-793}$. The water-soluble boron-containing compound boranocarbonate can slowly liberate CO under physiological conditions with a $t_{1/2} = 21$ minutes$^{794-796}$. Boranocarbonate CORMs have recently been developed by Professor Alberto and collaborators at the University of Zurich$^{694}$. The prototype sodium boranocarbonate (CORM-A1) does not contain a transition metal carbonyl but a carboxylic group that is converted to CO through hydrolysis with a strictly controlled slow release of CO dependent on pH and temperature. The gradual mild vasodilatation and hypotensive effects produced by
CORM-A1 can be amplified with the concomitant use of guanylate cyclase sensitizers. Recently, a new light-sensitive CORM has been developed that can deliver CO directly to target tissues without significant formation of carboxyhemoglobin and a low toxicity profile as compared to that of boron-containing compounds. This water-soluble CORM-S1 is based on iron and cysteamine and releases CO under irradiation with visible light spectrum whilst maintaining overall stability in the dark.

Recently developed CO-delivery systems based on aggregate micelle molecule forms of metal carbonyl complexes have demonstrated slowed diffusion in tissues and an improved ability to target specific tissue. The CO release at distal tissue drainage sites was slower than that achieved with equivalent CORM-3 treatment. The LPS-induced NF-κB activation of human monocytes was successfully attenuated after treatment with CO-releasing micelles whereas CORM-3 exposure did not elicit a beneficial response.

A recent novel concept using iron complexes that release CO via an enzyme-catalysed reaction has been introduced, termed enzyme-triggered CO-releasing molecules (ET-CORMs).

To maximise the potential for therapeutic applications of CO the issues that require continued study include the development of novel CORMs with ideal delivery mechanisms, a greater understanding of CO release from carrier molecules, improved analytical techniques to monitor CO release and further investigation of the cellular uptake of CORMs. A further potential pharmaceutical use of CORMs stems from their multiple modes of biological activity that are being progressively identified with the burgeoning scientific interest in CORMs.

### 3.3.6 Beneficial effects of CORMs

Carbon monoxide releasing molecules (CORMs) have the potential for therapeutic benefit in the prevention and cure of diseases involving inflammatory, infectious, thrombotic and apoptotic processes. Experimental data from *in vivo* animal models demonstrate the effectiveness of CORM therapy in various conditions including acute liver failure, multiple sclerosis, rheumatoid arthritis, stroke, NSAID-induced gastric ulcers, hypertension and
infection by antibiotic-resistant strains of bacteria\textsuperscript{802, 803}. Protective anti-inflammatory effects of CORMs reported in the literature to date include attenuation of the acute inflammatory response both \textit{in vitro} and \textit{in vivo} and mitigation of neuronal inflammatory responses in microglia\textsuperscript{292, 804-807}, reduction of immunological histamine release from guinea pig mast cells and human neutrophils\textsuperscript{808, 809} and moderation of hepatic leucocyte sequestration and the systemic inflammatory response during severe burn injury\textsuperscript{810}. In studies of the cardiovascular system there is published evidence that CORM treatment provides cardioprotection against both ischaemia and myocardial infarction\textsuperscript{238, 721, 732, 782}, reduction of cardiac graft rejection and positive inotropic effects on the heart\textsuperscript{782, 811}, anti-hypertensive effects and inhibition of platelet aggregation\textsuperscript{694, 788} and vasodilatation and anti-apoptotic effects in the cerebral circulation\textsuperscript{812-814}. Organ-specific effects demonstrated include the improvement of graft function in kidney transplantation when CORMs are used in the organ preservation fluid during cold ischaemia, and renal protection against cisplatin-induced nephrotoxicity\textsuperscript{720, 784}. CORM exposure has recently been shown to decrease photocarcinogenesis in the skin of hairless mice\textsuperscript{815}.

Recent \textit{in vitro} studies have observed that CORMs are capable of potent anti-proliferative effects in a model of pancreatic fibrosis with inhibition of pancreatic stellate cell proliferation\textsuperscript{714, 816}.

Methylene chloride, a compound developed as a cytochrome P450-dependent CO-donor, reduced post-transplant atherosclerosis in rat aorta allografts. Methylene chloride also protected mice against lipopolysaccharide-induced liver injury and prevented experimentally induced autoimmune encephalitis and arthritis in experimental rats.

Recent interest in the development of CORMs has provided a useful tool to investigate the intracellular signalling pathways involved in CO activity and its mechanisms of action\textsuperscript{671, 722, 817}. CO release from CO donors affects the function and activity of several heme-dependent proteins that have a central role in cellular homeostatic processes\textsuperscript{671} including cell proliferation, angiogenesis, the production of reactive oxygen species and mitochondrial respiration\textsuperscript{371, 718, 719, 722}. The activity of NADPH-oxidase in human neutrophils and airway smooth muscle cells is inhibited by CO release from CORM treatment, thereby attenuating the oxidative stress response. The heme-dependent
NADPH-oxidase enzyme is a major player in triggering and propagating oxidative stress via the production of superoxide anion\(^{722}\) \(808\). Future studies are required to identify metal-containing intracellular proteins with a preferential affinity for CO that represent signal transduction targets for the beneficial effects of CO activity.

### 3.3.7 CORMs and inflammation

Experimental murine models of sepsis have demonstrated important anti-inflammatory effects elicited by CORM treatment. Intravenous administration of CORM-2 at 8 mg per kg immediately after caecal ligation and perforation reduced the consequent induction of microbial sepsis. Accumulation of polymorphonuclear leucocytes, activation of transcription factor, up regulation of NF-κB and expression of adhesion molecule ICAM-1 were all reduced in mouse liver tissue 24 hours after induction of sepsis with CORM treatment\(^{818}\). In a rat model of acute hepatic ischaemia-reperfusion injury a single systemic infusion with CORM-2 at 8 mg per kg protected the liver from ischaemia-reperfusion injury with reduced levels of serum aspartate transaminase enzyme and an improved liver histology score. Rat livers that were re-perfused with CORM-2 after portal vein clamping demonstrated reduced levels of apoptosis with down-regulation of caspase-3 activation and increased expression of the anti-apoptotic protein Bcl-2\(^{819}\).

Recent work has aimed to identify CORM candidates that specifically target acute liver injury with evaluation of dose-dependent efficacy in a murine model of acetaminophen (paracetamol)-induced severe liver damage. A suitable molybdenum carbonyl CORM (ALF-794) was developed to preferentially distribute to the liver where CO release occurs through liver metabolism and enables delivery of a highly specific therapeutic dose of CO with low systemic toxicity\(^{820}\). Additional recent studies used a murine model of ulcerative colitis in TCR-α deficient mice. Mice treated with the CO-releasing molecule-186 (ALF186) demonstrated an improvement of active colitis with alterations of interleukin expression. Exposure to CO suppressed colonic IL-1β, IL-4 and tumour necrosis factor production while increasing secretion of IL-10 protein. Induction of IL-10 expression was mediated via pathway dependent upon HO-1 activity\(^{821}\).
3.3.8 CORM experiments in lung physiology and sepsis

The beneficial effects of CORMs in models of sepsis are well-documented. Moderation of the inflammatory response is achieved using CORM treatment with a reduction in cytokine release and oxidative stress demonstrated in macrophages and endothelial cells stimulated by lipopolysaccharide endotoxin. Changes in endothelial cell properties have been elicited by CORM treatment in septic and thermally-injured mice with decreases in nuclear factor-xB activation, expression of ICAM-1 adhesion protein and tissue granulocyte infiltration. These experiments demonstrate attenuation of the systemic inflammatory response and prolonged survival mediated by CO released from CORMs in vivo. Studies by Mizuguchi and colleagues used a murine model of lung sepsis. CORM-3 at 3 mg per kg was given to treatment subjects 15 minutes after sepsis was induced by caecal ligation and puncture. Bronchoalveolar lavage (BAL) fluid was obtained following a 6-hour period to measure accumulation of PMNs by myeloperoxidase assay and protein content of BAL fluid to assess increases in lung vascular permeability. Results demonstrated a reduction in the accumulation of polymorphonuclear leucocytes (PMN) and preservation of vascular permeability in the septic lung associated with systemic administration of CORM-3.

Reported studies collectively show that the CORM-dependent release of CO reduces mortality in septic mice and suggest a therapeutic potential of CORMs to prevent organ failure and death in severe sepsis.

3.3.9 Mitochondrial dynamics

Recent investigations have addressed the concept that mitochondria have an important role in signal transduction pathways mediated by CO activity. Studies reported by Lancel et al. looked at the potential of CORMs to preserve mitochondrial function in a murine model of peritonitis induced by caecal ligation and perforation (CLP). Functional mitochondrial studies were performed and plasma nitrite/nitrate levels, tumour necrosis factor and interleukin-10 levels were measured to evaluate the systemic inflammatory response. CORM-3 treatment in CLP-induced mice prevented the adverse effects of sepsis on mitochondrial membrane potential, respiratory control ratio and cellular redox status. Administration of CORM-3 during sepsis also elicited a mild oxidative stress
response that stimulated mitochondrial biogenesis with corresponding increases in (PPAR-γ-) coactivator-1α protein expression and mitochondrial DNA copy number\textsuperscript{826}.

Recent studies indicate that increased mitochondrial biogenesis is an important mechanism by which CORMs and their derived CO gas exert beneficial protective effects against cardiomyopathy and cardiac dysfunction in sepsis\textsuperscript{461,826}. Water-soluble CORM-3 has been shown to affect the function and bioenergetic parameters of isolated heart mitochondria by the delivery of low micro molar concentrations of CO gas. CORM-3 treatment regulated the production of reactive oxygen species via uncoupling of mitochondrial respiration and decreased mitochondrial membrane potential at concentrations that did not inhibit cytochrome c oxidase\textsuperscript{827,828}. The CO-mediated effects observed were diminished by pharmacological agents known to inhibit mitochondrial uncoupling\textsuperscript{827}.

Strategies attempting to modulate mitochondrial uncoupling and metabolism are exploited in the development of therapeutic applications for certain pathological conditions. Thyroid hormones, steroid hormones and fatty acids could play a vital role in control of mitochondrial respiration. Current work suggests that CORM treatment and exposure to liberated CO gas provides a significant novel regulator of mitochondrial function.

3.3.10 Metal carbonyl chemistry

Organometallic carbonyl complexes are an established chemical class of metal compounds primarily used as precursors to other organometallic compounds or catalysts\textsuperscript{829,830}. Carbon monoxide is well known to bind as a ligand to most transition metals in low oxidation states. The majority of organometallic carbonyl complexes are sensitive to water reactions and oxidation by oxygen and are consequently not stable under ambient atmospheric conditions of humid air. Traditional organometallic carbonyl chemistry reactions proceed largely in dry solvents and under inert atmospheres and are essentially incompatible with biological systems. Notable exceptions include technetium (Tc) and rhenium (Re) transition metal complexes designed to be used as pharmaceuticals during radiological imaging\textsuperscript{831}. These are stable complexes that are excreted in essentially their intact form without releasing CO\textsuperscript{832}. 

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If the CORM principle is to be successfully applied to pharmaceutical development then the limitations of organometallic complex chemistry need to be overcome. CORM drugs need to demonstrate sufficient stability in formulations appropriate for clinical administration such as pill form, liquid suspension or solution for intravenous injection. The ancillary non-CO ligands that stabilise the metal carbonyl complex need to be of sufficiently small molecular size to ensure that the corresponding CORMs have a size reasonable for drug use with a molecular weight (MW) less than 500 – 600 g per mol. Central requirements of CORM therapeutic candidates are stability under ambient conditions, solubility in aqueous solutions and the ability of the compound to release its CO ligand under controlled conditions. Targeted CO release can be achieved by selecting appropriate chemical structures for the stabilising ancillary non-CO ligands of the complexes. Specific ligands can be used that enable common mechanisms for the activation of pro-drugs to be employed.

CORM-3 has demonstrated encouraging biological activity in several in vitro and in vivo biological systems. However the chemical instability of CORM-3 in aqueous solutions represents a major limitation for pharmaceutical applications.

### 3.3.11 Molybdenum and other transition metals

There are various reported attempts to evaluate appropriate CO-binding transition metals with corresponding ancillary ligands for CORM development. Metals including manganese, cobalt and chromium were quickly discounted for known toxicities or specific chemical limitations of compound stability. CORMS have been developed using complexes with iron, ruthenium and rhenium and a variety of ancillary ligands with equivocal success. A consistent difficulty with CORM synthesis is attaining adequate profiles of stability and CO release in air or in aqueous solutions required for acceptable pharmaceutical use. Formation of molybdenum tricarbonyl complexes with lithium isocyanoacetate ligands has recently been described by Beck and co-workers. These complexes have a small molecular size and appear to be soluble and stable in water under ambient conditions.

Molybdenum-based CORMs formed with derivatives of isocyanoacetate ligands have recently been synthesised and shown to benefit from certain appropriate pharmaceutical
properties. Molybdenum carbonyl CORMs developed for the treatment of liver diseases have demonstrated preferential distribution to the liver after systemic administration where bioactivation of the drug enables specific CO delivery to the diseased tissue target \(^{820}\).

A novel approach reported recently by Zobi et al. promotes the use of cyanocobalamin (vitamin B\(_{12}\)) as a biocompatible scaffold for CORMs containing rhenium (Re) transition metal. The authors synthesised Rhenium-vitamin B\(_{12}\) bioconjugates (B\(_{12}\)-ReBr\(_2\)(CO)\(_2\)H\(_2\)O) that afforded myocardial protection against experimental ischaemia-reperfusion injury \(^{786}\). These organometallic complexes have favourable half-lives for biological and therapeutic applications and the resulting oxidised product after CO release (rhenium pertechnetate, ReO\(_4^−\)) is one of the least toxic of the rarest inorganic compounds \(^{786}\). The Re-bioconjugates developed have improved biocompatible characteristics as compared to their parent complexes. They have improved solubility in water and are more stable in aqueous aerobic media \(^{840}\).

The therapeutic possibilities of rhenium bioconjugates could apply particularly to the liver where vitamin B\(_{12}\) is stored and metabolised. Potential applications include the prevention and treatment of inflammatory liver disorders, treatment of liver injury and suppression of graft rejection in liver transplantation \(^{732}^{775}^{781}\).

3.3.12 Challenges of CORM clinical therapeutics

A central question in approaches to CO therapy is whether CORMs can directly deliver gaseous CO to living cells or organisms in a safe, accurate and measurable fashion. The physiological properties and toxicity profile of the CORM chemical scaffold and transition metal components as well as the released CO gas must all be carefully considered.

The principal advantages of CORMs over CO inhalation as a therapeutic approach include the precise controlled delivery of CO liberated from CORMs at desired concentrations via all possible routes of administration. Gaseous CO can only be administered effectively by inhalation and CO toxicity is a limiting factor. CORMs can utilize specific chemical engineering to trigger targeted delivery of small doses of CO gas to diseased tissue without significantly raising CO levels in other tissues. This should mitigate the overall
systemic adverse effects of CO inhalation. Administration of CORMs directly bypasses the pulmonary system and effectively avoids the biological trap of deoxyhemoglobin which is inevitably and rapidly converted to HbCO in the lung following CO gas inhalation. Formation of HbCO (carboxyhemoglobin) reduces the availability of deoxyhemoglobin and compromises tissue oxygen delivery. CO has an affinity for hemoglobin 240 times that of $O_2^{632}$. CO delivery to selected target tissue achieved with CORMs results in only slight increases in HbCO levels similar to endogenous CO production in disease states$^{670}$.

Published data are available on adverse systemic CO effects using pharmacologically effective doses of CORM-3 to reduce myocardial infarction and CORM-A1 to improve renal haemodynamics after acute renal failure in vivo$^{695, 841}$. Using CORMs in these experiments did not increase measured HbCO levels to a threshold considered to be dangerous (10%).

Working with organometallic complexes in medicinal chemistry offers unique advantages due to their marked adaptability and reliable methods of synthesis. The kinetics of CO release can be finely tuned by modifying the ancillary ligand coordinated to the transition metal centre or by altering the chemical behaviour according to ambient pH or temperature$^{694, 717}$. Significant progress in the development of CORM pharmaceuticals relies upon formulating compounds that are stable in ambient conditions and in vivo and can be specifically tailored to achieve precise delivery of CO gas to target tissue sites. The remaining product after CO release should ideally have biologically relevant properties. The end-product should be non-toxic or have an acceptable and well-characterised toxicity profile. Pharmacokinetics, efficacy profiles and manufacturing considerations are some of the issues to be addressed in ongoing work$^{842-844}$. 

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3.4 The Role of Bilirubin

3.4.1 Bilirubin biochemistry and physiological properties

The breakdown of heme by heme oxygenase enzyme produces equimolar amounts of free iron, carbon monoxide and biliverdin. Biliverdin is a green linear tetrapyrrrole bile pigment that is rapidly reduced to bilirubin by the action of biliverdin reductase. Bilirubin is excreted in bile and urine and is responsible for the yellow colour of urine as urobilin and the brown colour of faeces via its conversion to stercobilin. Bilirubin consists of an open chain of four pyrrole-like rings to form a tetrapyrrrole molecule. When bilirubin is oxidised it reverts to become biliverdin once again in a cycle that suggests a physiological role for bilirubin as a cellular antioxidant. Landmark studies reported by Stocker and colleagues provided evidence for the important antioxidant activities of bilirubin and changed the traditional view of bilirubin as a potentially cytotoxic lipid-soluble waste product simply requiring excretion.

At a low concentration bilirubin scavenges reactive oxygen species in vitro thereby reducing oxidant-mediated cellular damage and attenuating oxidant stress. These findings suggested that bilirubin was one of the most important endogenous antioxidants present in serum, displaying more effective peroxyl radical scavenging than α-tocopherol that was considered to be the most powerful antioxidant molecule available. Further in vitro studies demonstrated that bilirubin was more effective at protecting lipids from peroxidative damage than other endogenous antioxidants, protected human low density lipoprotein against oxidation by radicals and directly suppressed hydroxyl radicals generated by L-DOPA-mediated DNA cleavage. Bilirubin has been shown to partially prevent the effects of potent oxidant agents in human erythrocytes, protect neuronal cells against hydrogen peroxide-induced damage and protect vascular smooth muscle cells against oxidative stress.

Biliverdin and bilirubin are reducing molecules and hence potential antioxidants. At physiological concentrations in mammalian serum unconjugated bilirubin is an efficient scavenger of singlet oxygen and acts as a reducing agent for certain peroxidases including horseradish peroxidase and prostaglandin H synthase in the presence of hydrogen peroxide and organic hydroperoxides. Bilirubin has beneficial effects in pulmonary
and vascular diseases, improves renal haemodynamics and reduces blood pressure in an animal model of hypertension\textsuperscript{850, 851}. Recent studies have demonstrated antiviral activity of HO-1-derived bilirubin \textit{in vitro}\textsuperscript{852, 853}.

The physiological activity of bilirubin and biliverdin may be especially relevant to the prevention of cell death mediated by oxidative and nitrosative stress\textsuperscript{189, 748, 854}. Bilirubin at high concentration can be demonstrated to be toxic to the central nervous system in neonates. The syndrome of kernicterus is a severe irreversible form of bilirubin neurotoxicity that has become extremely rare with the advent of phototherapy to treat neonatal jaundice\textsuperscript{855}. In microvascular endothelial cells, lower physiological bilirubin concentrations can induce apoptosis and this situation is exacerbated under hyperglycaemic conditions. Endothelial cells of the blood-brain barrier are particularly sensitive to these effects of bilirubin and may contribute to its relative neurotoxicity\textsuperscript{856}.

Exposure to both bilirubin and biliverdin was shown to repress the pro-inflammatory effect of zinc protoporphyrin-mediated HO inhibition in rodent models of ischaemia-reperfusion injury. The anti-inflammatory effect of HO induction was thereby restored to the system\textsuperscript{234}. Further studies have reported protective effects of biliverdin therapy on ischemia and reperfusion injury in rat livers\textsuperscript{463}. A recent study using a rodent model of lipopolysaccharide endotoxin-induced acute lung injury and caecal ligation and puncture indicated that biliverdin administration provided a systematic defence against lethal endotoxaemia and effectively abrogated the inflammatory response. Exposure to the end-products of HO activity once again substituted for the effects observed with induction of HO-1 enzyme\textsuperscript{857}.

Heme oxygenase protects the intracellular environment from the toxic effects of heme. The other major enzyme in the heme degradation pathway is biliverdin reductase that has an important role in glucose uptake and the stress response. The activity of biliverdin reductase can produce multiple effects in cell signal transduction and the regulation of gene expression. The first suggestion that biliverdin affords intrinsic cytoprotection independent of heme degradation was provided by the use of short-interfering RNA to silence human biliverdin reductase and HO-1 enzyme. Knockdown of biliverdin reductase in cultured human embryonic kidney cells increased the sensitivity of the cells to...
apoptotic cell death mediated by arsenite treatment whereas cells transfected with HO-1 siRNA were unaffected\(^\text{858}\). It should be remembered that biliverdin is rapidly converted to bilirubin by biliverdin reductase in mammals and is therefore short-lived \textit{in vivo}. Nevertheless recent evidence supports the role of human biliverdin reductase in cellular signalling, cytoprotection and cytotoxicity and as a transcription factor in the regulation of gene expression\(^\text{859} \ 860\). Human biliverdin reductase has been identified as an upstream activator of the insulin growth factor-1 (IGF-1) and mitogen-activated protein kinase (MAPK) signalling pathways and is essential for MAPK–extracellular signal-regulated kinase (ERK)1/2 signalling. The enzyme is recognised to be a heme transporter of ERK1/2 and hematin as a pivotal component of gene expression during the cellular stress response\(^\text{861} \ 862\).

Bilirubin can exert either cytotoxic or cytoprotective effects depending on the concentration of its free fraction in serum or tissue, the nature of the target cell or tissue, and the cellular redox state. The central nervous system is particularly sensitive to the neurotoxic effects of bilirubin\(^\text{849} \ 863\). Studies in the cardiovascular system have supported a cytoprotective role for HO-1-derived bilirubin\(^\text{864} \ 865\). The cardioprotective effect observed in humans with moderate hyperbilirubinaemia such as that which occurs in Gilbert’s syndrome may result from the antioxidant activity of bilirubin. Biliverdin and bilirubin have been demonstrated to preserve endothelial cell integrity, prevent endothelial cell death and sloughing, enhance vascular reactivity, and prevent vascular restenosis\(^\text{696} \ 866-870\).

In experimental diabetes, bilirubin is implicated in reducing oxidative stress by increasing the bioavailability of nitric oxide needed for endothelial cell integrity and the inhibition of NADPH oxidase to reduce the diabetes-mediated generation of oxidants and uncoupling of endothelial nitric oxide synthase\(^\text{684} \ 871\). Increased levels of bilirubin can provide antiproliferative effect in smooth muscle cells and protect experimental rats from the development of atherosclerosis\(^\text{222}\). Studies have shown that vascular smooth muscle cell proliferation and consequent neointima formation was reduced in hyperbilirubinaemic subjects. This experimental finding is supported by epidemiological reports that describe a lower incidence of atherosclerosis in humans with a higher normal endogenous serum bilirubin level compared to individuals with low normal bilirubin levels\(^\text{866}\).
Bilirubin can inhibit the proliferation of medial smooth muscle cells following arterial injury leading to reduced neointima formation. The prevention of neointimal thickening mediated by bilirubin is associated with alterations in the expression of cell cycle regulatory proteins with a significant decrease in cyclin D1 and cyclin A expression and up regulation of p53 protein causing an increase in the percentage of cells in the G0/G1 phase of the cell cycle. Bilirubin causes a marked decrease in ERK 1/2 activity and an increase in p21 expression that blocks proliferation and migration of human arterial smooth muscle cells in a concentration-dependent manner without affecting cell viability.872

There is a lower incidence of atherosclerosis associated with individuals with Gilbert’s syndrome who have a congenital deficiency of glucuronyl transferase and elevated serum bilirubin levels. Glucuronyl transferase enzyme facilitates bilirubin metabolism by catalysing its conjugation with glucuronic acid in the liver and making it water-soluble for excretion in secreted bile.873-875 Several recent human studies have suggested a protective role of bilirubin against coronary artery disease with a reported inverse relationship between serum bilirubin level and the incidence of ischaemic stroke.257 859 876 Retrospective and prospective human studies have indicated serum bilirubin to be associated with important cardiovascular risk factors including diabetes mellitus, metabolic syndrome, arterial hypertension and body mass index.877 There is a lack of consensus in the therapeutic potential of manipulating serum bilirubin levels for the prevention or treatment of cardiovascular and related diseases.258 876 Studies assessing surrogate markers of atherosclerosis such as measuring the thickness of carotid intima-media and determining endothelial dysfunction by flow-mediated dilatation have shown beneficial effects of increased bilirubin concentrations.259 A greater risk of ischaemic heart disease has been reported to be associated with longer GT-repeat HO-1 gene promoter polymorphisms via a reduction in serum bilirubin concentration.878

Bilirubin has been shown to regulate cellular redox status and reactive oxygen species and influence the activity of NADPH oxidase enzyme. In the respiratory system increased levels of pulmonary HO-1 protein can be a potential biomarker of chronic silicosis. Induction of HO-1 enzyme by hemin treatment attenuates silicosis-induced lung injury and disease progression via a reduced production of reactive oxygen species and associated oxidative damage.879 Bilirubin can increase tolerance to islet cell allografts by
a mechanism that includes its anti-inflammatory and antioxidative properties with reduced neutrophil adhesion and down-regulation of adhesion molecule expression.\textsuperscript{880, 881}

The potential role of bilirubin in cancer is not clearly understood, with available evidence often contradictory. It has been suggested that biliverdin acts as a pro-mutagenic agent with promotion of neoplastic cell formation in liver epithelial cell cultures exposed to aflatoxin B\textsubscript{1}.\textsuperscript{882} More recently a cohort study of a Belgian population demonstrated that high serum bilirubin concentrations were associated with low cancer mortality. Study authors propose that this observation could be due to the antioxidant effects of bilirubin preventing oxidative damage-induced mutagenesis.\textsuperscript{883} A novel anti-cancer effect of bilirubin was documented by Rao and colleagues in 2006. Studies using the human gastric adenocarcinoma cell line TMK-1 demonstrated both antioxidant and pro-oxidant activity of bilirubin. Cell viability assays by Trypan blue exclusion showed almost a 50% reduction in cell proliferation after 48 hours incubation with bilirubin. Treating cell lines with bilirubin caused an increase in intracellular radicals and cell cycle arrest at the G\textsubscript{0}/G\textsubscript{1} phase of the cell cycle. There was evidence of impaired mitochondrial function with DNA damage characteristic of apoptosis induced by bilirubin exposure.\textsuperscript{884}

Additional \textit{in vitro} studies showed a cytostatic and pro-apoptotic activity of bilirubin in a variety of cell lines including colonic, pancreatic and hepatocellular carcinoma and malignant melanoma.\textsuperscript{885} Bilirubin inhibited tumour cell proliferation by causing cell cycle arrest the G\textsubscript{0}/G\textsubscript{1} phase after 24 hours of incubation and a significant increase in apoptosis after 72 hours of bilirubin treatment as compared to controls. These effects were dependent upon phosphorylation of ERK 1/2 by upstream activated kinase pathways. Using a specific MEK inhibitor to effectively suppress ERK 1/2 activation abolished the anti-proliferative effects of bilirubin on colonic adenocarcinoma cells. The same study describes an \textit{in vivo} model using mice that were injected subcutaneously with human colorectal carcinoma cell line and observation of tumour growth after treatment with either phosphate-buffered saline control or 25 mg kg\textsuperscript{-1} body weight bilirubin. Dramatically slower tumour growth was observed in mice treated with intraperitoneal bilirubin when compared to controls.\textsuperscript{885}
These recent novel findings and lack of consensus on cytoprotective roles of bilirubin provide a study hypothesis to investigate the activity of bilirubin in oesophagogastric cancer cells. Studies were designed to determine the effect of bilirubin on the proliferation of gastric and oesophageal cancer cell lines.
CHAPTER 4: RESULTS

Investigating Oxidative Stress and Altered Heme Oxygenase Activity

4.1 Oxidative stress and the role of heme oxygenase

4.1.1 Cell proliferation 24 hour experiments – Figure 4.1.1

The combined results of the MTT assay for the gastric cell lines demonstrate no obvious reduction in cell proliferation after 24-hour treatment with \( \text{H}_2\text{O}_2 \). There was a reduction in MTT activity at higher \( \text{H}_2\text{O}_2 \) concentrations that was not statistically significant for either AGS (\( p = 0.081 \)) or HGC-27 (\( p = 0.075 \)) cell lines.

In contrast to the gastric cell lines 24-hour treatment with \( \text{H}_2\text{O}_2 \) caused a significant increase in OE-33 cell proliferation at concentrations of 10, 20 and 200 µM (\( p < 0.05 \)). There was an observed decrease in proliferation of normal squamous epithelial cell line HET-1A after 24-hour exposure to \( \text{H}_2\text{O}_2 \) at a dose of 200 µM that was not statistically significant (\( p = 0.062 \)).

Treatment with NAC had a significant effect on AGS cell proliferation after 24 hours with convincing effects at doses of 5 mM, 10 mM and 20 mM (\( p \leq 0.001 \)). Although there was an observed reduction in the average MTT assay for AGS cells exposed to NAC at doses of 0.5 mM and 1 mM this effect was not statistically significant (\( p > 0.05 \)). Similar results were observed with HGC-27 cell lines with significant reduction in cell growth after 24-hour treatment with NAC at doses of 1 – 20 mM (\( p \leq 0.003 \)).

Morphological cell changes after 48 hours included rounding of cells and a reduction in cell confluence with visible necrotic cells detached from the cell monolayer.

Treatment of OE-33 cells with increasing doses of NAC from 0.1 – 20mM demonstrated a statistically significant reduction in cell proliferation at a dose of 10 – 20 mM (\( p = 0.015 \)). Higher concentrations of NAC (10, 20 mM) caused cellular morphological changes with cells becoming refractile and losing cell-cell contact.
For HET-1A cell lines there was a less marked reduction in cell proliferation after 24 hour treatment with NAC at increasing doses with no statistically significant effect observed ($p = 0.062$).

**4.1.2 Cell proliferation time course experiments – Figure 4.1.2**

Extended exposure of AGS cells to NAC at doses of 1, 5, 10 and 20 mM demonstrated a persistent reduction in cell proliferation that was significant at doses of 10 and 20 mM up to 72 hours incubation (Figure 4.1.2a). Extending the experiments to 168 hours showed a peak in exponential cell growth of control cells at 72 hours with cell numbers levelling out at 96 hours and falling at 120 hours and beyond. This observed effect was due to cells becoming confluent and subsequently overcrowded on the growth surface with resulting detachment from monolayer. Consistent effects of significantly reduced cell proliferation were seen with prolonged NAC treatment particularly at doses of 10 and 20 mM ($p \leq 0.017$, Figure 4.1.2a).

Extended treatment with NAC at doses of 1, 5, 10 and 20 mM produced quite different effects on cell growth in benign HET-1A cells compared to malignant OE-33 epithelial cells (Figures 4.1.2b – c). The malignant cells showed a clear reduction in cell growth over 24 – 72 hours with NAC doses of 10 and 20 mM with statistically significant effects over all time points with NAC at 20 mM. By contrast the benign epithelial cell growth was similar to control cells across all time points and doses of NAC except for a reduction in MTT assay after 72 hours of exposure to NAC at 20 mM concentration ($p = 0.047$). Extending the experiments to 168 hours in OE-33 cell lines demonstrated a plateau of cell growth in untreated controls after 72 hours with a reduction in cell counts by 144 hours as cells became detached. Consistent effects of reducing OE-33 cell proliferation were seen with NAC treatment at all concentrations beyond 72 hours ($p = 0.008$).

Chemotherapy studies demonstrated the ability of NAC to improve the anti-proliferative effects of chemotherapy in malignant OE-33 cells. The combination of 10mM NAC with cisplatin and 5-FU enabled a significant reduction in the IC$_{10}$ and IC$_{50}$ doses for both reagents ($0.1 \, \mu g mL^{-1}$ and $2.875 \, \mu g mL^{-1}$ respectively for cisplatin and $0.07 \, \mu g mL^{-1}$ and $6.5 \, \mu g mL^{-1}$ respectively for 5-FU, $p < 0.05$, Figures 4.1.2d – e).
Figure 4.1.1 Cell proliferation experiments: 24-hour data

Figure 4.1.1a: Effect of H$_2$O$_2$ on AGS & HGC-27 growth at 24 hours

**Effect of H$_2$O$_2$ on AGS growth at 24 hours**

![Graph showing the effect of H$_2$O$_2$ on AGS growth at 24 hours.](image)

$p = 0.081$ Kruskal-Wallis Independent Samples test

**Effect of H$_2$O$_2$ on HGC-27 growth at 24 hours**

![Graph showing the effect of H$_2$O$_2$ on HGC-27 growth at 24 hours.](image)

$p = 0.075$ Kruskal-Wallis Independent Samples test

Figure 4.1.1a: Cells were treated with H$_2$O$_2$ 0.2 – 200 µM for 24h in 24-well plates and cell viability assessed with MTT assay. Results are expressed as a percentage of vehicle control; values represented as means ± SEM; $n = 12$. Cell growth was not significantly affected by H$_2$O$_2$ treatment.
Figure 4.1.1b: Effect of H2O2 on OE-33 & HET-1A growth at 24 hours

* $p < 0.05$ Kruskal-Wallis Independent Samples test

$p = 0.062$ Kruskal-Wallis Independent Samples test

Figure 4.1.1b: Cells were treated with H2O2 0.2 – 200 µM for 24h in 24-well plates and cell viability assessed with MTT assay. Results are expressed as a percentage of vehicle control; values represented as means ± SEM; $n = 12$. HET-1A cell growth was not significantly affected by H2O2 treatment whereas OE-33 growth increased with H2O2 exposure.
Figure 4.1.1c: Effect of NAC on AGS & HGC-27 growth at 24 hours

*\( p < 0.0005 \), + \( p = 0.001 \), ‡ \( p = 0.003 \) Kruskal-Wallis Independent Samples test

Figure 4.1.1c: Cells were treated with NAC 0.1 – 20 mM for 24h in 24-well plates and cell viability assessed with MTT assay. Results are expressed as a percentage of vehicle control; values represented as means ± SEM; \( n = 12 \). Growth of AGS and HGC-27 cells was significantly inhibited by NAC 5 – 20 mM.
Figure 4.1.1d: Effect of NAC on OE-33 & HET-1A growth at 24 hours

+ $p = 0.015$ Kruskal Wallis Independent Samples test

$p = 0.062$ Kruskal-Wallis Independent Samples test

Figure 4.1.1d: Cells were treated with NAC 0.1 – 20 mM for 24h in 24-well plates and cell viability assessed with MTT assay. Results are expressed as a percentage of vehicle control; values represented as means ± SEM; $n = 12$. Growth of OE-33 but not HGC-27 cells was significantly inhibited by NAC 10 – 20 mM.
Figure 4.1.2 Cell proliferation experiments: Time-course data

Figure 4.1.2a: Effect of NAC on AGS growth curve

$\rho \leq 0.017$ Kruskal-Wallis Independent Samples test

$
\ddagger \ddagger \ddagger \ddagger \ddagger$

$\ddagger p < 0.05$ Kruskal-Wallis Independent Samples test

Figure 4.1.2a: AGS cells were treated with NAC 1 – 20 mM for 24 – 168h in 24-well plates and cell viability assessed with MTT assay and cell counts. Values represented as means ± SEM; $n = 12$. Growth of AGS cells was significantly reduced by NAC 10 and 20 mM over 24 – 72 hours. Effective inhibition of cell proliferation is observed up to 96 hours.
Figure 4.1.2b: Effect of NAC on OE-33 growth curves

OE-33 Growth Curve with NAC

- Control
- 1 mM
- 5 mM
- 10 mM
- 20 mM

Cell number x 10^4

24 h 48 h 72 h 96 h 120 h 144 h 168 h

$\text{p} = 0.008$ Kruskal-Wallis Independent Samples test

Effect of NAC on OE-33 cell growth

MTT Absorbance % control

$N$-Acetylcysteine [mM]

24 hrs 48 hrs 72 hrs

+ $\text{p} = 0.035$, ‡ $\text{p} = 0.0005$, † $\text{p} = 0.047$ Kruskal-Wallis Independent Samples test

Figure 4.1.2b: OE-33 cells were treated with NAC 1 – 20 mM for 24 – 168h in 24-well plates and cell viability assessed with MTT assay and cell counts. Values represented as means ± SEM; $n = 12$. Growth of OE-33 cells was significantly reduced by NAC 10 and 20 mM over 24 – 72 hours. Effective inhibition of cell proliferation is observed up to 120 hours.
Figure 4.1.2c: Effect of NAC on HET-1A growth time course

![Effect of NAC on HET-1A growth](image)

\( p = \text{NS except where stated} \)

\( ^\dagger p = 0.047 \) Kruskal-Wallis Independent Samples test

Figure 4.1.2c: HET-1A cells were treated with NAC 1 – 20 mM for 24 – 72h in 24-well plates and cell viability assessed with MTT assay. Results are expressed as a percentage of vehicle control and values represented as means ± SEM; \( n = 12 \). Growth of HET-1A cells was significantly inhibited only by NAC 20 mM at the 72 h time point.
Figure 4.1.2d: Effect of NAC on OE-33 response to Cisplatin

OE-33 Growth curve Cisplatin

![Chart showing OE-33 growth curve with Cisplatin and NAC treatments]

$p < 0.05$ Kruskal-Wallis Independent Samples test

Figure 4.1.2d: OE-33 cells were treated with cisplatin ± NAC 10 mM in 24-well plates for 48h and cell viability assessed with MTT assay over an extended time course. Values represented as means ± SEM; $n = 12$. The IC$_{10}$ and IC$_{50}$ doses of cisplatin were significantly reduced by concomitant NAC treatment.

Figure 4.1.2e: Effect of NAC on OE-33 response to 5-FU

OE-33 Growth curve 5-FU

![Chart showing OE-33 growth curve with 5-FU and NAC treatments]

$p < 0.05$ Kruskal-Wallis Independent Samples test

Figure 4.1.2e: OE-33 cells were treated with 5-FU ± NAC 10 mM in 24-well plates for 48h and cell viability assessed with MTT assay over an extended time course. Values represented as means ± SEM; $n = 12$. The IC$_{10}$ and IC$_{50}$ doses of 5-FU were significantly reduced by concomitant NAC treatment.
4.1.3 Heme oxygenase protein expression

NAC and H₂O₂ treatment did not influence constitutive expression of HO-2 protein in malignant gastric epithelial cells. Western blot analysis (western blots 4.1.3.1) showed no evidence of induction of HO-1 expression by NAC or H₂O₂ in either AGS or HGC-27 gastric cell line. Constitutive HO-2 protein was expressed equally in both cell lines and was unchanged by experimental conditions. HO protein blots correlated well with tubulin controls.

NAC and H₂O₂ exposure did not cause up-regulation of HO-1 protein in either of the oesophageal cell lines studied (western blots 4.1.3.2). There was equal expression of constitutive HO-2 protein in both OE-33 and HET-1A cell lines under basal and experimental conditions. There appears to be reduced expression of HO-2 protein in OE-33 cells treated with NAC at 1 and 20 mM concentration. This can be explained by the tubulin blots demonstrating a proportional reduced expression in the corresponding bands and is most likely to represent a relative lack of protein for this lysate sample and unequal loading onto the gel for electrophoresis.

Western blot analyses were performed in triplicate for each cell line studied. HO-1 protein has molecular weight of 32 kDa, HO-2 protein 36 kDa and β-Tubulin 55 kDa. Representative blots are shown with additional Western blots included in Appendix IV.
Figure 4.1.3 Western Blots

Western Blots 4.1.3.1: H$_2$O$_2$ and NAC Gastric cell lines

AGS H$_2$O$_2$ and NAC protein expression

![Western Blot Image](image1)

M Con 2 10 20 50 1 5 10
H$_2$O$_2$ µM NAC mM

HGC-27 H$_2$O$_2$ and NAC protein expression

![Western Blot Image](image2)

M Con 2 10 20 50 1 5 10
H$_2$O$_2$ µM NAC mM

Figure 4.1.3.1: Protein expression after treatment with H$_2$O$_2$ and NAC in gastric cell lines. Cells were seeded into 24-well plates and protein lysates harvested at 24h. Gel electrophoresis and Western blotting were performed as described. Blots demonstrate unchanged expression of HO-2 with no induction of HO-1 protein in AGS or HGC-27 cells. M = marker, Con = control
Western Blots 4.1.3.2: $\text{H}_2\text{O}_2$ and NAC Oesophageal cell lines

OE-33 $\text{H}_2\text{O}_2$ and NAC protein expression

HET-1A $\text{H}_2\text{O}_2$ and NAC protein expression

Figure 4.1.3.2: Protein expression after treatment with $\text{H}_2\text{O}_2$ and NAC in oesophageal cell lines. Cells were seeded into 24-well plates and protein lysates harvested at 24h. Gel electrophoresis and Western blotting were performed as described. Blots demonstrate unchanged expression of HO-2 with no induction of HO-1 protein in OE-33 or HET-1A cells with unequal loading of OE-33 lysate samples in the NAC 1 mM and 20 mM lanes. M = marker, Con = control
4.1.4 Effect of NAC on heme oxygenase enzyme activity

Assays of total heme oxygenase enzyme activity were performed on AGS lysate samples after 24 and 48 hours of treatment with NAC at 5 and 10 mM concentrations. The results show a statistically significant reduction in total HO activity measured in cells treated with NAC at 10 mM concentration for 24 and 48 hours ($p < 0.05$, Figure 4.1.4).

Figure 4.1.4: Effect of NAC on HO enzyme activity time course

![Figure 4.1.4](image)

Figure 4.1.4: Effect of NAC treatment on total HO enzyme activity. AGS cells were seeded into 175 cm$^3$ flasks and lysates harvested after 24 and 48h NAC treatment as described. Specific colorimetric assay demonstrated a significant reduction in HO activity expressed as pmoles per bilirubin formed per mg of protein pellet per 60 min ($p < 0.05$). Values are represented as means ± SD; $n = 3$. 
4.2 Oxidative stress and HO activity: Apoptosis studies

4.2.1 Apoptosis Studies: Annexin V and Propidium Iodide staining

Apoptosis experiments were performed in quadruplicate using cells seeded into 6-well plates with 3 wells per sample. Cell staining with Annexin V-FITC identified cells with exposed phosphatidylserine translocated to the external cell membrane at the onset of apoptosis. Propidium iodide (PI) staining identified necrotic and damaged cells with permeable plasma cell membranes with exclusion of PI by viable cells.

Controls were set up with unstained cells, ethanol-treated cells to permeabilise the cell membrane for PI staining and with staurosporine-treated cells to induce apoptosis for annexin V positive control staining. These samples were used to set the gates and quadrant regions for flow cytometry data analysis (see Appendix V). All experiment cell samples were then stained with both annexin V and PI at 24 hours and 48 hours to evaluate a possible process of apoptosis. Representative flow results from one experiment are shown for each sample investigated together with the combined data for AGS gastric carcinoma cells (Figure 4.2.1).

Cells treated with NAC at 10 mM concentration demonstrated a significantly increased affinity for annexin V binding after 24 hours as compared to untreated control cells over the same time period (40.28% versus 0.05%, p = 0.013, Figure 4.2.1). At 48 hours the treated cells become permeable to PI as membrane integrity is lost (41.07% versus 0.02%, p = 0.013). At 48 hours there were significantly fewer viable cells and significantly increased late apoptotic or necrotic cells in samples treated with NAC. The observed movement of treated cells over time through annexin V positive and PI negative to annexin V and PI positive suggests a process of early apoptosis progressing to late apoptosis and eventual cell death.
Figure 4.2.1: Annexin V binding and NAC treatment

AGS cells were treated with NAC 10 mM for 24 and 48h in 6-well plates and stained with Annexin V-FITC and PI for flow cytometry analysis. The rate of apoptosis (R6) and late apoptosis / necrosis (R4) is expressed relative to vehicle controls with values represented as means ± SD; n = 4; logarithmic scale. The percentage of cells counted in the apoptosis phase after 24h was 0.05% (+/- 0.006) in control cells versus 40.28% (+/- 3.575) in NAC-treated samples and the percentage in the late apoptotic / necrotic phase after 48h was 0.02% (+/- 0.01) in controls versus 41.07% (+/- 3.89) in NAC-treated cells (p = 0.013).
Table 4.2.1: Summary of Annexin V binding and NAC treatment

<table>
<thead>
<tr>
<th>Cell sample</th>
<th>Measurement</th>
<th>R3</th>
<th>R4</th>
<th>R5</th>
<th>R6</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control 24h</strong></td>
<td>Cell Count</td>
<td>689</td>
<td>12</td>
<td>30556</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>2.2</td>
<td>0.04</td>
<td>97.71</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td><strong>NAC 10mM 24h</strong></td>
<td>Cell Count</td>
<td>18</td>
<td>72</td>
<td>18280</td>
<td>12401</td>
<td>0.013</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>0.06</td>
<td>0.24</td>
<td>59.43</td>
<td>40.28</td>
<td></td>
</tr>
<tr>
<td><strong>Control 48h</strong></td>
<td>Cell Count</td>
<td>671</td>
<td>6</td>
<td>29394</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>2.23</td>
<td>0.02</td>
<td>97.57</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td><strong>NAC 10mM 48h</strong></td>
<td>Cell Count</td>
<td>34</td>
<td>12407</td>
<td>11911</td>
<td>5893</td>
<td>0.013</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>0.11</td>
<td>41.07</td>
<td>39.36</td>
<td>19.46</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2.1: Apoptosis studies in AGS cells treated with NAC 10 mM for 24 and 48h in 6-well plates. Summary data from quadruplicate experiments is displayed (means, n = 4). Quadrantic regions include: R3 – necrotic cell debris; R4 – late apoptosis or necrosis; R5 – viable cells; R6 – apoptosis. At 24h there is a significantly higher number of apoptotic cells and at 48h a significantly higher proportion of late apoptosis or necrosis in cells treated with NAC as compared to control samples.
4.2.2 Apoptosis Studies: Cell cycle analysis

Analysis of cellular DNA content using PI staining provided information about the position of analysed cells on the cell cycle. Experiments were performed in triplicate using cells seeded into 6-well plates. Representative flow results from one experiment are shown for each sample investigated together with the combined data for AGS gastric carcinoma cells (Figure 4.2.2).

Untreated control cell samples demonstrated the majority of cells in the G₀/G₁ phase (52.06%) with 2.41% of cells measured in the sub-G₀ region. There was no sub-G₀ peak demonstrated with the control cell samples (Figure 4.2.2). The coefficient of variation (CV) of the G₀/G₁ peak was 7.42 and the ratio of G₀/G₁ to G₂/M cells was 2.02, indicating good doublet discrimination.

Cells treated with NAC at 5 mM concentration for 24 hours showed a reduction in the proportion of cells in G₀/G₁ phase (36.85%) and an increase in cell population in the G₂/M phase (31.74%) with 7.4% of cells measured in the sub-G₀ region. The CV of the G₀/G₁ peak was 7.83 again indicating good quality DNA measurements.

Cells treated with NAC at 10 mM concentration for 24 hours showed a significant reduction in the proportion of cells in G₀/G₁ phase (23.44%) and significantly increased numbers of cells in the G₂/M phase (44.7%) and sub-G₀ region (11.49%). There was a sub-G₀ peak population of cells visible (Figure 4.2.2). The CV of the G₀/G₁ peak was 8.08 indicating acceptable doublet discrimination and accuracy of DNA measurement.

Cells in the sub-G₀ region are undergoing the process of apoptosis and the appearance of cells in this region was clearly demonstrated after 24-hour treatment with NAC at 10 mM concentration (p = 0.02). There was a significant increase in the proportion of cells measured in the G₂/M phase to indicate G₂/M cell cycle arrest after 24h treatment with NAC 10 mM (p = 0.02).
Figure 4.2.2: AGS cells were treated with NAC 5 and 10 mM for 24h in 6-well plates, stained with PI and analysed using flow cytometry. An increase in cells counted at G2/M arrest and sub G0 phase is seen with NAC treatment. Values are the percentage of total cell count per sample represented as means ± SD; n = 3.
4.2.3 Apoptosis Studies: Apo-BrdU DNA fragmentation assay

The Apo-BrdU assay measures the incorporation of brominated deoxyuridine triphosphate nucleotides into DNA strand breaks. DNA fragmentation is a hallmark of apoptosis in mammalian cells that is recognised as one of the later stages of the process.

Positive and negative control cells provided with the Apo-BrdU assay kit were used to set the gating regions and reliably identify positive and negative-stained cells. Apoptotic cells show an increased fluorescence with increased FITC staining as the incorporated BrdU nucleotide sites are detected by FITC-labelled anti-BrdU antibody. The positive control sample cells demonstrated increased FITC fluorescence compared to negative control cells (33.84% versus 0.82%, Appendix VI).

DNA fragmentation assay experiments were performed in triplicate using cells seeded into 6-well plates. Representative flow results from one experiment are shown for each sample investigated together with the combined data for AGS gastric carcinoma cells (Figure 4.2.3).

Control cell samples demonstrated minimal FITC staining after 24 hours with 0.5% in the positive R8 region (Figure 4.2.3). Cells treated with NAC at 10 mM concentration for 24 hours showed a clear increase in fluorescence with 27.39% positive for FITC staining ($p = 0.05$, Figure 4.2.3). Extended exposure to NAC at 10 mM concentration caused a further significant increase in fluorescence when compared to untreated cells over the same time period (64.1% versus 0.57% respectively, $p = 0.05$). These results indicate that one of the hallmarks of apoptosis occurs in AGS cell lines after treatment with NAC at 10 mM concentration for 24 and 48 hours.
Figure 4.2.3 Apoptosis Studies: Apo-BrdU labelling of DNA fragments

Figure 4.2.3: ApoBrdU assay performed on AGS cells 24 and 48h after treatment with NAC 10 mM. DNA fragmentation is measured by increased fluorescence in the FITC channel (R8). The rate of apoptosis is expressed as percentage of controls with values represented as means ± SD; n = 3; logarithmic scale.
Table 4.2.3: Summary of ApoBrdU DNA fragmentation assays AGS cells

<table>
<thead>
<tr>
<th>Cell sample</th>
<th>Measurement</th>
<th>REGION</th>
<th>Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R8</td>
<td>R9</td>
</tr>
<tr>
<td><strong>Control 24h</strong></td>
<td>Cell Count</td>
<td>97</td>
<td>19192</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>0.5</td>
<td>99.02</td>
</tr>
<tr>
<td><strong>NAC 10mM 24h</strong></td>
<td>Cell Count</td>
<td>5502</td>
<td>14464</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>27.39</td>
<td>72.01</td>
</tr>
<tr>
<td><strong>Control 48h</strong></td>
<td>Cell Count</td>
<td>119</td>
<td>20662</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>0.57</td>
<td>98.98</td>
</tr>
<tr>
<td><strong>NAC 10mM 48h</strong></td>
<td>Cell Count</td>
<td>13689</td>
<td>6738</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>64.1</td>
<td>31.55</td>
</tr>
</tbody>
</table>

Table 4.2.3: ApoBrdU assay performed on AGS cells 24 and 48 h after treatment with NAC 10 mM. DNA fragmentation is measured by increased fluorescence in the FITC channel (R8). There is a significant time-dependent increase in the proportion of cells counted with DNA fragmentation in samples treated with NAC 10 mM ($p = 0.05$). Summary data from triplicate experiments is displayed (means, $n = 3$).
4.2.4 Apoptosis Studies: Caspase 3/7 activity assay

The Apo-ONE Homogenous Caspase-3/7 assay (Promega) was performed after 24 hour treatment with NAC at doses of 5 mM and 10 mM. Experiments were performed in triplicate using cells seeded into 96-well opaque black plates. The X-Y scatter of absorbance data were analysed to identify a linear portion of the graph indicating a steep part of the signal curve between particular cycle numbers for each assay. Values within these time points were normalised to MTT assay to obtain the relative caspase activity per 1000 cells.

Cell proliferation experiments were performed concurrently in 96-well plates with an identical experiment design to provide cell numbers per well derived from MTT assay as described above. The relative caspase activity per 1000 cells for experiment samples was obtained after normalisation to cell number for each experiment well.

The signal curve generated by absorbance data over 60 minutes from one experiment is shown in Figure 4.2.4a. Combined results of relative caspase activity from triplicate experiments demonstrate an increase in fold caspase-3/7 induction after 24 hours exposure to NAC with statistical significance at a dose of 10 mM ($p = 0.022$, Figure 4.2.4b).
Figure 4.2.4 Apoptosis Studies: Caspase 3/7 activity assay

Figure 4.2.4a: Caspase activity at 24 hours AGS cells

Figure 4.2.4a: Evolution of kinetic absorbance data over 60 minutes during caspase 3/7 assay. AGS cells were seeded into black 96-well plates and treated with NAC 5 – 10 mM for 24h. Apo-ONE® Caspase-3/7 Reagent was added to each well and fluorescence measured after 1h incubation, collecting data over 250 plate reading cycles to generate complete signal curves.

Figure 4.2.4b: Relative caspase activity in AGS cells

Figure 4.2.4b: Effect of NAC treatment on caspase-3/7 activity. AGS cells were seeded into 96-well plates and Apo-ONE® Caspase-3/7 assay performed after 24h. Results demonstrate a significant increase in relative caspase-3/7 activity per 1000 cells with NAC 10 mM ($p = 0.022$). Values are represented as means ± SD; $n = 12$. 
4.3 The role of heme oxygenase activity on cancer cell growth

Study aims

The aim of these experiments was to investigate the influence of HO enzyme isoforms HO-1 and HO-2 on the regulation of cancer cell growth in cultured cell lines.

Methods

Experiments were designed to evaluate the influence of pharmacological modulation of HO enzyme activity on cell proliferation. Cells were treated with the HO enzyme substrate Hemin at a dose range between 2 – 200 µM to cause induction of HO-1 isoenzyme. Cells were also treated with the competitive HO enzyme inhibitor Zinc Protoporphyrin (Zinc PP) at a dose range of 0.1 – 40 µM. The cell response to treatment with vehicle agent sodium hydroxide (NaOH) was evaluated prior to reagent experiments.

MTT assay was performed at 24 hours and at time points of 24, 48 and 72 hours. Cell counts were also performed during the time course experiments. Experiments were extended to 168 hours (7 days) with AGS and OE-33 cell lines to further assess observed effects with selected reagents. Cell lysates from experiment wells were collected for protein, and expression of heme oxygenase 1 and 2 isoforms assessed by western blot analysis. Heme oxygenase activity assay was performed on representative experiment samples. Apoptosis studies were performed to evaluate mechanisms of reduced cell growth using caspase activity assay.

Results are expressed as percentage of control for each individual experiment. Statistical analysis used the non-parametric Kruskal-Wallis Independent samples test or Mann-Whitney U test for 2 independent samples to look for significant differences between average percent of control values. When histograms of continuous variable data demonstrated a normal distribution, one-way ANOVA or the Students t test for 2 independent samples were used to compare means.
4.3.1 Effect of treatment with reagent vehicle NaOH – Figure 4.3.1

Treatment of AGS, HGC-27, OE-33 and HET-1A cells seeded into 24-well plates with 1 mM of NaOH had no significant effect on cell proliferation as measured by MTT assay after 24, 48 and 72 hours. This was in contrast to the observed effects of treatment with hemin 100 µM and zinc protoporphyrin 20 µM over the same time course (Figure 4.3.1). These findings supported the use of NaOH as a vehicle for hemin and zinc reagents. NaOH was used to dissolve hemin and zinc reagents. The maximal final concentration of NaOH in experiment wells was 1 mM.
Figure 4.3.1 Cell proliferation experiments: Effect of vehicle on cell growth

Figure 4.3.1a: Effect of NaOH vehicle on AGS growth

[Graph showing effect of vehicle on AGS cell growth]

Figure 4.3.1b: Effect of NaOH vehicle on HGC-27 growth

[Graph showing effect of vehicle on HGC-27 cell growth]

Figure 4.3.1a – b: Cells were seeded into 24-well plates and treated with NaOH, hemin and zinc PP for 24 – 72h with cell proliferation assessed by MTT assay. NaOH vehicle had no observed effect on cell proliferation for AGS and HGC-27 cells in contrast to significant growth reductions seen with zinc 20 µM. Results are expressed as a percentage of untreated control; values represented as means ± SEM; n = 12.
Figure 4.3.1c: Effect of NaOH vehicle on OE-33 growth

Figure 4.3.1d: Effect of NaOH vehicle on HET-1A growth

Figure 4.3.1c – d: Cells were seeded into 24-well plates and treated with NaOH, hemin and zinc PP for 24 – 72h with cell proliferation assessed by MTT assay. NaOH vehicle had no observed effect on cell proliferation for OE-33 and HET-1A cells in contrast to significant growth reductions seen with high-dose hemin (100µM) and zinc 20µM. Results are expressed as a percentage of untreated control; values represented as means ± SEM; n = 12.
4.3.2 Influence of pharmacological induction of HO by Hemin: 24-hour data

Treating AGS cells with increasing concentrations of hemin for 24 hours in 5 separate experiments with a total of 18 wells per reagent produced no significant overall difference in cell growth ($p = 0.457$). For HGC-27 cells, hemin treatment had a variable effect on cell growth. At a low concentration of 2 µM there was an increase in MTT assay at 24 hours. Treatment with hemin at 150 µM concentration caused a significant reduction in MTT assay after 24 hours ($p = 0.029$). No other hemin concentrations produced a significant effect in HGC-27 cells.

Treating OE-33 cells with increasing concentrations of hemin for 24 hours in 5 separate experiments with a total of 27 wells per reagent produced no significant difference in cell growth ($p = 0.417$). For the normal oesophageal squamous epithelial cell line HET-1A, hemin caused a significant reduction in MTT assay at doses of 50 µM and above under the same experimental conditions ($p \leq 0.003$).
Figure 4.3.2 Cell proliferation experiments: 24-hour data Hemin

Figure 4.3.2a: Effect of Hemin on AGS cell growth at 24h – Summary of 5 experiments total 18 wells per reagent

<table>
<thead>
<tr>
<th>Hemin [µM]</th>
<th>MTT Absorbance % control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100 ± 5</td>
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<tr>
<td>2</td>
<td>95 ± 4</td>
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<tr>
<td>10</td>
<td>92 ± 3</td>
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<td>90 ± 2</td>
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<td>50</td>
<td>88 ± 1</td>
</tr>
<tr>
<td>100</td>
<td>86 ± 0</td>
</tr>
<tr>
<td>150</td>
<td>84 ± 0</td>
</tr>
<tr>
<td>200</td>
<td>82 ± 1</td>
</tr>
</tbody>
</table>

$p = 0.457$ Kruskal-Wallis test

Figure 4.3.2b: Effect of Hemin on HGC-27 cell growth at 24h – Summary of 5 experiments total 18 wells per reagent

<table>
<thead>
<tr>
<th>Hemin [µM]</th>
<th>MTT Absorbance % control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>2</td>
<td>95 ± 4</td>
</tr>
<tr>
<td>10</td>
<td>92 ± 3</td>
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<td>90 ± 2</td>
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<td>50</td>
<td>88 ± 1</td>
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<tr>
<td>100</td>
<td>86 ± 0</td>
</tr>
<tr>
<td>150</td>
<td>84 ± 0</td>
</tr>
<tr>
<td>200</td>
<td>82 ± 1</td>
</tr>
</tbody>
</table>

†$p = 0.029$ Kruskal-Wallis test

Figure 4.3.2a – b: Cells were seeded into 24-well plates, treated with Hemin 2 – 200 µM for 24h and cell proliferation assessed by MTT assay. Hemin had no consistent significant effect on cell proliferation for AGS and HGC-27 cells with the exception of high-dose hemin at 150 µM causing reduced MTT assay in HGC-27 cells. Results are expressed as a percentage of vehicle control; values represented as means ± SEM; n = 18.
Figure 4.3.2c: Effect of Hemin on OE-33 cell growth at 24h – Summary of 5 experiments total 27 wells per reagent

Effect of Hemin on OE-33 cell growth at 24hrs

Hemin [µM] 0 20 40 60 80 100 120
control 2 10 20 50 100 150 200

MTT Absorbance % control

$p = 0.417$ Kruskal-Wallis test

Figure 4.3.2d: Effect of Hemin on HET-1A cell growth at 24h – Summary of 5 experiments total 27 wells per reagent

Effect of Hemin on HET-1A cell growth at 24hrs

Hemin [µM] 0 20 40 60 80 100 120
control 2 10 20 50 100 150 200

MTT % control

*p = 0.04 †p = 0.01 Kruskal-Wallis test

Figure 4.3.2c – d: Cells were seeded into 24-well plates, treated with Hemin 2 – 200 µM for 24h and cell proliferation assessed by MTT assay. Hemin had no significant effect on cell proliferation for OE-33 cells but caused significant reductions in HET-1A cell growth at doses 50 – 200 µM. Results are expressed as a percentage of vehicle control; values represented as means ± SEM; n = 18.
4.3.3 Hemin time course data

Treatment with hemin at 20, 50 and 100 µM concentrations for 24, 48 and 72 hours caused no significant reduction in AGS cell growth measured by MTT assay at any time point observed. Exposing HGC-27 cells to hemin at 20, 50 and 100 µM for 24, 48 and 72 hours caused a significant reduction in MTT assay only with a hemin concentration of 100 µM and a treatment period of 48 hours (\(p = 0.028\)). These data represent four independent experiments with a total of 15 wells per reagent.

Identical experiments with oesophageal cell lines demonstrated no significant changes in OE-33 cell growth at any time point but significantly reduced HET-1A proliferation at a hemin concentration of 100 µM (\(p = 0.03\)).
Figure 4.3.3 Cell proliferation experiments: Time-course data Hemin

Figure 4.3.3a: Effect of Hemin on AGS cell growth – time course

![Effect of Hemin on AGS cell growth](image)

Figure 4.3.3b: Effect of Hemin on HGC-27 cell growth – time course

![Effect of Hemin on HGC-27 cell growth](image)

Figure 4.3.3a – b: Cells were seeded into 24-well plates and treated with Hemin 20 – 100 µM for 24 – 72h with cell proliferation assessed by MTT assay. Hemin had no significant effects on AGS cell proliferation. High-dose hemin at 100 µM caused reduced MTT assay in HGC-27 cells at 48h. Results are expressed as a percentage of vehicle control; values represented as means ± SEM; n = 15.
Figure 4.3.3c: Effect of Hemin on OE-33 cell growth – time course

![Graph showing effect of Hemin on OE-33 cell growth](image)

$p = \text{NS}$

Figure 4.3.3d: Effect of Hemin on HET-1A cell growth – time course

![Graph showing effect of Hemin on HET-1A cell growth](image)

$^\dagger p = 0.03$

Figure 4.3.3c – d: Cells were seeded into 24-well plates and treated with Hemin 20 – 100 µM for 24 – 72h with cell proliferation assessed by MTT assay. Hemin had no significant effects on OE-33 cell proliferation. High-dose hemin at 100 µM caused reduced MTT assay in HET-1A cells at all time points. Results are expressed as a percentage of vehicle control; values represented as means ± SEM; n = 15.
4.3.4 Influence of competitive inhibition of HO by Zinc Protoporphyrin: 24-hour data

Incubating AGS cells for 24 hours with increasing concentrations of zinc protoporphyrin in 5 individual experiments with a total of 18 wells per reagent resulted in cell growth inhibition. MTT assay was significantly reduced at zinc concentrations of 10, 20 and 40 µM ($p < 0.0005$). Adding zinc to HGC-27 cells for 24 hours had no significant effect at concentrations of 0.1, 0.5 and 1 µM. Treatment with higher zinc doses of 10, 20 and 40 µM caused a significant reduction in cell proliferation as measured by MTT assay ($p \leq 0.001$).

Oesophageal OE-33 and HET-1A cell lines were treated with zinc at concentrations of 0.1 – 40 µM in 3 independent experiments with a total of 18 wells per reagent. There was no significant effect on cell growth at lower zinc concentrations. MTT assay was significantly reduced for OE-33 cells after 24 hours of treatment with zinc at concentrations of 10, 20 and 40 µM ($p < 0.0005$). Significant reductions in MTT assay for HET-1A cells were only seen at a zinc concentration of 40 µM ($p = 0.012$). Treatment with zinc protoporphyrin 10 µM or 20 µM produced consistent reductions in cell proliferation with statistical significance only in malignant gastric and oesophageal adenocarcinoma cells and not in control oesophageal squamous epithelium.
Figure 4.3.4 Cell proliferation experiments: 24-hour data Zinc

Figure 4.3.4a: Effect of Zinc on AGS cell growth at 24 hours – *Summary of 5 experiments total 18 wells per reagent*

![Effect of Zinc PP on AGS growth at 24hrs](image)

*Effect of Zinc PP on AGS growth at 24hrs

<table>
<thead>
<tr>
<th>Zinc Protoporphyrin [µM]</th>
<th>MTT Absorbance % control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.1</td>
<td>80</td>
</tr>
<tr>
<td>0.5</td>
<td>60</td>
</tr>
<tr>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
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<tr>
<td>20</td>
<td>5</td>
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<td>40</td>
<td>0</td>
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</table>

*p = 0.01 †p < 0.0005

Figure 4.3.4b: Effect of Zinc on HGC-27 cell growth at 24 hours – *Summary of 5 experiments total 18 wells per reagent*

![Effect of Zinc PP on HGC-27 growth at 24hrs](image)

*Effect of Zinc PP on HGC-27 growth at 24hrs

<table>
<thead>
<tr>
<th>Zinc Protoporphyrin [µM]</th>
<th>MTT Absorbance % control</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
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<tr>
<td>0.1</td>
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<td>20</td>
<td>5</td>
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<tr>
<td>40</td>
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</table>

*p = 0.05 †p ≤ 0.001

* †

Figure 4.3.4a – b: Cells were seeded into 24-well plates, treated with zinc PP 0.1 – 40 µM for 24h and cell proliferation assessed by MTT assay. Zinc PP caused significant cell growth inhibition in AGS and HGC-27 cells at doses 10 – 40 µM (*p ≤ 0.001). Results are expressed as a percentage of vehicle control; values represented as means ± SEM; n = 18.
Figure 4.3.4c: Effect of Zinc on OE-33 cell growth at 24 hours – Summary of 4 experiments total 18 wells per reagent

![Graph showing the effect of Zinc PP on OE-33 cell growth at 24hrs.](image)

†p < 0.0005 Kruskal-Wallis test

Figure 4.3.4d: Effect of Zinc on HET-1A cell growth at 24 hours – Summary of 4 experiments total 18 wells per reagent

![Graph showing the effect of Zinc PP on HET-1A cell growth at 24hrs.](image)

†p = 0.012 Kruskal-Wallis test

Figure 4.3.4c – d: Cells were seeded into 24-well plates, treated with zinc PP 0.1 – 40 µM for 24h and cell proliferation assessed by MTT assay. Zinc PP caused significant cell growth inhibition in OE-33 cells at doses 10 – 40 µM (p < 0.0005) and in HET-1A cells only at 40 µM concentration (p = 0.012). Results are expressed as a percentage of vehicle control; values represented as means ± SEM; n = 18.
4.3.5 Zinc time course data

Treatment with zinc PP at 10, 20 and 40 µM concentrations for 24, 48 and 72 hours in 4 independent experiments caused significant decreases in AGS cell growth at zinc PP concentrations of 20 and 40 µM (p < 0.03). Exposing HGC-27 cells to zinc under the same experimental conditions produced similar significant reductions in cell proliferation as measured by MTT assay for various time points (Figure 4.3.5).

Identical independent time-course experiments performed in oesophageal cell lines demonstrated contrasting results between benign and malignant cells. Cell proliferation measured by MTT assay was significantly reduced in HET-1A cells only with a zinc PP concentration of 40 µM. Growth of OE-33 cells was significantly reduced at all observed time points with all experimental concentrations of zinc protoporphyrin (p ≤ 0.038).
Figure 4.3.5 Cell proliferation experiments: Time-course data Zinc

Figure 4.3.5a: Effect of Zinc on AGS cell growth over time – Summary of 4 experiments total 12 wells per reagent

![Effect of Zinc PP on AGS cell growth](image)

<table>
<thead>
<tr>
<th>Zinc Protoporphyrin [µM]</th>
<th>MTT Absorbance % control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc 10</td>
<td>91 ± 10</td>
</tr>
<tr>
<td>Zinc 20</td>
<td>88 ± 10</td>
</tr>
<tr>
<td>Zinc 40</td>
<td>82 ± 10</td>
</tr>
</tbody>
</table>

†p < 0.03  *p = 0.002  ‡p < 0.0005

Figure 4.3.5b: Effect of Zinc on HGC-27 cell growth over time – Summary of 4 experiments total 12 wells per reagent

![Effect of Zinc PP on HGC-27 cell growth](image)

<table>
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<tr>
<th>Zinc Protoporphyrin [µM]</th>
<th>MTT Absorbance % control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc 10</td>
<td>86 ± 10</td>
</tr>
<tr>
<td>Zinc 20</td>
<td>76 ± 10</td>
</tr>
<tr>
<td>Zinc 40</td>
<td>74 ± 10</td>
</tr>
</tbody>
</table>

†p = 0.013  *p < 0.05

Figure 4.3.5a – b: Cells were seeded into 24-well plates and treated with zinc PP 10 – 40 µM for 24 – 72h with cell proliferation assessed by MTT assay. Zinc PP caused significant cell growth inhibition in AGS and HGC-27 cell lines at concentrations of 20 and 40 µM at various time points. Results are expressed as a percentage of vehicle control; values represented as means ± SEM; n = 12.
Figure 4.3.5c: Effect of Zinc on OE-33 cell growth over time – Summary of 4 experiments total 12 wells per reagent

![Effect of Zinc PP on OE-33 cell growth](image)

**Zinc Protoporphyrin [µM]**
- Zinc 10
- Zinc 20
- Zinc 40

**MTT Absorbance % control**
- 100
- 80
- 60
- 40
- 20
- 0

**24 hrs**
![Graph](image)

- **Zinc 10**: 89 ± 7
- **Zinc 20**: 82 ± 7
- **Zinc 40**: 79 ± 7

**48 hrs**
![Graph](image)

- **Zinc 10**: 70 ± 7
- **Zinc 20**: 66 ± 7
- **Zinc 40**: 55 ± 7

**72 hrs**
![Graph](image)

- **Zinc 10**: 69 ± 7
- **Zinc 20**: 60 ± 7
- **Zinc 40**: 54 ± 7

†p < 0.0005 *p < 0.02 ‡p = 0.038

Figure 4.3.5d: Effect of Zinc on HET-1A cell growth over time – Summary of 4 experiments total 12 wells per reagent

![Effect of Zinc PP on HET-1A cell growth](image)

**Zinc Protoporphyrin [µM]**
- Zinc 10
- Zinc 20
- Zinc 40

**MTT Absorbance % control**
- 100
- 80
- 60
- 40
- 20
- 0

**24 hrs**
![Graph](image)

- **Zinc 10**: 100 ± 7
- **Zinc 20**: 88 ± 7
- **Zinc 40**: 73 ± 7

**48 hrs**
![Graph](image)

- **Zinc 10**: 88 ± 7
- **Zinc 20**: 63 ± 7
- **Zinc 40**: 54 ± 7

**72 hrs**
![Graph](image)

- **Zinc 10**: 51 ± 7
- **Zinc 20**: 57 ± 7
- **Zinc 40**: 41 ± 7

†p < 0.05 *p < 0.02

Figure 4.3.5c – d: Cells were seeded into 24-well plates and treated with zinc PP 10 – 40 µM for 24 – 72h with cell proliferation assessed by MTT assay. Zinc PP caused significant cell growth inhibition in OE-33 cells at all time points with all concentrations. MTT assay was significantly reduced in HET-1A cells only with a zinc PP concentration of 40 µM. Results are expressed as a percentage of vehicle control; values represented as means ± SEM; n = 12.
4.3.6 Cell count data

Cell count data for AGS cells demonstrate a non-significant decrease in cell numbers after treatment with hemin at 20, 50 and 100 µM concentrations for 24 hours \((p = 0.099)\). There was no real change in HGC-27 cell numbers under the same conditions \((p = 0.598)\). Cell counts for both AGS and HGC-27 cells showed a decrease in cell number after exposure to zinc at concentrations of 5, 10 and 20 µM for 24 hours \((p \leq 0.014)\).

4.3.7 Extended growth curves

Prolonged exposure of epithelial cells to zinc at concentrations of 20 and 40 µM produced similar results in AGS and OE-33 cell lines. Extending the experiments to 168 hours showed a peak in exponential cell growth of control cells at 96 hours with cell numbers levelling out and falling beyond 120 hours. This observed effect was due to cells becoming over-confluent and crowded on the growth surface with resulting detachment from monolayer. Consistent effects of significantly reduced cell proliferation in both AGS \((p < 0.0002)\) and OE-33 \((p < 0.001)\) cells were seen with zinc treatment at 20 and 40 µM at all time points beyond 24 hours (Figure 4.3.7).
Figure 4.3.6 Cell Count Data

Figure 4.3.6a: Effect of Hemin on AGS & HGC-27 cell counts at 24 hours

\[ p = 0.099 \] Independent Samples Kruskal-Wallis test AGS cells
\[ p = 0.598 \] Independent Samples Kruskal-Wallis test HGC-27 cells

Figure 4.3.6b: Effect of Zinc on AGS & HGC-27 cell counts at 24 hours

\[ p = 0.004 \] Independent Samples Kruskal-Wallis test AGS cells
\[ p = 0.014 \] Independent Samples Kruskal-Wallis test HGC-27 cells

Figure 4.3.6: Gastric cell lines were seeded into 24-well plates and treated with hemin [20, 50, 100 µM] and zinc PP [5, 10, 20 µM] for 24h. Cell growth was assessed by cell counting using Trypan blue exclusion. Significantly fewer cells were counted after exposure to zinc PP at concentrations of 5, 10 and 20 µM for 24 hours \((p \leq 0.014)\).
Figure 4.3.7 Extended Growth Curves: Zinc experiments

Figure 4.3.7a: Extended growth curves with AGS cells exposed to Zinc

\[ p < 0.0002 \] all time points Independent Samples Kruskal-Wallis test

Figure 4.3.7b: Extended growth curves with OE-33 cells exposed to Zinc

\[ p < 0.001 \] all time points Independent Samples Kruskal-Wallis test

Figure 4.3.7a – b: AGS and OE-33 cells were treated with zinc PP 20 and 40 µM for 24 – 168h in 24-well plates and cell viability assessed with MTT assay and cell counts. Significant inhibition of cell proliferation is observed throughout the period of exponential cell growth in both cell lines (up to 96h AGS cells and 120h OE-33 cells). Values represented as means ± SEM; \( n = 9 \).
4.3.8 Effect of Hemin and ZnPP on HO protein expression

Western blot protein analysis showed an increase in HO-1 isoform expression with increasing doses of hemin (20, 50 and 100 µM) in all cell lines investigated. Hemin treatment had no effect on HO-2 protein expression in any of the cell lines (western blots 4.3.8). This demonstrates the response of the inducible HO-1 isoform to hemin, while constitutive HO-2 expression is unchanged. Tubulin expression was equivalent in experimental cell lysates demonstrating adequate protein loading onto electrophoresis gels.

Addition of zinc PP at 5 and 10 µM concentrations caused up-regulation of HO-1 protein in AGS and HGC-27 cells after 24 hours (western blots 4.3.8.1). Expression of HO-2 protein was equal in gastric cell lines and unchanged with zinc treatment. Exposure of OE-33 and HET-1A cells to zinc protoporphyrin caused a mild decrease in HO-2 isoform expression after 24 hours of cell treatment with zinc at 100 µM concentrations (western blots 4.3.8.2). There is slightly decreased β-tubulin expression in the HET-1A sample loaded into the zinc 100 µM lane that may account for the apparent reduction in HO-2 expression. Probing the blots for tubulin otherwise demonstrated equal protein loading of cell lysates.

In OE-33 cells HO-1 protein expression was less marked and only in response to the highest dose of zinc protoporphyrin at 100 µM. HO-1 protein expression was readily induced in HET-1A cells by treatment with zinc at 20, 40 and 100 µM.
Figure 4.3.8 Western Blots

Western Blots 4.3.8.1: Hemin and Zinc PP Gastric Cancer cell lines

AGS Hemin and Zinc PP protein expression

HGC-27 Hemin and Zinc PP protein expression

Figure 4.3.8.1: Protein expression in gastric cell lines after treatment with hemin and zinc PP. Cells were seeded into 24-well plates and protein lysates harvested at 24h. Gel electrophoresis and Western blotting were performed as described. Blots demonstrate unchanged expression of HO-2 and increased induction of HO-1 protein with increasing doses of hemin and zinc PP. M = marker, Con = control
Western Blots 4.3.8.2: Hemin and Zinc Oesophageal Cancer cell lines

OE-33 Hemin and Zinc PP protein expression

HET-1A Hemin and Zinc PP protein expression

Figure 4.3.8.2: Protein expression in oesophageal cell lines after treatment with hemin and zinc PP. Cells were seeded into 24-well plates and protein lysates harvested at 24h. Gel electrophoresis and Western blotting were performed as described. Blots demonstrate slightly reduced expression of HO-2 in response to zinc PP 100 µM. HO-1 protein expression was readily induced by hemin and by zinc PP treatment in HET-1A cells. M = marker, Con = control
4.3.9 Heme Oxygenase Enzyme Activity Assay

Cellular HO activity was measured by performing a specific colorimetric assay. The results demonstrate an increase in total HO enzyme activity after treatment with hemin 40 µM for 6, 12 and 24 hours consistent with the demonstrable up regulation of HO-1 protein. The results show a significant decrease in total HO enzyme activity after treatment with zinc 40 µM for 6 and 12 hours when compared to untreated control cells (Figure 4.3.9). These findings are consistent with competitive inhibition of heme oxygenase enzyme by zinc protoporphyrin.
**Figure 4.3.9 Heme Oxygenase Enzyme Activity Assay**

**Figure 4.3.9a: Effect of Hemin and Zinc on HO enzyme activity time course**

![Graph showing the effect of hemin and zinc on HO enzyme activity](image1)

**Figure 4.3.9b: Effect of Hemin and Zinc on HO enzyme activity time course**

![Graph showing the effect of hemin and zinc on HO enzyme activity](image2)

†p ≤ 0.05 Independent Samples Kruskal-Wallis test compared to control

*p < 0.002 Independent Samples Kruskal-Wallis test all samples

Figure 4.3.9: Effect of hemin and zinc PP on total HO enzyme activity. AGS cells were seeded into 175 cm$^3$ flasks and lysates harvested after 6, 12 and 24h treatment as described. Specific colorimetric assay demonstrated a significant increase in HO activity with HO-1 induction associated with hemin and a significant reduction in HO activity after brief exposure to zinc 40 µM. Values are represented as means ± SD; n = 3.
4.3.10 Apoptosis Studies: Caspase 3/7 activity assay

The Apo-ONE Homogenous Caspase-3/7 assay (Promega) was performed after 12 hour treatment with hemin 20 µM and zinc 20 µM and 40 µM. Evolution of the assay data over 100 minutes is shown in Figure 4.3.10a.

Cell proliferation assay was performed concurrently in 96-well plates with an identical experiment design to provide cell numbers per well derived from MTT assay performed as described above. The relative caspase activity per 1000 cells for experiment samples was obtained after normalisation to MTT assay and untreated control cells. Results demonstrate an increase in fold caspase-3/7 induction after 12 hours treatment with zinc protoporphyrin with statistical significance at a dose of 20 µM ($p = 0.035$, Figure 4.3.10b). There was no increase in caspase-3/7 induction after 12 hours exposure to hemin.
Figure 4.3.10 Apoptosis Studies: Caspase 3/7 activity assay

Figure 4.3.10a: Caspase activity at 12 hours AGS cells

Figure 4.3.10a: Evolution of kinetic absorbance data over 100 minutes during caspase 3/7 assay. AGS cells were seeded into black 96-well plates and treated with hemin or zinc PP for 12h. Apo-ONE® Caspase-3/7 Reagent was added to each well and fluorescence measured after 1h incubation, collecting data over 250 plate reading cycles to generate complete signal curves.

Figure 4.3.10b: Relative caspase activity in AGS cells

Figure 4.3.10b: Effect of hemin and zinc treatment on caspase-3/7 activity. AGS cells were seeded into 96-well plates and Apo-ONE® Caspase-3/7 assay performed after 12h. Results demonstrate a significant increase in relative caspase-3/7 activity per 1000 cells with zinc 40 µM (p = 0.035). Values are represented as means ± SD; n = 12.
4.4 Investigating a Specific Role for HO-2 Enzyme in Cell Growth

To investigate a specific effect of the HO-2 isoform protein short interfering RNA (siRNA)-mediated HO-2 gene knockdown experiments were performed.

4.4.1 Establishing protocols for adequate transfection of siRNA complex

4.4.1.1 Toxicity

Although the manufacturer claims that the transfection reagent has a mild cytotoxicity profile, initial experiments were designed to establish the effects of different concentrations of Lipofectamine on cellular proliferation.

4.4.1.2 Lipofectamine protocol

Lipofectamine experiments were set up in 24-well plates for AGS cells at concentrations of 0.5, 1.0 and 1.5 µl. Cells in culture were diluted with fresh medium without antibiotics and seeded at 1.25 x 10^4 cells per well in 24-well plates, such that cells were 30-40% confluent at the time of adding transfection reagent. Lipofectamine was added diluted in Opti-MEM medium at concentrations of 0.5, 1.0 and 1.5 µl per well. After 10 hours the reagent medium was washed out and replaced with fresh serum-complete medium. MTT assay was performed at 72 hours to determine the effect of the transfection reagent on cell proliferation.

4.4.1.3 Lipofectamine results

MTT assay was not significantly different for AGS cells at concentrations of 0.5, 1.0 and 1.5µl per well. There did not appear to be a toxic effect on AGS cells with the recommended concentrations of Lipofectamine at 72 hours (Figure 4.4.1a).

4.4.1.4 Transfection efficiency

The next stage was to assess the efficacy of oligonucleotide transfection using the chosen Lipofectamine transfection reagent. In order to assess uptake of double stranded nucleic acid into the cells, a fluorescently tagged oligonucleotide was transfected with the Lipofectamine. The labelled RNAi duplex recommended for use with Lipofectamine RNAiMAX was BLOCK-iT™ AlexaFluor Red Fluorescent Control obtained from Invitrogen.
This is a double-stranded RNA molecule with the same length, charge and configuration as standard siRNA, whose sequence is not homologous to any known gene to avoid non-specific cellular events caused by its introduction into the cytoplasm.

Cells were seeded into a 24-well plate at 1.25 x 10^4 cells per well. Lipofectamine and AlexaFluor duplexes were first diluted in Opti-MEM medium then combined and left to incubate for 20 minutes at room temperature. This allows the RNAi duplex-Lipofectamine transfection complex to form. The complexes were added to the wells to give a range of final concentrations as shown:

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Control</th>
<th>Control</th>
<th>Control</th>
<th>Control</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimem</td>
<td>Lipofectamine 0.5µl</td>
<td>Lipofect 0.5µl + AlexaFluor10nM</td>
<td>Lipofect 0.5µl + AlexaFluor20nM</td>
<td>Lipofect 0.5µl + AlexaFluor40nM</td>
<td></td>
</tr>
<tr>
<td>Optimem</td>
<td>Lipofectamine 1.0µl</td>
<td>Lipofect 1.0µl + AlexaFluor10nM</td>
<td>Lipofect 1.0µl + AlexaFluor20nM</td>
<td>Lipofect 1.0µl + AlexaFluor40nM</td>
<td></td>
</tr>
<tr>
<td>Optimem</td>
<td>Lipofectamine 1.5µl</td>
<td>Lipofect 1.5µl + AlexaFluor10nM</td>
<td>Lipofect 1.5µl + AlexaFluor20nM</td>
<td>Lipofect 1.5µl + AlexaFluor40nM</td>
<td></td>
</tr>
</tbody>
</table>

After 10 hours the medium in the wells was replaced with fresh serum-complete medium. MTT assay was performed at 72 hours to determine any effects on cell growth. To assess the RNA uptake, wells were examined under inverted fluorescent microscopy after replacing the culture medium with warm PBS.

A separate 6-well experiment plate was set up with AlexaFluor oligo at concentrations of 1, 5, 10, 20 and 40nM. Lipofectamine was used at 4.2µl per well, equivalent to 1µl per well in a 24-well plate. Microscopy slide coverslips were placed in the base of the well and coating medium applied prior to cell seeding. This enabled subsequent removal of the coverslip onto a slide with the cell monolayer intact and facilitated fluorescent microscopy examination.
4.4.1.5 AlexaFluor results

Although there was a trend towards reduced cell growth in cells treated with higher concentrations of AlexaFluor oligo with a higher concentration of Lipofectamine, there were no significant differences between MTT assay results in any of the experimental combinations (Figure 4.4.1b).

AlexaFluor Red-labelled oligo uptake on fluorescent microscopy was assessed comparing one concentration of Lipofectamine (1µl) and various oligonucleotide concentrations. There was a distinct improvement in uptake with AlexaFluor 10nM as opposed to 1nM or 5nM, with intracellular red staining clearly visible at x20 and x40 magnifications (Figure 4.4.1c).

These results demonstrate effective transfection of the labelled siRNA using Lipofectamine reagent at 1µl per well and oligo at 10nM concentration.

4.4.1.6 siRNA and Negative Universal Control

In order to assess potential effects of the negative universal control (NUC) on the cell lines of interest, experiments were set up using AGS and HGC-27 cells exposed to various concentrations of NUC for a 72 hour period.

4.4.1.7 NUC Oligonucleotide protocol

Cells were suspended in fresh medium without antibiotics and seeded at 1.25 x 10⁴ cells per well in 24-well plates, such that cells were 30-40% confluent at the time of adding transfection reagent. Lipofectamine was used at 1 µl per well, and NUC oligo was added to give a final concentrations of 1, 2, 5, 10, 20 and 40 nM per well. The NUC was supplied as a stock solution at 20 µM concentration. A calculated volume of stock NUC solution was added to 50 µl of Opti-MEM reduced serum medium and mixed gently. Lipofectamine reagent was mixed before use, and then 1 µl added to 50 µl Opti-MEM medium. The diluted NUC duplex and transfection reagent were combined and allowed to incubate for up to 20 minutes at room temperature to allow complexes to form. The total volume of 100 µl was added to 500 µl of antibiotic-free medium in the wells to give a total final volume of 600 µl. Medium was changed after 8 – 10 hours in all wells. MTT
assay and cell counts were performed at 72 hours to assess any effects on cell proliferation.

4.4.1.8 Negative Universal Control results

For AGS cells there was a reduction in cell growth 72 hours after transfection with negative universal control at a concentration of 40 nM. MTT assay was reduced ($p = 0.069$) and cell counts significantly reduced at 72 hours ($p = 0.024$). There was no effect with NUC 10 nM and no significant effects with NUC 5 nM or 20 nM (Figures 4.4.1d & 4.4.1e).

Identical experiments using HGC-27 cells demonstrated similar results with a significantly reduced cell growth at 72 hours after transfection with NUC at 40 nM ($p = 0.021$). HGC-27 cells appeared to be more sensitive to transfection with the control oligonucleotide, with all concentrations above 1 nM causing a reduction in MTT assay at 72 hours (average % control < 90) but these observed effects only reached significance at a concentration of NUC 40 nM. There were no significant differences in MTT assay outcomes for HGC-27 cells between NUC concentrations of 5, 10 or 20 nM (Figure 4.4.1f).
Figure 4.4.1 Establishing protocols for adequate transfection of siRNA complex

Figure 4.4.1a: Effect of Lipofectamine on AGS cell growth at 72 hours

$\rho = 0.151$ Independent samples Kruskal-Wallis test

Figure 4.4.1a: AGS cells were treated with 0.5 – 1.5 µL lipofectamine transfection reagent in 24-well plates for 10h. MTT assay performed at 72h showed no significant effect on cell proliferation. Results are expressed as a percentage of vehicle control; values represented as means ± SEM; $n = 12$.

Figure 4.4.1b: Effect of siRNA controls on AGS cell growth at 72 hours

$\rho = 0.058$ Independent samples Kruskal-Wallis test

Figure 4.4.1b: AGS cells were treated with lipofectamine transfection reagent ± AlexaFluor RNAi duplex at various concentration combinations. MTT assay performed at 72h showed no significant effect on cell proliferation despite a trend toward reduced cell growth with high-dose combinations. Results are expressed as a percentage of vehicle control; values represented as means ± SEM; $n = 3$. 
Figure 4.4.1c Fluorescent microscopy of siRNA transfection controls

Lipofectamine 1µl + AlexaFluor 1nM x20    Lipofectamine 1µl + AlexaFluor 5nM x20

Figure 4.4.1c: Fluorescent microscopy to assess transfection efficiency at various oligonucleotide concentrations. Intracellular red staining is displayed at x20 and x40 magnifications. Transfection of labelled AlexaFluor duplex is effective at 10 nM concentration with 1 µL lipofectamine reagent per well.

Figure 4.4.1d: Effect of siRNA NUC on AGS cell counts

Figure 4.4.1d: AGS cells seeded into 24-well plates were treated with lipofectamine reagent plus scrambled negative universal control duplex (NUC). Cell counts performed at 72h showed no significant effect on cell proliferation except for high dose NUC 40 nM. Values represented as means ± SEM; n = 12.
Figure 4.4.1e: Effect of siRNA NUC on AGS cell growth

Figure 4.4.1e: AGS cells seeded into 24-well plates were treated with lipofectamine transfection reagent plus NUC. MTT assay performed at 72h showed no significant effect on cell proliferation. Results are expressed as a percentage of vehicle control; values represented as means ± SEM; n = 12.

\[ p = 0.069 \text{ Independent Samples Kruskal-Wallis test} \]

Figure 4.4.1f: Effect of siRNA NUC on HGC-27 cell growth

Figure 4.4.1f: HGC-27 cells seeded into 24-well plates were treated with lipofectamine transfection reagent plus NUC. MTT assay performed at 72h showed no significant effect on cell proliferation except for high dose NUC 40 nM. Results are expressed as a percentage of vehicle control; values represented as means ± SEM; n = 12.

\[ \dagger p = 0.021 \]
4.4.2 Efficacy of supplied siRNA and HO-2 knockdown

4.4.2.1 siRNA Oligonucleotide protocol

The siRNA targeted to the HO-2 gene was supplied as three individual non-overlapping sequences (Invitrogen). The next stage was to determine which individual siRNA resulted in effective knockdown of the HO-2 gene transcript.

The Stealth™ Select siRNA targeted to the HO-2 gene (HMOX2) were delivered freeze-dried and desalted by normal phase chromatography with a total amount of 20 nmole. This amount was resuspended in 1 ml RNase-free water previously treated with diethylpyrocarbonate that inactivates RNase enzymes. This gave 0.02 µmole in 1 ml, a solution of 20 µM concentration. From the stock solution 0.3 µl was added to 50 µl Opti-MEM medium and mixed gently. A volume of 1 µl Lipofectamine RNAiMAX was added to 50 µl Opti-MEM medium and mixed gently, then added to the diluted siRNA oligonucleotide. This mixture was left to incubate for 20 minutes at room temperature to enable transfection complexes to form. The total volume of 100 µl was added to 500 µl of antibiotic-free medium in the wells to give a final siRNA concentration of 10 nM in each experimental well of a 24-well plate. The amount of siRNA duplex used in each well was 6 pmole. Because transfection complexes were added to more than one well in each experiment, a working stock of siRNA with lipofectamine was prepared at x6 concentration by serial dilution, with 100 µl of the stock added to each siRNA experiment well.

The medium in every well was replaced with fresh serum-complete medium 8 – 10 hours after transfection. Samples were collected for RNA isolation and analysis 24, 48 and 72 hours after transfection. Purified RNA was obtained from collected samples and reverse transcriptase polymerase chain reaction performed for qualitative and quantitative assessment of mRNA knockdown achieved. Cell lysates were collected for protein assay and subsequent Western blotting to assess HO-2 and HO-1 protein expression.
4.4.2.2 RNA purification results

Quality control with PCR using GAPDH primers demonstrated that without using the Turbo DNase enzyme (Ambion) there were clear PCR products visible on ethidium bromide gels. There were no bands visible for samples treated with DNase enzyme (Figure 4.4.2a). This process was repeated for every sample of total RNA isolated and purified to ensure no contaminating DNA was present prior to making complementary DNA.

4.4.2.3 Reverse transcriptase polymerase chain reaction results

Analysis of the RT-PCR products demonstrated effective HO-2 gene knockdown after transfection with two out of the three supplied siRNA duplexes. The most effective was siRNA 02, with a complete absence of product band identifiable from PCR with the HMOX2 primers. By contrast there were equal bands visible from each RNA sample after RT-PCR with GAPDH primers (Figure 4.4.2b). For all subsequent siRNA experiments the siRNA 02 duplex was used for HO-2 gene knockdown.

Agarose gel analysis of RT-PCR products demonstrated effective knockdown of HO-2 mRNA after transfecting cells with targeted siRNA for 24, 48 and 72 hours. There were no visible bands after PCR with HMOX2 primers at 24 and 72 hours, and a markedly reduced band at 48 hours when compared to PCR products with GAPDH primers. The quality of the results is compromised by the appearance of reduced HO-2 mRNA after 72 hours of lipofectamine treatment, although this may reflect a reduction of total RNA in this sample (Figure 4.4.2c). Experiments were repeated and PCR products evaluated using agarose gel electrophoresis for both AGS and HGC-27 cell lines. Although analysis demonstrated effective knockdown of HO-2 mRNA after transfecting cells with siRNA duplexes there was inconsistent findings with PCR products using GAPDH primers (see Appendix VII). These results suggested a compromise in RNA quality with possible DNA contamination in the eluted RNA samples. Treatment with Turbo DNase enzyme was repeated and any samples with visible DNA contamination were discarded along with any corresponding complementary DNA stored. Effective knockdown of HO-2 mRNA using the selected siRNA duplex was demonstrated in both AGS and HGC-27 cells with relative preservation of GAPDH mRNA (Figures 4.4.2d & 4.4.2e).
Figure 4.4.2 RNA sample preparation and PCR Products

Figure 4.4.2a: DNA contamination & DNase treatment

Figure 4.4.2a: PCR products using GAPDH primers demonstrated effective removal of DNA in samples treated with DNase enzyme. Clear bands are visible from samples not treated with DNase. Isolated RNA was therefore treated with DNase prior to making complementary DNA. Lanes: 1=Control, 2=Lipofectamine, 3=NUC 10nM, 4=siRNA 01 10nM, 5=siRNA 02 10nM, 6=siRNA 03 10nM ALL WITH DNase treatment; 7=Lipofectamine, 8=NUC 10nM, 9=siRNA 01 10nM, 10=siRNA 02 10nM, 11=siRNA 03 10nM ALL WITHOUT DNase treatment.

Figure 4.4.2b: siRNA select duplexes

Figure 4.4.2b: PCR products demonstrate effective HO-2 gene knockdown after transfection with two out of the three supplied siRNA duplexes with the most effective siRNA 02 (lane 5). Lanes: 1=NUC 10nM, 2=NUC 10nM, 3=NUC 10nM, 4=siRNA duplex 01 10nM, 5=siRNA duplex 02 10nM, 6=siRNA duplex 03 10nM.
Figure 4.4.2c: PCR products siRNA knockdown HMOX2 and GAPDH AGS cells

Lanes
1 = Lipofectamine 24hrs  
2 = NUC 10nM 24hrs  
3 = siRNA 10nM 24hrs  
4 = Lipofectamine 48hrs  
5 = NUC 10nM 48hrs  
6 = siRNA 10nM 48hrs  
7 = Lipofectamine 72hrs  
8 = NUC 10nM 72hrs  
9 = siRNA 10nM 72hrs

Figure 4.4.2c: All subsequent siRNA experiments used the siRNA 02 duplex for HO-2 gene knockdown. Analysis of RT-PCR products demonstrated effective knockdown of HO-2 mRNA after 24, 48 and 72 hours. The appearance of reduced HO-2 mRNA after 72 hours of lipofectamine treatment may reflect a reduction of total RNA in this sample with a visible reduction in GAPDH band for this sample.
Figure 4.4.2d: Repeated analysis of RT-PCR products demonstrated effective knockdown of HO-2 mRNA using the selected siRNA duplex in AGS cells. HO-2 knockdown appears to be most effective at 48 – 72h with relative preservation of GAPDH mRNA.
Figure 4.4.2e: Repeated analysis of RT-PCR products demonstrated effective knockdown of HO-2 mRNA using the selected siRNA duplex in HGC-27 cells. HO-2 mRNA is reduced even after 24h in HGC-27 cells with relative preservation of GAPDH mRNA.
### 4.4.3 Real-time Quantitative PCR

In order to clarify siRNA-mediated knockdown of the heme oxygenase target mRNA, real-time quantitative PCR analysis was performed using pre-optimised TaqMan® assays for human heme oxygenase 1 (Hs00157965_m1) and heme oxygenase 2 (Hs00157969_m1).

The purity and concentration of all RNA samples eluted for real-time PCR analysis was estimated UV absorbance measurement with a spectrophotometer. The measured $A_{260}/A_{280}$ ratio value was between 1.8 and 2.1 for all samples, indicating a pure RNA sample for experimental purposes (Table 4.4.3).

To provide accurate RNA quantification prior to reverse transcriptase PCR a sensitive RNA-binding stain was used called Quant-iT™ RiboGreen® RNA assay (Invitrogen) with construction of standard curves against a ribosomal standard (Figure 4.4.3a). A total of 40 ng RNA was used for cDNA synthesis for each sample. The cDNA samples were diluted to 1 ngmL$^{-1}$ with pure water and a total of 10 ng used in each PCR amplification reaction.

The comparative $C_T$ method was used to calculate the expression of target HO-1 and HO-2 mRNA in AGS and OE-33 cell samples from siRNA experiments. The expression level of the HO-1 and HO-2 genes of interest were normalised to an endogenous 18S ribosomal RNA gene to normalise for variation in the amount and quality of RNA between samples.

In AGS experiments siRNA-mediated knockdown of HO-2 using two separate oligonucleotides (siRNA duplex 01 and siRNA duplex 02) produced a clear reduction in relative HO-2 mRNA expression and a significant effect with siRNA duplex 02 ($p = 0.004$, Figure 4.4.3b). There was a significant corresponding increase in relative expression of HO-1 mRNA (Figure 4.4.3c). Very similar results were seen for the OE-33 cells with significantly reduced HO-2 expression and significantly increased HO-1 expression after transfection with targeted siRNA oligonucleotides (Figures 4.4.3d & 4.4.3e). Relative RNA expression results are a summary of 3 experiments with total of 9 RNA samples used per reagent. The results confirm efficient knockdown of HO-2 mRNA from the siRNA experiment protocols used and indicate a concurrent up regulation of HO-1 mRNA.
4.4.3 Real-time Quantitative PCR Results

Table 4.4.3: RNA quantification by Ultraviolet Absorbance – $A_{260}:{A_{280}}$ ratio

<table>
<thead>
<tr>
<th>Sample</th>
<th>OD 280 nm</th>
<th>OD 260 nm</th>
<th>Purity</th>
<th>RNA [ngµL$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGS cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.33</td>
<td>0.671</td>
<td>2.033333</td>
<td>402.6</td>
</tr>
<tr>
<td>NUC</td>
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<tr>
<td>siRNA 01</td>
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<td>0.411</td>
<td>2.004878</td>
<td>246.6</td>
</tr>
<tr>
<td>siRNA 02</td>
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<td>0.401</td>
<td>1.975369</td>
<td>240.6</td>
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<tr>
<td>Control</td>
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<td>1.029</td>
<td>2.074597</td>
<td>617.4</td>
</tr>
<tr>
<td>NUC</td>
<td>0.411</td>
<td>0.848</td>
<td>2.06326</td>
<td>508.8</td>
</tr>
<tr>
<td>siRNA 01</td>
<td>0.273</td>
<td>0.557</td>
<td>2.040293</td>
<td>334.2</td>
</tr>
<tr>
<td>siRNA 02</td>
<td>0.248</td>
<td>0.514</td>
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<td>308.4</td>
</tr>
<tr>
<td>OE33 cells</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
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<td>2.069069</td>
<td>413.4</td>
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<td>0.654</td>
<td>2.04375</td>
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<tr>
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<td>0.357</td>
<td>2.051724</td>
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</tr>
<tr>
<td>siRNA 02</td>
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<tr>
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<td>2</td>
<td>642</td>
</tr>
<tr>
<td>NUC</td>
<td>0.41</td>
<td>0.845</td>
<td>2.060976</td>
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</tr>
<tr>
<td>siRNA 01</td>
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<td>0.587</td>
<td>2.066901</td>
<td>352.2</td>
</tr>
<tr>
<td>siRNA 02</td>
<td>0.275</td>
<td>0.498</td>
<td>1.810909</td>
<td>298.8</td>
</tr>
</tbody>
</table>

Table 4.4.3: UV absorbance measurement of all RNA samples eluted for real-time PCR analysis estimated the $A_{260}:{A_{280}}$ ratio value between 1.8 and 2.1 indicating a pure RNA sample.
Figure 4.4.3a: RNA quantification by RiboGreen Assay

Standard Curve

![Standard Curve All Samples](image)

\[ y = 24853x + 12.315 \]
\[ R^2 = 0.9945 \]

RNA concentrations

<table>
<thead>
<tr>
<th>Sample</th>
<th>SYBR - Blank</th>
<th>RNA [ngµL(^{-1})]</th>
<th>Mean</th>
<th>% CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20563.789</td>
<td>595.488</td>
<td>565.275</td>
<td>4.645</td>
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<td></td>
<td>18922.654</td>
<td>548.004</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>19072.285</td>
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<td></td>
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</tr>
<tr>
<td>NUC</td>
<td>21343.844</td>
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<td>680.308</td>
<td>8.162</td>
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<tr>
<td></td>
<td>24111.793</td>
<td>698.142</td>
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<td></td>
<td>25030.52</td>
<td>724.724</td>
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<td></td>
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<td>421.004</td>
<td>7.065</td>
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<td></td>
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<td></td>
<td>14203.291</td>
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<td>491.3</td>
<td>4.514</td>
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<tr>
<td></td>
<td>16841.518</td>
<td>487.791</td>
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</tbody>
</table>

CV = coefficient of variation

Figure 4.4.3a: Accurate RNA quantification was provided by RiboGreen® RNA assay standard curves prior to reverse transcriptase PCR. A total of 40 ng RNA was used for cDNA synthesis for each sample calculated from derived RNA concentrations.
Figure 4.4.3b: Relative HO-2 mRNA expression after 48 hrs AGS cells

![Bar chart showing HO-2 expression after 48 hrs AGS cells](chart1.png)

Figure 4.4.3c: Relative HO-1 mRNA expression after 48 hrs AGS cells

![Bar chart showing HO-1 expression after 48 hrs AGS cells](chart2.png)

Figure 4.4.3b – c: Expression of HO-1 and HO-2 mRNA normalised to endogenous 18S ribosomal RNA using the comparative C\(_T\) method. Knockdown of HO-2 in AGS cells using two separate oligonucleotides (siRNA 01 and siRNA 02) produced a significant reduction in relative HO-2 mRNA expression with a corresponding increase in relative expression of HO-1 mRNA. Values represented as means ± SEM; \(n = 9\).
Figure 4.4.3d: Relative HO-2 mRNA expression after 48 hrs OE33 cells

![Bar chart showing relative HO-2 mRNA expression with error bars.](image)

+ p = 0.05  †p = 0.005

Figure 4.4.3e: Relative HO-1 mRNA expression after 48 hrs OE33 cells

![Bar chart showing relative HO-1 mRNA expression with error bars.](image)

†p = 0.002

Figure 4.4.3d – e: Expression of HO-1 and HO-2 mRNA normalised to endogenous 18S ribosomal RNA using the comparative C\_t method. Knockdown of HO-2 in OE-33 cells using two separate oligonucleotides (siRNA 01 and siRNA 02) produced a significant reduction in relative HO-2 mRNA expression with a corresponding increase in relative expression of HO-1 mRNA. Values represented as means ± SEM; n = 9.
4.4.4 Effect of siRNA knockdown on heme oxygenase protein expression

Western blot analysis of protein expression demonstrates a reduction in HO-2 protein in AGS cells treated with HMOX2 siRNA 10nM after 24 and 48 hours. Probing the blots for HO-1 protein demonstrates that the protein is up-regulated in cells treated with siRNA targeted toward the HO-2 isoform. HO-1 expression increases with time from 24 hours to 48 hours. Differential HO protein expression in experiment wells was supported by assessment of β-tubulin that demonstrated relatively equal expression and adequate protein loading of gel lanes (western blots 4.4.4.1). The siRNA oligonucleotides 01 and 02 are most effective at reducing HO-2 protein bands at 24 hours and all 3 siRNA duplexes reduced the expression of HO-2 protein with marked HO-1 up-regulation at 48 hours. There is a faint HO-1 band appearing 48 hours after transfection with the scrambled oligonucleotide (NUC, negative universal control). This may represent a non-specific induction of HO-1 protein due to off-target effects of the transfection process that did not have an observed effect on cell phenotype.

Further western blot analysis confirms effective reduction in HO-2 protein at 24, 48 and 72 hours using siRNA duplex 02 with accompanying up-regulation of HO-1 protein at each time point. Expression of HO-1 protein is slightly increased at 24 hours with further time-dependent increases over 48 and 72 hours (western blots 4.4.4.2). Adequate protein loading is demonstrated across samples with equal expression of β-tubulin protein.

Western blots from siRNA experiments with OE-33 cells demonstrate reduced expression of HO-2 protein 24 and 48 hours after transfection with siRNA duplex at 10 nM concentration. Increased expression of HO-1 protein is seen after 24 and 48 hours using siRNA oligonucleotides 01 and 02. The siRNA duplex 02 appears to be more effective than duplex 01 in achieving HO-2 knockdown and consequent HO-1 induction. Expression of β-tubulin protein is slightly reduced in the lysate sample from cells treated with siRNA 10 nM for 24 hours and otherwise demonstrates equal protein loading (western blots 4.4.4.3).
Figure 4.4.4 Western Blots

Western Blots 4.4.4.1: siRNA HO-2 knockdown in AGS cells

AGS siRNA mediated HO-2 knockdown – anti-HO1 & anti-HO-2

![Western Blot Image]

Figure 4.4.4.1: Protein expression in gastric cell lines after siRNA transfection with 3 anti-HO-2 siRNA duplexes. Cells were seeded into 6-well plates, protein lysates harvested at 24 – 48h and Western blotting performed as described. Blots demonstrate effective reduction of HO-2 expression with increased induction of HO-1 protein after 48h transfection. M = marker, NUC = negative universal control
Western Blots 4.4.4.2: siRNA HO-2 knockdown in AGS cells

AGS siRNA mediated HO-2 knockdown – selected siRNA duplex 02

![Western Blot Images]

Lanes
1 = siRNA 02 10nM 24h  2 = siRNA 02 10nM 48h  3 = siRNA 02 10nM 72h
4 = NUC 10nM 24h  5 = NUC 10nM 48h  6 = NUC 10nM 72h
7 = Control 24h  8 = Control 48h  9 = Control 72h

Figure 4.4.4.2: Protein expression in gastric cell lines after siRNA transfection with anti-HO-2 siRNA 02 duplex. Cells were seeded into 6-well plates, protein lysates harvested at 24 – 48h and Western blotting performed as described. Blots demonstrate effective knockdown of HO-2 protein and selective induction of HO-1 protein after 24 – 72h transfection. M = marker, NUC = negative universal control.
Western Blots 4.4.4.3: siRNA HO-2 knockdown in OE-33 cells

OE-33 siRNA mediated HO-2 knockdown and HO-1 upregulation

Figure 4.4.4.3: Protein expression in oesophageal cell lines after siRNA transfection with anti-HO-2 siRNA duplex. Cells were seeded into 6-well plates, protein lysates harvested at 24 – 48h and Western blotting performed as described. Blots demonstrate reduced HO-2 expression and selective up-regulation of HO-1 protein after 24 – 48h transfection. M = marker, NUC = negative universal control.
4.4.5 The effect of siRNA HO-2 knockdown on heme oxygenase enzyme activity

A specific colorimetric HO enzyme assay was performed on AGS cell lysate samples after 12, 24 and 48 hours treatment with siRNA targeted toward HO-2 protein. Results from triplicate experiments demonstrate a significant reduction in total heme oxygenase activity after 24 and 48 hours. However the combined activity of HO-2 and HO-1 enzyme proteins was significantly increased after 12 hours. This may be due to an increased HO-1 expression in response to siRNA-mediated HO-2 knockdown (Figure 4.4.5).

Figure 4.4.5 Heme Oxygenase Enzyme Activity Assay

Figure 4.4.5: Effect of HO-2 knockdown on HO enzyme activity time course

Figure 4.4.5: Effect of siRNA-mediated HO-2 knockdown on total HO enzyme activity. AGS cells were seeded into 175 cm² flasks and lysates harvested 12, 24 and 48h after transfection as described. Specific colorimetric assay demonstrated a significant reduction in HO activity associated with HO-2 knockdown at 24 and 48h but a marked increase in combined activity of HO enzymes after 12h. Values represented are means ± SD; n = 3.
4.4.6 The effect of siRNA-mediated HO-2 knockdown on cell growth

4.4.6.1 Cell proliferation protocol

Experiments were set up to assess the effect of HO-2 gene knockdown on cell proliferation in the cultured cell lines. Cells were seeded at $1.25 \times 10^4$ cells per well in 24-well plates 24 hours prior to adding transfection complexes, at which time the cells were 30 – 40% confluent. Lipofectamine was used at a concentration of 1 µL per well. Six pmole of the selected siRNA duplex and negative universal control duplex were used to give a final concentration of 10 nM per well. The medium in every well was replaced with fresh serum-complete medium 8 – 10 hours after transfection. MTT assay and cell counts were performed at 24, 48 and 72-hour time points after transfection.

4.4.6.2 Cell proliferation results – MTT assay

A total of 3 independent experiments were performed on AGS cells. The results demonstrate a significantly reduced MTT assay at 48 and 72 hours in cells transfected with HMOX2 siRNA as compared to negative universal control (Figure 4.4.6.1). There was no effect observed at the 24 hour time point but significantly reduced cell proliferation at 48 and 72 hours in siRNA-transfected cells as compared to cells treated with NUC at 10 nM concentration (% of control 63.9% vs. 92.0% at 48 hours and 59.7% vs. 90.5% at 72 hours, respectively; $p < 0.0005$). There was no advantage in treating the cells with oligonucleotide complexes at a higher dose of 20 nM.

Three separate experiments were performed on the HGC-27 cell line with transfection performed using HMOX2 siRNA at concentrations of 10 nM and at a lower dose of 5 nM. MTT assay results show significant reduction of cell proliferation at 48 and 72 hours in cells treated with HMOX2 siRNA (Figure 4.4.6.2). Cells transfected with HMOX2 siRNA 10 nM compared to those transfected with NUC 10 nM had significantly reduced cell growth at 48 hours (92.5% control vs. 73.6% control, $p = 0.007$) and at 72 hours (87.5% control vs. 40.8% control, $p = 0.01$). The lower dose of 5 nM was equally as effective in reducing cell growth, with no significant differences between the two concentrations in MTT assay performed at 48 ($p = 0.82$) and 72 hours ($p = 0.42$).
Cell growth experiments were also performed to assess the effects of HO-2 knockdown in oesophageal cell lines HET-1A and OE-33. There was a significant reduction in MTT assay for OE-33 cells 48 and 72 hours after transfection with HMOX2 siRNA 10 nM compared to corresponding controls (Figure 4.4.6.3). There were similar effects seen in HET-1A cells at 24 and 48 hours, although cell proliferation measured by MTT assay appeared to recover in these cells by 72 hours (Figure 4.4.6.4).

4.4.6.3 Cell proliferation results – cell counts

Cell counts performed for siRNA experiments supported the finding of a clear effect on cell proliferation (see Appendix VIII). There were significantly fewer live AGS cells counted at 24, 48 and 72 hours from wells treated with siRNA 10 nM as compared to those transfected with NUC 10 nM \( (p < 0.05) \). In HGC-27 experiments there were significant reductions in cell counts with HO-2 gene knockdown at 48 and 72 hours when compared to negative universal control \( (p < 0.004) \). In two cell counts performed from OE-33 cell experiments, there was a marked reduction in live cells treated with siRNA at 48 hours and 72 hours. OE-33 siRNA experiment cell count data are insufficient for statistical significance. HO-2 knockdown in AGS, HGC-27 and OE-33 cells produced effects on cell counts that are more dramatic than those seen with MTT. The discrepancy between cell count numbers and the equivalent MTT results is likely to be explained by the technical aspects and relatively cumbersome nature of cell counting with the major factor being fewer experiments performed leading to greater variability and an increased likelihood of observer error.

During all cell counts for the siRNA set of experiments there were very few dead cells counted in every well, so few that any suggestive comparison from the counting technique was inappropriate.
Figure 4.4.6 MTT Cell Proliferation Assay – Time course experiments

Figure 4.4.6.1: Effect of HO-2 knockdown on AGS cell growth

Effect of HO-2 knockdown on AGS growth

<table>
<thead>
<tr>
<th></th>
<th>MTT Absorbance % control</th>
</tr>
</thead>
<tbody>
<tr>
<td>NUC 10nM</td>
<td>95 ± 1</td>
</tr>
<tr>
<td>siRNA 10nM</td>
<td>92 ± 2</td>
</tr>
<tr>
<td>siRNA 20nM</td>
<td>91 ± 3</td>
</tr>
</tbody>
</table>

‡p < 0.005 †p = 0.002 *p = 0.006 Independent Samples Kruskal-Wallis test

Figure 4.4.6.1: Effect of siRNA-mediated HO-2 knockdown on AGS cell growth. Cells were seeded into 24-well plates and MTT assay performed 24, 48 and 72h after transfection as described. HO-2 knockdown caused significantly reduced cell proliferation at 48 and 72 hours as compared to cells treated with NUC (negative universal control) oligonucleotide. Results are expressed as a percentage of vehicle control; values represented as means ± SEM; n = 15.
Figure 4.4.6.2: Effect of HO-2 knockdown on HGC-27 cell growth

Effect of HO-2 knockdown on HGC-27 growth

<table>
<thead>
<tr>
<th></th>
<th>24hrs</th>
<th>48hrs</th>
<th>72hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>NUC 10nM</td>
<td>94</td>
<td>92</td>
<td>88</td>
</tr>
<tr>
<td>SiRNA 5nM</td>
<td>87</td>
<td>75</td>
<td>43</td>
</tr>
<tr>
<td>SiRNA 10nM</td>
<td>95</td>
<td>74</td>
<td>41</td>
</tr>
</tbody>
</table>

†p = 0.07 †p = 0.01 *p = 0.05 Independent Samples Kruskal-Wallis test

Figure 4.4.6.2: Effect of siRNA-mediated HO-2 knockdown on HGC-27 cell growth. Cells were seeded into 24-well plates and MTT assay performed 24, 48 and 72h after transfection as described. HO-2 knockdown caused significantly reduced cell proliferation at 48 and 72 h. Results are expressed as a percentage of vehicle control; values represented as means ± SEM; n = 15.
Figure 4.4.6.3: Effect of HO-2 knockdown on OE-33 cell growth

Figure 4.4.6.3: Effect of siRNA-mediated HO-2 knockdown on OE-33 cell growth. Cells were seeded into 24-well plates and MTT assay performed 24, 48 and 72h after transfection as described. HO-2 knockdown caused significant reductions in MTT assay at 48 and 72h in OE-33 cells. Results are expressed as a percentage of vehicle control; values represented as means ± SEM; n = 12.
Figure 4.4.6.4: Effect of HO-2 knockdown on HET-1A cell growth

Figure 4.4.6.4: Effect of siRNA-mediated HO-2 knockdown on HET-1A cell growth. Cells were seeded into 24-well plates and MTT assay performed 24, 48 and 72h after transfection as described. HO-2 knockdown caused less dramatic effects on cell growth although statistically significant reductions in MTT assay at 24 and 48h. Cell proliferation measured appeared to recover in HET-1A cells by 72h. Results are expressed as a percentage of vehicle control; values represented as means ± SEM; n = 12.

†p < 0.05 ‡p < 0.02 Independent Samples Kruskal-Wallis test
4.4.7 Cell Morphology after siRNA-mediated HO-2 knockdown

Cell phase photographs taken in the same field at x20 magnification demonstrate reduced cell confluence in HMOX2 siRNA-treated cells in 24-well plates after 48 hours.

Figure 4.4.7 Cell Morphology after siRNA-mediated HO-2 knockdown

Figure 4.4.7.1 Control cells at 48 hours x20

Figure 4.4.7.2 NUC 10nM treated cells at 48 hours x20

Figure 4.4.7.3 siRNA 10nM treated cells at 48 hours x20
Apoptosis experiments were performed in quadruplicate using AGS gastric carcinoma cells. Representative results from one experiment are shown for each sample investigated together with the combined data for AGS gastric carcinoma cells (Figure 4.4.8).

Controls were set up to set gating parameters with unstained cells, ethanol-treated cells to permeabilise the cell membrane for PI staining and with staurosporine-treated cells to induce apoptosis for annexin V positive control staining (see Appendix V).

All experiment cell samples were then stained with both annexin V and PI at 24, 48 and 72 hours to evaluate a possible process of apoptosis.

Cells treated with HMOX2 siRNA at 10 nM concentration demonstrated a significantly increased affinity for annexin V binding after 24 hours as compared to cells transfected with NUC 10 nM over the same time period (28.82% versus 0.01%, \( p = 0.004 \), Figures 4.4.8a – b). At 48 hours the siRNA treated cells become permeable to PI as membrane integrity is lost (51.87% versus 0.03%, \( p = 0.015 \)). At 72 hours there were significantly fewer viable cells (1.42% versus 99.12%, \( p = 0.001 \)) and significantly increased late apoptotic or necrotic cells (96.97% versus 0.02%, \( p = 0.003 \)) in samples transfected with HMOX2 siRNA compared to NUC. The observed movement of siRNA-treated cells over time through annexin V positive and PI negative to annexin V and PI positive suggests a process of early apoptosis progressing to late apoptosis and eventual cell death.
Figure 4.4.8a: Annexin V binding and siRNA-mediated HO-2 knockdown

AGS cells were transfected with anti-HO-2 siRNA in 6-well plates and stained with Annexin V-FITC and PI for flow cytometry analysis at 24, 48 and 72h. The percentage of cells counted in the apoptosis phase after 24h was 0.01% (+/- 0.009) in NUC-transfected cells versus 28.82% (+/- 6.425) in cells with HO-2 knockdown. There is progression over time toward late apoptosis or necrosis at 72h in siRNA-treated cells. Values are represented as means ± SD; n = 4.
Figure 4.4.8b: Annexin V binding and siRNA-mediated HO-2 knockdown

Figure 4.4.8b: AGS cells were transfected with anti-HO-2 siRNA in 6-well plates and stained with Annexin V-FITC and PI for flow cytometry analysis at 24, 48 and 72h. The rate of apoptosis (R6) and late apoptosis / necrosis (R4) is expressed relative to negative universal control (NUC) with values represented as means ± SD; n = 4. There is a significant increase in the proportion of apoptotic cells counted at 24h progressing to late apoptosis or necrosis at 72h.

Table 4.4.8: Summary of Annexin V binding and siRNA-mediated HO-2 knockdown

<table>
<thead>
<tr>
<th>Cell sample</th>
<th>Measurement</th>
<th>Region</th>
<th>Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R3</td>
<td>R4</td>
</tr>
<tr>
<td>NUC 10nM 24h</td>
<td>Cell Count</td>
<td>641</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>2.02</td>
<td>0.04</td>
</tr>
<tr>
<td>siRNA 10nM 24h</td>
<td>Cell Count</td>
<td>96</td>
<td>667</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>0.33</td>
<td>2.3</td>
</tr>
<tr>
<td>NUC 10nM 48h</td>
<td>Cell Count</td>
<td>506</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>1.45</td>
<td>0.03</td>
</tr>
<tr>
<td>siRNA 10nM 48h</td>
<td>Cell Count</td>
<td>34</td>
<td>12521</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>0.14</td>
<td>51.87</td>
</tr>
<tr>
<td>NUC 10nM 72h</td>
<td>Cell Count</td>
<td>152</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>0.6</td>
<td>0.02</td>
</tr>
<tr>
<td>siRNA 10nM 72h</td>
<td>Cell Count</td>
<td>156</td>
<td>20230</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>0.75</td>
<td>96.97</td>
</tr>
</tbody>
</table>

Table 4.4.8: Apoptosis studies in AGS cells treated with anti-HO-2 siRNA for 24 – 72h in 6-well plates. Summary data from 4 experiments is displayed (means, n = 4). Quadrantic regions: R3 – necrotic cell debris; R4 – late apoptosis or necrosis; R5 – viable cells; R6 – apoptosis. (*) = late apoptosis or necrosis.
4.4.9 Apoptosis studies: Cell Cycle Analysis

Analysis of cellular DNA content using PI staining provided information about the position of analysed cells on the cell cycle. Representative results and combined data from 2 independent experiments are presented below.

Forty-eight hours after transfection with NUC oligonucleotide at 10 nM the majority of cells were in the G\textsubscript{0}/G\textsubscript{1} phase (55.08%) or the G\textsubscript{2}/M phase (27.65%) with 17.27% of cells measured outside the respective R4 and R5 regions representing cellular debris or late apoptotic cells. There was no sub-G\textsubscript{0} peak demonstrated with the NUC treated cell samples (Figure 4.4.9a). The coefficient of variation (CV) of the G\textsubscript{0}/G\textsubscript{1} peak was 6.85 and the ratio of G\textsubscript{0}/G\textsubscript{1} to G\textsubscript{2}/M cells was 1.99, indicating good doublet discrimination.

Cell samples transfected with HMOX2 siRNA duplex at 10 nM concentration for 24 hours demonstrated a reduction in the proportion of cells in G\textsubscript{0}/G\textsubscript{1} phase (42.06%) or G\textsubscript{2}/M phase (10.62%) with 47.32% of cells measured outside the respective R4 and R5 regions. There was a sub-G\textsubscript{0} population of cells visible (Figure 4.4.9b). The CV of the G\textsubscript{0}/G\textsubscript{1} peak was 3.53 and the ratio of G\textsubscript{0}/G\textsubscript{1} to G\textsubscript{2}/M cells was 3.96 indicating reduced quality of DNA measurement and interference by increased cellular debris. After 48 hours the siRNA-treated sample showed a further reduction in the proportion of cells in G\textsubscript{0}/G\textsubscript{1} phase (25.59%) or G\textsubscript{2}/M phase (16.47%) with an increase in cellular debris or apoptotic cells to 57.94% (Figure 4.4.9c). The CV of the G\textsubscript{0}/G\textsubscript{1} peak was 4.17 and the ratio of G\textsubscript{0}/G\textsubscript{1} to G\textsubscript{2}/M cells was 1.55 with a broad G\textsubscript{0}/G\textsubscript{1} peak indicating reduced accuracy in DNA measurement.
Figure 4.4.9 Apoptosis studies: Cell Cycle Analysis

**Figure 4.4.9a: NUC 10nM treated cells 48 hours DNA analysis**

<table>
<thead>
<tr>
<th>Region</th>
<th>Cell Count</th>
<th>% Histogram</th>
<th>Median</th>
<th>CV</th>
<th>Skew</th>
</tr>
</thead>
<tbody>
<tr>
<td>R4</td>
<td>3059</td>
<td>55.08</td>
<td>67</td>
<td>6.85</td>
<td>0.17</td>
</tr>
<tr>
<td>R5</td>
<td>1536</td>
<td>27.65</td>
<td>126</td>
<td>3.7</td>
<td>-0.03</td>
</tr>
<tr>
<td>Total</td>
<td>5555</td>
<td>100</td>
<td>75</td>
<td>6.85</td>
<td>0.32</td>
</tr>
</tbody>
</table>

**Figure 4.4.9b: siRNA 10nM treated cells 24 hours DNA analysis**

<table>
<thead>
<tr>
<th>Region</th>
<th>Cell Count</th>
<th>% Histogram</th>
<th>Median</th>
<th>CV</th>
<th>Skew</th>
</tr>
</thead>
<tbody>
<tr>
<td>R4</td>
<td>895</td>
<td>42.06</td>
<td>51</td>
<td>3.53</td>
<td>0.01</td>
</tr>
<tr>
<td>R5</td>
<td>226</td>
<td>10.62</td>
<td>104</td>
<td>0.83</td>
<td>0.08</td>
</tr>
<tr>
<td>Total</td>
<td>2128</td>
<td>100</td>
<td>48</td>
<td>16.95</td>
<td>0.61</td>
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</tbody>
</table>
Figure 4.4.9c: siRNA 10nM treated cells 48 hours DNA analysis

<table>
<thead>
<tr>
<th>Region</th>
<th>Cell Count</th>
<th>% Histogram</th>
<th>Median</th>
<th>CV</th>
<th>Skew</th>
</tr>
</thead>
<tbody>
<tr>
<td>R4</td>
<td>889</td>
<td>25.59</td>
<td>60</td>
<td>4.17</td>
<td>0.13</td>
</tr>
<tr>
<td>R5</td>
<td>572</td>
<td>16.47</td>
<td>105</td>
<td>0.92</td>
<td>0.24</td>
</tr>
<tr>
<td>Total</td>
<td>3474</td>
<td>100</td>
<td>54</td>
<td>15.89</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Figure 4.4.9a – c: AGS cells were stained with PI and analysed using flow cytometry 24 and 48h after transfection for siRNA-mediated HO-2 knockdown. A reduction of cells counted in the G0/G1 phase and the sub-G0 phase is observed with HO-2 knockdown. Quality control parameters indicate reduced quality of DNA measurement and interference by increased cellular debris. Values are the percentage of total cell count per sample and total cell counts represented as means; \( n = 3 \).
4.4.10 Apoptosis studies: Apo-BrdU labelling of DNA fragments

Incorporation of brominated deoxyuridine triphosphate nucleotides into DNA strand breaks was measured by the Apo-BrdU assay technique to assess DNA fragmentation as a late hallmark of apoptosis in cell samples studied. Positive and negative control cells provided with the Apo-BrdU assay kit were used to set the gating regions and reliably identify positive and negative-stained cells as described above. The positive control sample cells demonstrated DNA fragmentation with increased FITC fluorescence compared to negative control cells (34.06% versus 1.03%, see Appendix VI).

Representative results and combined data from triplicate experiments are displayed in Figure 4.4.10 and Table 4.4.10. Cells treated with negative universal control oligonucleotide at 10 nM showed minimal FITC staining after 24 hours with 1.08% in the positive R8 region. Cells transfected with HMOX2 siRNA at 10 nM concentration demonstrated a clear increase in fluorescence after 24 hours with 29.63% positive for FITC staining and a significant reduction in the proportion of non-apoptotic cells ($p = 0.047$, Figure 4.4.10). After 48 hours there is little evidence of DNA fragmentation in NUC-treated cells but a significant increase in FITC staining in HMOX2 siRNA-treated cells (2.47% versus 39.54%, $p = 0.038$) and a further reduction in the proportion of non-apoptotic cells in samples treated with HO-2 knockdown.

These results indicate that one of the hallmarks of apoptosis is detectable in AGS cell lines 24 hours after siRNA-mediated knockdown of HO-2 mRNA and that this process progresses up to 48 hours after transfection.
Figure 4.4.10: Apoptosis Studies: Apo-BrdU labelling of DNA fragments

**Figure 4.4.10:** ApoBrdU assay was performed on AGS cells 24 and 48h after transfection for HO-2 knockdown. DNA fragmentation is measured by increased fluorescence in the FITC channel (R8). The rate of apoptosis is expressed as percentage of controls with values represented as means ± SD; n = 3; logarithmic scale. †p = 0.047, ‡p = 0.038, Independent Samples Kruskal-Wallis test.
Table 4.4.10: Summary of ApoBrdU DNA fragmentation assays AGS cells

<table>
<thead>
<tr>
<th>Cell sample</th>
<th>Measurement</th>
<th>R8</th>
<th>R9</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NUC 10nM 24h</strong></td>
<td>Cell Count</td>
<td>215</td>
<td>19622</td>
<td></td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>1.08</td>
<td>98.72</td>
<td></td>
</tr>
<tr>
<td><strong>siRNA 10nM 24h</strong></td>
<td>Cell Count</td>
<td>5772</td>
<td>13598</td>
<td>0.047</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>29.63</td>
<td>69.8</td>
<td></td>
</tr>
<tr>
<td><strong>NUC 10nM 48h</strong></td>
<td>Cell Count</td>
<td>542</td>
<td>21011</td>
<td></td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>2.47</td>
<td>95.79</td>
<td></td>
</tr>
<tr>
<td><strong>siRNA 10nM 48h</strong></td>
<td>Cell Count</td>
<td>8254</td>
<td>11883</td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>39.54</td>
<td>56.92</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.4.10: Apoptosis studies with DNA fragmentation assay in AGS cells treated with anti-HO-2 siRNA for 24 – 48h in 6-well plates. Summary data from triplicate experiments is displayed (means, n = 3). DNA fragmentation is measured by increased fluorescence in the FITC channel (R8) with no FITC binding in viable cells with intact DNA (R9).

4.4.11 Apoptosis Studies: Caspase 3/7 activity assay

The Apo-ONE Homogenous Caspase-3/7 assay (Promega) was performed 24 hours after transfection of AGS cells with HMOX2 siRNA at 10 nM concentration and appropriate controls. Assay reagent was added to each well and fluorescence measured after 1h incubation, collecting data over 250 plate reading cycles to generate complete signal curves. Evolution of the assay data over 180 minutes is shown in Figure 4.4.11a.

Cell proliferation assay was performed concurrently in 96-well plates with an identical experiment design to provide cell numbers per well derived from MTT assay performed as described above. The relative caspase activity per 1000 cells for experiment samples was obtained after normalisation to MTT assay. Results demonstrate a statistically significant increase in fold caspase-3/7 induction 24 hours after siRNA-mediated HO-2 knockdown when compared to controls (p = 0.005, Figure 4.4.11b).
**Figure 4.4.11 Apoptosis Studies: Caspase 3/7 activity assay**

**Figure 4.4.11a: Caspase activity at 24 hours AGS cells**

Figure 4.4.11a: Evolution of kinetic absorbance data over 180 minutes during Apo-ONE® Caspase-3/7 assay. AGS cells were seeded into black 96-well plates and transfected with anti-HO-2 siRNA for 24h.

**Figure 4.4.11b: Relative caspase activity in AGS cells**

Figure 4.4.11b: Effect of HO-2 knockdown on caspase-3/7 activity. AGS cells were seeded into 96-well plates and Apo-ONE® Caspase-3/7 assay performed 24h after transfection with anti-HO-2 siRNA. Results demonstrate a significant increase in relative caspase-3/7 activity per 1000 cells with anti-HO-2 siRNA 10 nM ($p = 0.005$). Values are represented as means ± SD; n = 12.
4.5 Downstream Effectors of Heme Oxygenase Activity: Bilirubin

4.5.1 Cell Proliferation Assay – Time course experiments

Experiments to evaluate the effect of bilirubin on the proliferation of AGS cells produced consistent results. There was no significant effect on cell proliferation as measured by MTT assay with 24 hours of treatment with bilirubin at various doses ($p = 0.505$, Figure 4.5.1a). However, extending cell exposure to bilirubin at doses of 10 μM and 20 μM produced a significant reduction in MTT assay after 48 hours ($p = 0.001$, Figure 4.5.1b) and at 72 hours ($p = 0.002$, Figure 4.5.1c). These effects were reproducible even after replacement of bilirubin reagent medium with fresh serum-complete medium after 24 hours. Significant reductions in cell proliferation were observed at 48 hours after bilirubin treatment for 24 hours at 10 μM concentration ($p = 0.02$, Figure 4.5.1d) and at 72 hours after cells were exposed to bilirubin at 20 μM concentration for 24 hours ($p = 0.001$, Figure 4.5.1d).

Cell count data demonstrate a similar trend with reductions in viable cell counts 72 hours after treatment with bilirubin at 10 and 20 μM that did not reach statistical significance (Figure 4.5.1e).
Figure 4.5.1 Cell Proliferation Assay – Time course experiments

Figure 4.5.1a: Effect of Bilirubin on AGS cell growth at 24 hours

Cells were treated with bilirubin 0.1 – 30 µM for 24h in 24-well plates and cell viability assessed with MTT assay. Results are expressed as a percentage of untreated control; values represented as means ± SEM; n = 12. Cell growth at 24h was not significantly affected by bilirubin treatment (p = 0.505).

Figure 4.5.1b: Effect of Bilirubin on AGS cell growth at 48 hours

Cells were treated with bilirubin 0.1 – 30 µM for 48h in 24-well plates and cell viability assessed with MTT assay. Results are expressed as a percentage of untreated control; values represented as means ± SEM; n = 12. Cell growth at 48h was significantly reduced by bilirubin treatment at doses of 10 µM and 20 µM (p = 0.001).
**Figure 4.5.1c: Effect of Bilirubin on AGS cell growth at 72 hours**

Cells were treated with bilirubin 0.1 – 30 µM for 72h in 24-well plates and cell viability assessed with MTT assay. Results are expressed as a percentage of untreated control; values represented as means ± SEM; n = 12. Cell growth at 72h was significantly reduced by bilirubin treatment at doses of 10 µM and 20 µM (p = 0.002).

**Figure 4.5.1d: Effect of 24 hour Bilirubin exposure – time course data**

Cells were treated with bilirubin 5, 10 and 20 µM for 24h in 24-well plates before replacement of bilirubin reagent medium with fresh serum-complete medium. MTT assay was performed at 24, 48 and 72h. Results are expressed as a percentage of untreated control; values represented as means ± SEM; n = 12. Cell growth at 48h was significantly reduced by bilirubin treatment at 10 µM (p = 0.02) and at 72h by bilirubin 20 µM (p = 0.001).
Figure 4.5.1e: Cell count AGS cells time course experiments

$\rho = 0.081$ Independent Samples Kruskal-Wallis test

Figure 4.5.1e: Cells were treated with bilirubin 5, 10 and 20 µM for 24h in 24-well plates before replacement of bilirubin reagent medium with fresh serum-complete medium. Cell growth was assessed by cell counting using Trypan blue exclusion with reductions in viable cell counts demonstrated 72h after bilirubin treatment. Values are represented as means ± SEM; $n = 12$. 
4.5.2 Effect of Bilirubin after HO enzyme inhibition – MTT Assay

Experiments were performed to investigate the impact of bilirubin treatment for 24 hours at a concentration of 5 µM and 10 µM on the reversal or prevention of effects mediated by heme oxygenase inhibition or gene knockdown in AGS cells.

Results demonstrate a significant reduction in cell proliferation after treatment with zinc protoporphyrin 10 µM for 48 hours that was observed up to 72 hours. These effects were not reversed or prevented by treatment with bilirubin at 5 or 10 µM concentrations for 24 hours (Figure 4.5.2a).

Similar findings were obtained from experiments with specific siRNA-mediated knockdown of the HO-2 gene. Treatment with bilirubin at 5 and 10 µM concentrations for 24 hours did not prevent significant reductions in cell proliferation observed at 72 hours after siRNA transfection (Figure 4.5.2b).

Figure 4.5.2a: AGS cell proliferation with Zinc and Bilirubin – Time Course

![AGS cell proliferation with Zinc and Bilirubin](image.png)

Figure 4.5.2a: Cells seeded into 24-well plates were treated with zinc 10 µM for 48h before replacement of reagent medium with fresh serum-complete medium ± bilirubin 5 and 10 µM. MTT assay was performed at 24, 48 and 72h. Zinc effects on cell growth were not prevented by bilirubin treatment. Values are represented as means ± SEM; n = 15.
Figure 4.5.2b: AGS cell proliferation after HO-2 knockdown – Time Course

Effect of Bilirubin after HO-2 knockdown

![Graph showing MTT absorbance % control over time with different reagents and concentrations](image)

* *p = 0.008

Figure 4.5.2b: Cells seeded into 24-well plates were transfected with anti-HO-2 siRNA and incubated for 48h prior to replacement of culture medium with fresh serum-complete medium ± bilirubin 5 and 10 µM. MTT assay was performed at 24, 48 and 72h. Significant reductions in cell growth at 72h (p = 0.008) were not prevented by bilirubin treatment. Values are represented as means ± SEM; n = 15.

4.5.3 siRNA-mediated HO-2 knockdown – PCR products

Cells were seeded into 24-well plates and siRNA transfection protocol followed as described previously. After 48h incubation culture medium was replaced with fresh serum-complete medium ± bilirubin 5 µM and RNA samples eluted after a further 24h.

Agarose gel analysis of PCR products demonstrate a clear reduction in HMOX2 mRNA in samples eluted from cells after transfection with HMOX2 siRNA duplex 02 at 10 nM concentration. The reduction in HMOX2 mRNA is not prevented by treatment with bilirubin at 5 µM concentration for 24 hours. The HMOX2 siRNA duplex 03 does not appear to be effective in reducing HO-2 mRNA in these experiments. Quality and quantity of eluted RNA from experiment samples is indicated by equal expression of GAPDH control RNA (Figure 4.5.3).
Figure 4.5.3: PCR products siRNA knockdown HMOX2 and GAPDH AGS cells

Lanes
siRNA duplex 02:
1 = Control  2 = Vehicle  3 = Bilirubin 5µM
4 = NUC 10nM  5 = siRNA 10nM  6 = siRNA 10nM + Bilirubin 5µM 24h
siRNA duplex 03:
7 = Control  8 = Vehicle  9 = Bilirubin 5µM
10 = NUC 10nM  11 = siRNA 10nM  12 = siRNA 10nM + Bilirubin 5µM 24h

Figure 4.5.3: RT-PCR products of RNA samples eluted 72h after anti-HO-2 siRNA transfection ± 24h bilirubin treatment. Effective reduction of HO-2 mRNA is not prevented by treatment with bilirubin 5 µM.
4.5.4 Protein expression after siRNA HO-2 knockdown and Zinc PP treatment

Western blot analyses of protein expression show a clear reduction in HO-2 protein expression in cells transfected with HMOX2 siRNA after 24, 48 and 72 hours. HO-2 protein expression is not significantly reduced by treatment with bilirubin alone at 5 µM concentration for 24 hours. There is a reduction in control protein β-actin expression at 48 hours that is not proportional to the reduction in HO-2 protein expression at the same time point and represents unequal protein loading onto the gel (western blots 4.5.4).

**Western Blots 4.5.4: Bilirubin and siRNA HO-2 knockdown in AGS cells**

AGS Bilirubin and siRNA mediated HO-2 knockdown

![Western Blot Images](image)

Figure 4.5.4: Protein expression in gastric cell lines after siRNA transfection with anti-HO-2 siRNA ± 24h treatment with bilirubin 5µM. Cells were seeded into 6-well plates, protein lysates harvested at 24 – 72h and Western blotting performed as described. Blots demonstrate effective knockdown of HO-2 protein that was not prevented by bilirubin treatment. Expression of control β-actin demonstrates reduced protein loading at 48h. M = marker, NUC = negative universal control
4.5.5 Apoptosis studies: Annexin V and Propidium Iodide Staining

Apoptosis experiments were performed in triplicate using AGS gastric adenocarcinoma cells. Representative results from one experiment are shown for each sample investigated together with combined data from 3 individual experiments. Controls were set up with unstained cells, ethanol-treated cells and with staurosporine-treated cells for positive controls as described above. All experiment cell samples were then stained with both annexin V and PI at 36 hours and 48 hours to evaluate potential apoptosis.

Cells treated with bilirubin at 10 µM concentration demonstrated a significantly increased affinity for annexin V binding after 36 hours as compared to vehicle control cells over the same time period (19.72% versus 0.01%, \( p = 0.017 \), Figure 4.5.5 and Table 4.5.5). At 48 hours the bilirubin-treated cells become permeable to PI as membrane integrity is lost (32.86% versus 0.02%, \( p = 0.008 \)). At 48 hours there were significantly fewer viable cells (25.26% versus 97.68%, \( p = 0.024 \)) and significantly increased late apoptotic or necrotic cells in samples treated with bilirubin as compared to vehicle-treated cells. There is a demonstrable shift in the proportion of cells staining annexin V positive 36 hours after bilirubin exposure to staining both annexin V and PI after 48 hours (Figure 4.5.5).
Figure 4.5.5 Apoptosis studies: Annexin V and Propidium Iodide Staining

Figure 4.5.5: AGS cells were treated with bilirubin 10 µM in 6-well plates and stained with Annexin V-FITC and PI for flow cytometry analysis at 36 and 48h. The percentage of cells counted in the apoptosis phase (R6) after 24h was 0.01% (±0.01) in vehicle-treated cells versus 19.72% (±4.01) in cells treated with bilirubin 10 µM with progression over time toward late apoptosis or necrosis at 48h. Values represented as means ± SD; n = 3.
Table 4.5.5: Summary of Annexin V binding and Bilirubin 10 µM treatment

<table>
<thead>
<tr>
<th>Cell sample</th>
<th>Measurement</th>
<th>R3</th>
<th>R4</th>
<th>R5</th>
<th>R6</th>
<th>Apoptosis</th>
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<td>Cell Count</td>
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<td>12</td>
<td>29556</td>
<td>3</td>
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<tr>
<td></td>
<td>% Histogram</td>
<td>1.68</td>
<td>0.04</td>
<td>98.27</td>
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<td><strong>Bilirubin 36h</strong></td>
<td>Cell Count</td>
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<td>92</td>
<td>24504</td>
<td>4399</td>
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<td></td>
<td>% Histogram</td>
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<td>0.32</td>
<td>79.86</td>
<td>19.72</td>
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<tr>
<td><strong>Vehicle 48h</strong></td>
<td>Cell Count</td>
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<td>7</td>
<td>28382</td>
<td>72</td>
<td></td>
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<tr>
<td></td>
<td>% Histogram</td>
<td>2.05</td>
<td>0.02</td>
<td>97.68</td>
<td>0.25</td>
<td></td>
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<tr>
<td><strong>Bilirubin 48h</strong></td>
<td>Cell Count</td>
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<td>9673</td>
<td>7436</td>
<td>4262</td>
<td>0.024*</td>
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<tr>
<td></td>
<td>% Histogram</td>
<td>27.4</td>
<td>32.86</td>
<td>25.26</td>
<td>14.48</td>
<td></td>
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</table>

Table 4.5.5: Apoptosis studies in AGS cells treated with bilirubin 10 µM in 6-well plates and stained with Annexin V-FITC and PI at 36 and 48h. Summary data from triplicate experiments is displayed (means, n = 3). Quadrantic regions include: R3 – necrotic cell debris; R4 – late apoptosis or necrosis; R5 – viable cells; R6 – apoptosis. (*) = late apoptosis or necrosis.
4.5.6 Apoptosis studies: Cell Cycle Analysis

Cell cycle DNA analysis of vehicle control cells demonstrates the majority of cells were in the G₀/G₁ phase (54.34%) or the G₂/M phase (22.26%) after 36 hours, with 23.4% of cells measured outside the respective R4 and R5 regions representing cellular debris or late apoptotic cells. There was no sub-G₀ peak seen with vehicle-treated cell samples at 36 hours (Figure 4.5.6a). The coefficient of variation (CV) of the G₀/G₁ peak was 5.56 and the ratio of G₀/G₁ to G₂/M cells was 2.44, indicating good doublet discrimination.

Cell samples treated with bilirubin at 10 µM concentration for 36 hours demonstrate a reduction in the proportion of cells in G₀/G₁ phase (38.46%) or G₂/M phase (17.6%) with 43.94% of cells measured outside the respective R4 and R5 regions. There was a population of cells undergoing apoptosis visible in the sub-G₀ region at 36 hours (Figure 4.5.6b). The CV of the G₀/G₁ peak was 5.65 and the ratio of G₀/G₁ to G₂/M cells 2.19 indicating good quality of DNA measurement and accurate doublet discrimination.
Figure 4.5.6 Apoptosis studies: Cell Cycle Analysis

**Figure 4.5.6a: Vehicle control cells 36 hours DNA analysis 01.05.2010**

<table>
<thead>
<tr>
<th>Phase</th>
<th>Region</th>
<th>Cell Count</th>
<th>% Histogram</th>
<th>Median</th>
<th>CV</th>
<th>Skew</th>
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</thead>
<tbody>
<tr>
<td>G0/G1</td>
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<td>5.56</td>
<td>0.19</td>
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<tr>
<td>G2/M</td>
<td>R5</td>
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<td>22.26</td>
<td>116</td>
<td>4.38</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>2098</td>
<td>100</td>
<td>65</td>
<td>5.56</td>
<td>0.66</td>
</tr>
<tr>
<td>Apoptosis</td>
<td></td>
<td>491</td>
<td>23.4</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4.5.6b: Bilirubin 10µM treated cells 36 hours DNA analysis 01.05.2010**

<table>
<thead>
<tr>
<th>Phase</th>
<th>Region</th>
<th>Cell Count</th>
<th>% Histogram</th>
<th>Median</th>
<th>CV</th>
<th>Skew</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0/G1</td>
<td>R4</td>
<td>638</td>
<td>38.46</td>
<td>60</td>
<td>5.65</td>
<td>0.13</td>
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<tr>
<td>G2/M</td>
<td>R5</td>
<td>292</td>
<td>17.6</td>
<td>108</td>
<td>1.8</td>
<td>-0.01</td>
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<tr>
<td></td>
<td>Total</td>
<td>1659</td>
<td>100</td>
<td>64</td>
<td>5.65</td>
<td>0.6</td>
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<tr>
<td>Apoptosis</td>
<td></td>
<td>729</td>
<td>43.94</td>
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</tbody>
</table>

*Diagram showing cell counts and histograms for cell cycle analysis.*
4.5.7 Apoptosis studies: Apo-BrdU labelling of DNA fragments

The Apo-BrdU assay was performed to measure DNA fragmentation and indicate a late hallmark of apoptosis in cell samples studied. Positive and negative control cells provided with the Apo-BrdU assay kit were used to set the gating regions and identify positive and negative-stained cells as described above. The positive control sample cells demonstrated DNA fragmentation with increased FITC fluorescence compared to negative control cells (35.81% versus 0.84%, Appendix VI). There was no significant difference observed in FITC staining between cells treated with vehicle control for 36 hours and untreated control cells (0.64% versus 1.6% respectively, Appendix VI).

Cells treated with bilirubin 10 µM demonstrated a significant increase in DNA fragmentation with positive FITC staining and a significant reduction in the proportion of non-apoptotic cells when compared to vehicle control cells after 36 hours (34.7% versus 0.64%, \( p = 0.047 \), Figure 4.5.7).

After 48 hours there is little evidence of DNA fragmentation in vehicle-treated cells but a significant increase in FITC staining in bilirubin-treated cells (0.78% versus 86.63%, \( p = 0.047 \)). There is an observed reduction in the proportion of non-apoptotic cells in populations treated with bilirubin compared to controls (12.49% versus 98.63%, \( p = 0.104 \), Figure 4.5.7 and Table 4.5.7).

These results indicate that one of the hallmarks of apoptosis is specifically detectable in AGS cell lines 36 hours after treatment with bilirubin 10 µM and that this process progresses up to 48 hours after bilirubin treatment.
Figure 4.5.7 Apoptosis Studies: Apo-BrdU labelling of DNA fragments

Figure 4.5.7: ApoBrdU assay was performed on AGS cells 36 and 48h after treatment with bilirubin 10 µM in 6-well plates. DNA fragmentation is measured by increased fluorescence in the FITC channel (R8) with a significant increase at 36h in cells treated with bilirubin compared to vehicle controls (34.69% ±1.42 versus 0.64% ±0.09, \( p = 0.047 \)). The proportion of cells with DNA fragmentation increases by 48h in cells treated with bilirubin as compared to those treated with vehicle control (86.63% ±2.46 versus 0.78% ±0.12, \( p = 0.047 \)). Values are represented as means ± SD; \( n = 3 \).
Table 4.5.7: Summary of ApoBrdU DNA fragmentation assays AGS cells bilirubin treatment

<table>
<thead>
<tr>
<th>Cell sample</th>
<th>Measurement</th>
<th>Region</th>
<th>Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R8</td>
<td>R9</td>
</tr>
<tr>
<td><strong>Vehicle 36h</strong></td>
<td>Cell Count</td>
<td>137</td>
<td>21206</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>0.64</td>
<td>99.3</td>
</tr>
<tr>
<td><strong>Bilirubin 36h</strong></td>
<td>Cell Count</td>
<td>7130</td>
<td>13320</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>34.69</td>
<td>64.81</td>
</tr>
<tr>
<td><strong>Vehicle 48h</strong></td>
<td>Cell Count</td>
<td>154</td>
<td>19478</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>0.78</td>
<td>98.63</td>
</tr>
<tr>
<td><strong>Bilirubin 48h</strong></td>
<td>Cell Count</td>
<td>18764</td>
<td>2705</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>86.63</td>
<td>12.49</td>
</tr>
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Table 4.5.7: Apoptosis studies with DNA fragmentation assay in AGS cells treated with bilirubin 10 µM for 36 – 48h in 6-well plates. Summary data from triplicate experiments is displayed (means, n = 3). DNA fragmentation is measured by increased fluorescence in the FITC channel (R8) with no FITC binding in viable cells with intact DNA (R9).
4.6 Downstream Effectors of Heme Oxygenase Activity: Carbon Monoxide

4.6.1 MTT Cell Proliferation Assay – 24-hour experiments CORMS

The influence of carbon monoxide on the proliferation of oesophagogastric cancer cells and their expression of heme oxygenase isoenzymes was investigated.

Treatment for 24 hours with carbon monoxide-releasing molecules (CORM3) and their inactive controls (iCORM) produced consistent results with a specific effect on malignant cell lines. For the normal squamous epithelial cell line HET-1A there was no significant effect on cell proliferation measured by MTT assay after 24-hour treatment with iCORM or CORM3 at doses of 10, 20, 40 and 60 µM (Figure 4.6.1a). However the malignant adenocarcinoma cell lines behaved very differently after carbon monoxide exposure. Treatment with CORM3 for 24 hours caused a significant reduction in MTT assay in AGS cells compared to untreated controls whereas treatment with equivalent doses of inactive iCORM had no significant effect (p < 0.0005 versus p = 0.458 respectively, Figure 4.6.1b). Cell proliferation in OE-33 cells was similarly reduced after 24 hour exposure to CORM3 but not iCORM as compared to untreated controls (p < 0.005 versus p = 0.41 respectively, Figure 4.6.1c).

4.6.2 MTT Cell Proliferation Assay – Time course experiments CORMS

Extending cell proliferation experiments to 72 hours demonstrated persistent reductions in cell growth for malignant cells after CORM3 treatment. There were no significant effects on MTT assay for HET-1A cells treated with CORM3 for either 24 hours (p = 0.346), 48 hours (p = 0.712) or 72 hours (p = 0.979) as compared to equivalent treatment with iCORM controls (Figure 4.6.2a). For malignant OE-33 cells there was a significant reduction in cell growth at each time point with CORM3 but not iCORM (Figure 4.6.2b). Proliferation of the gastric adenocarcinoma AGS cells as measured by MTT assay was significantly reduced 24, 48 and 72 hours after treatment with CORM3 at doses of 20 and 40 µM when compared to equivalent doses of iCORM. Treatment with iCORM at a higher dose of 60 µM caused a reduction in MTT assay at 48 and 72 hours. The effects of inactive iCORM treatment at 60 µM were not significantly
different to the observed effects of active CORM3 treatment at the same dose after 24 and 48 hours (Figure 4.6.2c).

4.6.3 MTT Cell Proliferation Assay – Growth curves CORMS

Growth curves for the three cell lines investigated display the effects of CORM3 treatment versus untreated controls in reducing malignant cell growth as compared to unaffected benign oesophageal epithelial cells. There is no significant effect on the HET-1A cells but marked effects on OE-33 cells at all doses and on AGS cells at doses of 40 and 60 µM (Figures 4.6.3a – c).

Figure 4.6.1 MTT Cell Proliferation Assay – 24-hour experiments CORMS

Figure 4.6.1a: Effect of CORMS on HET-1A cell growth at 24 hours

![HET-1A 24-hour growth CORMs](image)

CORM [µM]

iCORM $p = 0.325$ all concentrations compared to control
CORM3 $p = 0.258$ all concentrations compared to control

Figure 4.6.1a: HET-1A cells were treated with CORMS 10 – 60 µM for 24h in 24-well plates and cell viability assessed with MTT assay. Results are expressed as a percentage of untreated control; values represented as means ± SEM; $n = 12$. Growth of HET-1A cells was not significantly affected by 24h CORM treatment.
Figure 4.6.1b: Effect of CORMs on AGS cell growth at 24 hours

Figure 4.6.1b: AGS cells were treated with CORMs 10 – 60 µM for 24h in 24-well plates and cell viability assessed with MTT assay. Results are expressed as a percentage of untreated control; values represented as means ± SEM; n = 12. Treatment with active CORM3 caused a significant reduction in AGS cell growth at 24h.

Figure 4.6.1c: Effect of CORMs on OE-33 cell growth at 24 hours

Figure 4.6.1c: OE-33 cells were treated with CORMs 10 – 60 µM for 24h in 24-well plates and cell viability assessed with MTT assay. Results are expressed as a percentage of untreated control; values represented as means ± SEM; n = 12. CORM3 caused a significant reduction in OE-33 cell growth at 24h.
Figure 4.6.2 MTT Cell Proliferation Assay – Time course experiments CORMS

Figure 4.6.2a: Effect of CORMS on HET-1A cell growth time course

Data 24 hours $p = 0.346$  Data 48 hours $p = 0.712$  Data 72 hours $p = 0.979$

Figure 4.6.2a: HET-1A cells were seeded into 24-well plates and treated with CORMS 10 – 60 µM for 24 – 72h with cell proliferation assessed by MTT assay. Results are expressed as a percentage of untreated control; values represented as means ± SEM; $n = 15$. Growth of HET-1A cells was not significantly affected by extended CORM treatment.
Figure 4.6.2b: Effect of CORMS on OE-33 cell growth time course

OE-33 time course iCORM

OE-33 time course CORM3

<table>
<thead>
<tr>
<th>iCORM versus CORM3*</th>
<th>24 hrs</th>
<th>48 hrs</th>
<th>72 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>[CORM]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 µM</td>
<td>p = 0.003</td>
<td>p = 0.009</td>
<td>p = 0.004</td>
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<tr>
<td>40 µM</td>
<td>p &lt; 0.0005</td>
<td>p = 0.001</td>
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<tr>
<td>60 µM</td>
<td>p = 0.011</td>
<td>p &lt; 0.0005</td>
<td>p = 0.011</td>
</tr>
</tbody>
</table>

*Independent Samples Kruskal-Wallis test

Figure 4.6.2b: OE-33 cells were seeded into 24-well plates and treated with CORMS 10 – 60 µM for 24 – 72h with cell proliferation assessed by MTT assay. Results are expressed as a percentage of untreated control; values represented as means ± SEM; n = 15. CORM3 caused significant cell growth inhibition compared to inactive CORM treatment in OE-33 cells at concentrations of 20 – 60 µM at all time points studied.
Figure 4.6.2c: Effect of CORMs on AGS cell growth time course

**AGS time course iCORM**

<table>
<thead>
<tr>
<th>iCORM [µM]</th>
<th>24 hrs</th>
<th>48 hrs</th>
<th>72 hrs</th>
</tr>
</thead>
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<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 µM</td>
<td><em>p &lt; 0.0005</em></td>
<td><em>p = 0.02</em></td>
<td><em>p = 0.007</em></td>
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<td>40 µM</td>
<td><em>p = 0.002</em></td>
<td><em>p = 0.001</em></td>
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</tr>
<tr>
<td>60 µM</td>
<td><em>p = NS</em></td>
<td><em>p = NS</em></td>
<td><em>p = 0.037</em></td>
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</table>

*Independent Samples Kruskal-Wallis test; NS = not significant at level 0.05

**AGS time course CORM3**

<table>
<thead>
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<th>CORM3 [µM]</th>
<th>24 hrs</th>
<th>48 hrs</th>
<th>72 hrs</th>
</tr>
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<tbody>
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<td>Control</td>
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</tr>
<tr>
<td>20 µM</td>
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</tbody>
</table>

Figure 4.6.2c: AGS cells were seeded into 24-well plates and treated with CORMs 10 – 60 µM for 24 – 72h with cell proliferation assessed by MTT assay. Results are expressed as a percentage of untreated control; values represented as means ± SEM; n = 15. CORM3 caused significant cell growth inhibition compared to inactive CORM treatment in AGS cells at concentrations of 20 and 40 µM at all time points studied as shown. Treatment with iCORM at a higher dose of 60 µM caused a reduction in MTT assay at 48 and 72h.
Figure 4.6.3 MTT Cell Proliferation Assay – CORM3 Growth Curves

Figure 4.6.3a: HET-1A growth curves with CORM3 treatment

Figure 4.6.3b: OE-33 growth curves with CORM3 treatment

Figure 4.6.3a – b: Growth curves for HET-1A and OE-33 cell lines demonstrate the effects of CORM3 treatment in reducing malignant cell proliferation as compared to unaffected benign oesophageal epithelial cells. There is no significant effect on the HET-1A cells but marked effects on OE-33 cells at all doses at 24 – 72h.
Figure 4.6.3c: AGS growth curves with CORM3 treatment

Figure 4.6.3c: Growth curves for AGS cells demonstrate significant effects of CORM3 treatment on AGS cell proliferation at doses of 40 and 60 µM at 48 – 72h.
4.6.4 Protein expression after CORM and bilirubin treatment

Western blot analyses of protein expression show no clear changes in constitutive HO-2 protein expression after 24h treatment with inactive CORMs. Up-regulation of inducible HO-1 protein occurs after 24 hours of treatment with active CORM3 at both 20 and 40 µM but no visible HO-1 induction with equivalent inactive iCORM treatment (western blots 4.6.4.1). Blots at 48 hours demonstrate induced expression of HO-1 protein after 48h treatment with active CORM3 but not inactive CORM 20 – 60 µM. There is a small reduction of HO-2 protein after 48h treatment with active CORM3 40 – 60 µM.

There are no marked changes in HO-2 protein expression after 24 hours treatment with bilirubin at 10 µM and 20 µM concentration and no effect from treatment with vehicle control. There is a slight reduction in HO-2 protein demonstrated after 48 hour treatment with bilirubin at 20 µM concentration. Exposure of AGS cells to bilirubin at 10 or 20 µM doses for 24 – 48 hours did not cause induction of HO-1 isoform (western blots 4.6.4.2).

Expression of control protein β-tubulin is unchanged across experimental samples analysed indicating adequate equal loading of lysate samples for gel electrophoresis.
Western Blots 4.6.4.1: CORMS and Bilirubin HO protein expression in AGS cells

CORM and Bilirubin treatment after 24 hours AGS cells

Figure 4.6.4.1: Protein expression in gastric cell lines after 24h treatment with bilirubin and CORMS. Cells were seeded into 6-well plates, protein lysates harvested at 24h and Western blotting performed as described. Blots demonstrate up-regulation of inducible HO-1 protein after 24h treatment with active CORM3 at both 20 and 40 µM but no visible HO-1 induction with equivalent inactive iCORM treatment. HO protein expression was unaffected by bilirubin treatment at 24h. Expression of β-tubulin is unchanged indicating equal loading of lysate samples for gel electrophoresis (M = marker).
Western Blots 4.6.4.2: CORMS and Bilirubin HO protein expression in AGS cells

CORM and Bilirubin treatment after 48 hours AGS cells

Figure 4.6.4.2: Protein expression in gastric cell lines after 48h treatment with bilirubin and CORMS. Cells were seeded into 6-well plates, protein lysates harvested at 48h and Western blotting performed as described. Blots demonstrate induced expression of HO-1 protein after 48h treatment with active CORM3 but not inactive CORM 20 – 60 μM. There is possible reduction of HO-2 protein after 48h treatment with bilirubin 20 μM and active CORM3 40 – 60 μM. Expression of β-tubulin indicates adequate loading of lysate samples for gel electrophoresis (M = marker).
4.6.5 Apoptosis studies with CORM treatment

4.6.6 Apoptosis studies: Annexin V and Propidium Iodide Staining

Apoptosis experiments were performed in triplicate using AGS gastric carcinoma cells. Representative results from one experiment are shown for each sample investigated together with combined data from 3 individual experiments. Staining with Annexin V-FITC identified cells with exposed phosphatidylserine translocated to the external cell membrane at the onset of apoptosis. Propidium iodide (PI) staining identified necrotic and damaged cells with permeable plasma cell membranes with exclusion of PI by viable cells.

Controls were set up with unstained cells, ethanol-treated cells and staurosporine-treated cells to induce apoptosis for annexin V positive control staining as described above. Experiment cell samples were treated with inactive iCORM or active CORM3 at 20 µM concentration and stained with both annexin V and PI at 24, 48 and 72 hours to evaluate potential apoptosis.

Cells treated with active CORM3 molecule at 20 µM demonstrated a significantly increased affinity for annexin V binding after 24 hours as compared to those treated with equivalent inactive iCORM (33.57% versus 0.02%, \( p = 0.001 \), Figures 4.6.6a – b). At 48 hours a significantly higher proportion of CORM3-treated cells lost plasma membrane integrity and became permeable to PI as compared to iCORM-treated cells (54.3% versus 0.02%, \( p = 0.028 \)). There was further progression of CORM3-treated cells with significantly fewer viable cells and significantly increased late apoptotic or necrotic cells measured at the 72-hour time point (Figures 4.6.6a – b and Table 4.6.6). This observed movement of CORM3-treated cells over time through annexin V positive and PI negative to annexin V and PI positive suggests a process of early apoptosis progressing to late apoptosis and eventual necrosis.
Figure 4.6.6a: AGS cells were treated with CORMs 20 µM in 6-well plates and stained with Annexin V-FITC and PI for flow cytometry analysis at 24 – 72h. The percentage of cells counted in the apoptosis phase (R6) after 24h was 0.02% (±0.03) in cells treated with inactive CORM 20 µM versus 33.57% (±5.1) in CORM3-treated cells. Active CORM3 treatment caused time-dependent progression toward late apoptosis or necrosis at 72h. Values represented as means ± SD; n = 3.
Table 4.6.6 Summary of Annexin V binding and CORM treatment

<table>
<thead>
<tr>
<th>Cell sample</th>
<th>Measurement</th>
<th>Region</th>
<th>Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>iCORM 24 hrs</td>
<td>Cell Count</td>
<td>R3</td>
<td>738</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>R4</td>
<td>2.63</td>
</tr>
<tr>
<td>CORM3 24 hrs</td>
<td>Cell Count</td>
<td>R5</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>R6</td>
<td>0.06</td>
</tr>
<tr>
<td>iCORM 48 hrs</td>
<td>Cell Count</td>
<td>R3</td>
<td>703</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>R4</td>
<td>1.84</td>
</tr>
<tr>
<td>CORM3 48 hrs</td>
<td>Cell Count</td>
<td>R5</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>R6</td>
<td>0.2</td>
</tr>
<tr>
<td>iCORM 72 hrs</td>
<td>Cell Count</td>
<td>R3</td>
<td>729</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>R4</td>
<td>2.38</td>
</tr>
<tr>
<td>CORM3 72 hrs</td>
<td>Cell Count</td>
<td>R5</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>R6</td>
<td>1.12</td>
</tr>
</tbody>
</table>

Table 4.6.6: Apoptosis studies in AGS cells treated with CORMS 20 µM and stained with Annexin V-FITC and PI at 24 – 72h. Summary data from triplicate experiments is displayed (means, n = 3). Quadrantic regions include: R3 – necrotic cell debris; R4 – late apoptosis or necrosis; R5 – viable cells; R6 – apoptosis. (*) = late apoptosis or necrosis.

Figure 4.6.6b: AGS cell apoptosis with CORMs

Figure 4.6.6b: AGS cells were treated with CORMS 20 µM in 6-well plates and stained with Annexin V-FITC and PI at 24 – 72h. The rate of apoptosis (R6) and late apoptosis / necrosis (R4) is expressed relative to inactive CORM controls with values represented as means ± SD; n = 3; logarithmic scale.
Incorporation of bromodeoxyuridine triphosphate nucleotides into DNA strand breaks was measured by the Apo-BrdU assay to assess DNA fragmentation as one of the later hallmarks of apoptosis in mammalian cells.

Positive and negative control cells provided with the Apo-BrdU assay kit were used to set the gating regions and validate data collection protocols to reliably identify positive and negative-stained cells. Apoptotic cells are characterised by an increased FITC staining as the incorporated BrdU nucleotide sites are detected by FITC-labelled anti-BrdU antibody. The positive control sample cells demonstrated increased FITC fluorescence compared to negative control cells as described above (33.84% versus 0.82%, Appendix VI).

Untreated experiment control cell samples demonstrated minimal FITC staining after 48 hours with 0.82% in the positive R8 region (Appendix VI). Cells treated with inactive iCORM had similarly little positive FITC staining at 48 hours (0.95%, Figure 4.6.7). Treatment with active CORM3 molecule caused a significant increase in fluorescence when compared to control cells over 48 hours indicating a significant increase in the detection of apoptotic cells (38.02% versus 0.95%, $p = 0.011$). Exposure to carbon monoxide from active CORM3 produced a significant increase in the proportion of apoptotic cells and a significant decrease in the proportion of non-apoptotic cells measured by the Apo-BrdU assay as compared to both untreated cells and those treated with inactive iCORM (Figure 4.6.7 and Table 4.6.7). These finding indicate that one of the hallmarks of apoptosis occurs in AGS cell lines after treatment with CORM3 at 20 µM concentration for 48 hours.
Figure 4.6.7 Apoptosis Studies: Apo-BrdU labelling of DNA fragments

Figure 4.6.7: ApoBrdU assay was performed on AGS cells 48h after treatment with CORMS in 6-well plates. DNA fragmentation is measured by increased fluorescence in the FITC channel (R8) with a significant increase at 48h in cells treated with CORM3 compared to those treated with inactive iCORM (38.02% ±0.87 versus 0.95% ±0.3, p = 0.011). Values are represented as means ± SD; n = 3.
Table 4.6.7: Summary of ApoBrdU DNA fragmentation assays AGS cells CORM treatment

<table>
<thead>
<tr>
<th>Cell sample</th>
<th>Measurement</th>
<th>REGION</th>
<th>Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R8</td>
<td>R9</td>
</tr>
<tr>
<td><strong>Control 48h</strong></td>
<td>Cell Count</td>
<td>169</td>
<td>19274</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>0.86</td>
<td>98.21</td>
</tr>
<tr>
<td><strong>iCORM 20µM 48h</strong></td>
<td>Cell Count</td>
<td>201</td>
<td>20684</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>0.95</td>
<td>97.83</td>
</tr>
<tr>
<td><strong>CORM3 20µM 48h</strong></td>
<td>Cell Count</td>
<td>7994</td>
<td>12679</td>
</tr>
<tr>
<td></td>
<td>% Histogram</td>
<td>38.02</td>
<td>60.3</td>
</tr>
</tbody>
</table>

Table 4.6.7: Apoptosis studies with DNA fragmentation assay in AGS cells treated with CORM3 and inactive iCORM at 20 µM concentration for 48h. Summary data from triplicate experiments is displayed (means, n = 3). DNA fragmentation is measured by increased fluorescence in the FITC channel (R8) with no FITC binding in viable cells with intact DNA (R9). There were no significant differences observed between control cells and iCORM-treated cells but significantly increased apoptosis and a reduction in viable cells associated with active CORM3 treatment.

### 4.6.8 Apoptosis Studies: Mitochondrial staining using JC-1 reagent

Activation of the mitochondrial permeability transition pore is an important step in the induction of apoptosis causing a loss of mitochondrial membrane potential ($\Delta \psi_m$) that can be measured using the JC-1 assay. The cationic JC-1 dye accumulates in the mitochondrial matrix of healthy cells and forms aggregates that emit red fluorescence. When the mitochondrial membrane potential collapses in apoptotic cells the JC-1 monomers remain in the cell cytoplasm and emit green as opposed to red fluorescence.

AGS cells treated with active CORM3 20 µM for 48 hours demonstrated a significant increase in green fluorescence as compared to iCORM-treated control cells after both samples were labelled with JC-1 (43.35% versus 8.19%, $p = 0.031$, Figure 4.6.8). Treatment with CORM3 for 48 hours produced a significant loss of red fluorescence after JC-1 labelling compared to equivalent treatment with iCORM (32.7% versus 78.7%, $p = 0.05$, Figure 4.6.8). These results indicate the induction of apoptosis in a significantly higher proportion of cells treated with active CORM3 for 48 hours as compared to cells treated with inactive iCORM.
Figure 4.6.8 Apoptosis Studies: Mitochondrial staining using JC-1 reagent

Figure 4.6.8: CORM-treated cells 48 hours JC-1 labelled

<table>
<thead>
<tr>
<th>Region</th>
<th>Cell Count</th>
<th>% Histogram</th>
<th>Median X</th>
<th>Median Y</th>
</tr>
</thead>
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<tr>
<td>R1</td>
<td>3612</td>
<td>78.69</td>
<td>619.67</td>
<td>536.31</td>
</tr>
<tr>
<td>R3</td>
<td>376</td>
<td>8.19</td>
<td>1027.46</td>
<td>76.27</td>
</tr>
<tr>
<td>Total</td>
<td>4590</td>
<td>100</td>
<td>642.46</td>
<td>464.16</td>
</tr>
</tbody>
</table>

<table>
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<th>Region</th>
<th>Cell Count</th>
<th>% Histogram</th>
<th>Median X</th>
<th>Median Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>10587</td>
<td>32.71</td>
<td>576.48</td>
<td>498.93</td>
</tr>
<tr>
<td>R3</td>
<td>14033</td>
<td>43.35</td>
<td>251.19</td>
<td>47.69</td>
</tr>
<tr>
<td>Total</td>
<td>32368</td>
<td>100</td>
<td>387.47</td>
<td>70.95</td>
</tr>
</tbody>
</table>

Figure 4.6.8: JC-1 labelling of cell samples treated with CORMS for 48h in 6-well plates. There is a significant increase in the proportion of cells counted with a lost Δψ_m (R3) in cells treated with active CORM3 as compared to those treated with inactive iCORM (43.4% ±2.46 versus 8.2% ±1.15, p = 0.031).
**4.6.9 Apoptosis Studies: Caspase 3/7 activity assay**

The Apo-ONE Homogenous Caspase-3/7 assay (Promega) was performed after 48 hours of treatment with CORMs at 20 µM and bilirubin at 20 µM concentration. Evolution of the assay data over 60 minutes is shown in Figure 4.6.9a. Cell proliferation assay was performed concurrently in 96-well plates with an identical experiment design to provide cell numbers per well derived from MTT assay performed as described above. The relative caspase activity per 1000 cells for experiment samples was obtained after normalisation to untreated control cells.

Results demonstrate a significant increase in fold caspase-3/7 induction after 48 hours exposure to active CORM3 as compared to inactive iCORM ($p < 0.05$, Figure 4.6.9b). Treatment with bilirubin produced a significant increase in caspase-3/7 activity after 48 hours as compared to vehicle control ($p < 0.05$, Figure 4.6.9b). These findings support the notion that the observed cell proliferation effects of both bilirubin and carbon monoxide are mediated by a process of apoptosis.
Figure 4.6.9 Apoptosis Studies: Caspase 3/7 activity assay

Figure 4.6.9a: Caspase activity at 48 hours in AGS cells

**Figure 4.6.9a:** Evolution of kinetic absorbance data over 1h Apo-ONE® Caspase-3/7 assay. AGS cells were seeded into black 96-well plates and treated with bilirubin, inactive iCORM and active CORM3 all at 20 µM concentration.

**Figure 4.6.9b:** Relative caspase activity in AGS cells

**Figure 4.6.9b:** Effect of HO downstream effectors on caspase-3/7 activity. AGS cells were seeded into 96-well plates and Apo-ONE® Caspase-3/7 assay performed 48h after addition of reagents. Results demonstrate a significant increase in relative caspase-3/7 activity per 1000 cells with bilirubin 20 µM and CORM3 20 µM after 48h ($p < 0.05$). Values are represented as means ± SD; $n = 12$. 
4.6.10 Heme Oxygenase Enzyme Activity Assay

Cellular HO activity was measured by performing a specific colorimetric assay as described above. The results demonstrate an increase in total HO enzyme activity after treatment with hemin 40 µM for 24 and 48 hours consistent with up regulation of HO-1 protein.

A decrease in total HO enzyme activity was observed after treatment with CORM3 molecule for 24 and 48 hours as compared to untreated control cells and cells treated with inactive iCORM. There was no difference between iCORM-treated cells and untreated controls. Although there was an observed reduction in measured HO activity after CORM3 treatment the data did not reach statistical significance ($p = 0.07$, Figures 4.6.10a – b).
Figure 4.6.10 Heme Oxygenase Enzyme Activity Assay

Figure 4.6.10a: Effect of CORMS on HO enzyme activity in AGS cells

![Effect of CORM on HO enzyme activity](image1)

Reagent [µM]

Control  | Hemin 40  | iCORM 20  | CORM3 20

pmoles Bill / mg prot / 60 min

- Control: 0
- Hemin 40: 200
- iCORM 20: 200
- CORM3 20: 100

24 hrs | 48 hrs

Figure 4.6.10b: Effect of CORMS on HO enzyme activity logarithmic scale

![Effect of CORM on HO enzyme activity](image2)

Reagent [µM]

Control  | Hemin 40  | iCORM 20  | CORM3 20

pmoles Bill / mg prot / 60 min

- Control: 10
- Hemin 40: 100
- iCORM 20: 100
- CORM3 20: 20

24 hrs | 48 hrs

†p = 0.07

Figure 4.6.10a – b: Effect of CORM treatment on total HO enzyme activity. AGS cells were seeded into 175 cm³ flasks and lysates harvested after 24 and 48h treatment with CORMS. Specific colorimetric assay demonstrated a significant increase in HO activity with HO-1 induction associated with hemin controls. A reduction in total HO enzyme activity was observed after CORM3 treatment that did not reach statistical significance (p = 0.07). HO activity is expressed as pmoles per bilirubin formed per mg of protein pellet per 60 min, values represented as means ± SD; n = 3.
CHAPTER 5: DISCUSSION

5.1 Oxidative stress and the role of heme oxygenase

5.1.1 Oxidative stress and cell proliferation

Gastric cell lines exposed to oxidative stress as simulated by H$_2$O$_2$ treatment show no significant reduction in cell proliferation after 24 hours. There was a reduction in MTT activity at higher H$_2$O$_2$ concentrations that was not statistically significant. By contrast, treating oesophageal OE-33 cells with the same reagent caused an increase in cell growth, while there were variable non-significant effects in the epithelial HET-1A cells. The findings suggest that malignant gastric cells are more susceptible than malignant oesophageal cells to inhibition of cell growth induced by oxidative stress. Growth inhibition may be mediated by an alternative pathway to the heme oxygenase system as the observed effects of experimental oxidative stress occurred independently of HO-1 protein expression. Oesophageal adenocarcinoma cells in culture appeared to be protected from adverse proliferation effects caused by oxidative stress simulated by H$_2$O$_2$, indeed the malignant cell line thrived under these conditions. The consistent levels of HO-1 and HO-2 expression do not indicate a protective role of HO in response to H$_2$O$_2$ exposure. Up-regulation of inducible HO-1 protein did not occur in response to experimental oxidative stress in any of the cell lines studied. This finding challenges the expectation that HO-1 enzyme plays an important role in the oxidative stress response for the cells investigated. However the lack of significant proliferation effects in AGS and OE-33 cells treated with H$_2$O$_2$ suggest that a cytoprotective response is not required, indicating that the cell biology specific to these malignant cells has adapted and has become robust enough to maintain healthy growth in conditions of oxidative stress.

Proliferation of AGS and HGC-27 cells was significantly affected by antioxidant treatment with N-acetylcysteine with morphological changes observed after 24 hour NAC treatment at 10 mM concentration. Consistent effects of significantly reduced cell proliferation were seen in prolonged NAC treatment of AGS cells with extended growth curves to seven days.
Treatment with NAC over 24 – 72 hours produced a marked contrast in growth effects in benign HET-1A cells compared to malignant OE-33 epithelial cells. The malignant cells showed statistically significant reductions in cell growth over 24 – 72 hours with NAC doses of 10 mM. By contrast growth of treated HET-1A cells was similar to untreated control cells except for an observed reduction in MTT assay after 72 hours of exposure to NAC at 20 mM concentration. Consistent effects of a significant reduction in OE-33 cell proliferation were seen with NAC treatment at all concentrations beyond 72 hours. The combination of 10mM NAC with cisplatin and 5-FU enabled a significant reduction in the IC\textsubscript{10} and IC\textsubscript{50} doses for both reagents (0.1 μg/mL\textsuperscript{-1} and 2.875 μg/mL\textsuperscript{-1} respectively for cisplatin and 0.07 μg/mL\textsuperscript{-1} and 6.5 μg/mL\textsuperscript{-1} respectively for 5-FU, \( p < 0.05 \)).

This is an important cancer-specific finding with anti-oxidant treatment causing significant effects on the growth of malignant cells without adverse effects in benign cells. This finding is in keeping with similar findings from a published study of NAC treatment in human signet ring gastric carcinoma cells\textsuperscript{886}. These results provide further evidence to support the idea that malignant cells thrive under conditions of oxidative stress as the anti-oxidant treatment proves detrimental to cell proliferation. Furthermore a link between chronic inflammation, oxidative stress and cancer can be demonstrated to be relevant for oesophagogastric cancer under experimental conditions: the oesophageal adenocarcinoma cell lines were susceptible to antioxidant treatment but not simulated conditions of oxidative stress. As discussed previously there is evidence for genomic instability associated with persistent acid reflux-induced inflammation in patients with Barrett’s that predisposes to the progression to invasive carcinoma\textsuperscript{490,887}. These experiments support this concept by demonstrating that the transformed malignant oesophageal adenocarcinoma cells are resistant to oxidative stress and susceptible to antioxidant treatment\textsuperscript{472,480}.

5.1.2 Oxidative stress and heme oxygenase protein expression

Western blot analysis of protein expression demonstrate that under basal conditions, all of the investigated cell lines express the constitutive heme oxygenase isoform HO-2 but do not express the inducible HO-1 isoform. Up-regulation of HO-1 protein was not caused
by treatment with either hydrogen peroxide or N-acetylcysteine in any of the cell lines investigated.

NAC and H$_2$O$_2$ treatment did not influence constitutive expression of HO-2 protein in AGS or HGC-27 gastric cell lines. There was comparable expression of constitutive HO-2 protein in both OE-33 and HET-1A cell lines under basal and experimental conditions but an apparent reduction in HO-2 protein in OE-33 cells treated with NAC at 10 mM concentration. However this finding may represent a relative lack of protein for this lysate sample as there is a reduction in the corresponding expression of control β-tubulin. The proportional reduced expression in corresponding bands represents a relative lack of protein for this lysate sample and unequal loading onto the gel for electrophoresis.

Results suggest that oxidative stress may have an important role in the growth of gastric and oesophageal epithelial cancer cells. This effect could be cancer cell-specific and related to a malignant cell phenotype as the effects were not observed in normal oesophageal epithelial cells. Further experiments were designed to define the cellular proliferation response to antioxidant treatment.

5.1.3 The effect of NAC on heme oxygenase enzyme activity

Assays of total heme oxygenase enzyme activity after NAC treatment demonstrated very interesting results with a statistically significant reduction in total HO activity measured in AGS cells treated with NAC at 10 mM concentration for 24 and 48 hours. This means that despite no demonstrable reduction in HO-2 protein expression there is a clear reduction in measurable total HO enzyme activity. This suggests that HO-2 enzyme activity is important for maintaining exponential cell proliferation in malignant AGS cells.

5.1.4 The effect of NAC on cellular apoptosis

Cell populations treated with NAC at 10 mM concentration demonstrated significantly increased evidence of early apoptosis after 24 hours when compared to untreated control cells over the same time period. At 48 hours there were significantly fewer viable cells and significantly increased late apoptotic or necrotic cells in samples treated with NAC. The observed movement of treated cells over time suggests a process of early apoptosis
progressing to late apoptosis and eventual cell death as a cancer-specific cellular response to NAC treatment.

Cell cycle studies demonstrated cells undergoing a process of apoptosis with a sub-$G_0$ peak population of cells visible after 24-hour treatment with NAC at 5 and 10 mM concentrations. There was a significant increase in the proportion of cells measured in the $G_2/M$ phase to indicate $G_2/M$ cell cycle arrest after 24h treatment with NAC 10 mM ($p = 0.02$). The measured coefficient of variation of the $G_0/G_1$ peak and the ratio of $G_0/G_1$ to $G_2/M$ cells indicated good quality doublet discrimination and accuracy of DNA measurement.

Studies show significantly increased DNA fragmentation in cells treated with NAC at 10 mM concentration for 24 hours. Extended exposure to NAC at 10 mM concentration for 48 hours caused a further significant increase in DNA fragmentation compared to untreated cells over the same time period. The findings indicate that one of the hallmarks of apoptosis occurs in AGS cell lines after treatment with NAC at 10 mM concentration for 24 and 48 hours. Caspase-3/7 assays demonstrate a significant increase in relative caspase activity after 24 hours in cells treated with NAC 10 mM compared to untreated control cells and provide additional supporting evidence for a process of apoptosis.

5.1.5 Oxidative stress and the role of heme oxygenase: Conclusions

Experiments performed to investigate oxidative stress provide evidence of a cancer-specific effect of antioxidant NAC treatment in reducing the proliferation of oesophagogastric cancer cells. This effect is independent of HO-1 induction and is associated with a reduction in HO-2 protein activity. Studies of apoptosis provide evidence for an increased induction and maintenance of the apoptotic process in cells treated with $N$-acetylcysteine.

There was no significant effect on the proliferation of gastric and oesophageal adenocarcinoma cells with oxidative stress simulated by $H_2O_2$ exposure. There were variable non-significant effects observed with normal squamous epithelial cells. There was no up-regulation of HO-1 protein in response to $H_2O_2$ exposure in any of the cell lines.
investigated indicating that HO-1 does not have a protective role in response to oxidative stress in these cells. Collectively the experiment findings suggest that the malignant cells studied have adapted to thrive under conditions of oxidative stress and indeed demonstrate adverse responses with antioxidant treatment. Consistent cancer-specific effects of significantly reduced cell proliferation were seen with NAC treatments that were independent of HO-1 up-regulation. Hallmark features of apoptosis including increased relative caspase-3/7 activity and DNA fragmentation are demonstrable after NAC treatment in malignant adenocarcinoma cells. NAC administration caused G2/M cell cycle arrest in cancer cell lines studied.

There is no associated induction of HO-1 protein so these events are mediated by an alternative pathway. Induction of HO-1 enzyme has been demonstrated to occur as a response to various conditions and stimuli including oxidative stress, endotoxin, inflammatory cytokines and hyperthermia. Increased enzyme activity is a positive cellular response to stressful conditions and has been demonstrated to confer cytoprotection through anti-oxidative, anti-apoptotic and anti-inflammatory actions. Constitutive over-expression of HO-1 enzyme is not a characteristic feature of the oesophagogastric cancer cells studied.

Inducible HO-1 expression is relatively low in most tissues and it has functional significance only in response to stressful stimuli. Constitutive HO-2 is believed to be responsible for basal HO activity contributing to cell homeostasis and normal cellular function both in maintaining intracellular heme levels and in the tonic production of biliverdin and CO that contribute to regulation of signalling pathways.

A salient finding from these studies is a clear reduction in measurable total HO enzyme activity after antioxidant treatment despite no demonstrable reduction in HO-2 protein expression or HO-1 protein induction. These results indicate that HO-2 enzyme activity is important for maintaining exponential cell proliferation in malignant AGS cells.

The anti-cancer effects of N-acetyl cysteine in vitro have been demonstrated by two recent studies. Treatment with NAC was able to selectively inhibit the growth of human signet ring gastric cancer cells in a time-dependent and dose-dependent manner by inducing apoptosis and DNA synthesis arrest within 48 hours of treatment. More
recent studies on a hepatocellular carcinoma cell line demonstrate the ability of NAC to reduce cell viability and to enhance the cytotoxic effect of the chemotherapeutic agent Interferon alpha. The implications of these published studies and the findings of experiments performed for this thesis relate to the potential of N-acetyl cysteine as a useful adjunct for the treatment of oesophagogastric cancer.

Results from oxidative stress experiments provide further evidence to support the idea that malignant cells thrive under conditions of oxidative stress with anti-oxidant treatment detrimental to cell proliferation. The role of reactive oxygen species (ROS) and redox regulation in the promotion and survival of cancer cells is only partially understood with both pro-tumourigenic and anti-cancer effects described. Production of reactive oxygen species have been demonstrated to enhance carcinogenesis in fibroblasts and promote survival of epithelial tumour cells. Conversely, an increase in ROS levels has been shown to lower the threshold for cellular apoptosis and increase the cytotoxicity of therapeutic agents. Chemosensitivity toward platinum-based drugs may be enhanced by co-treatment with agents that increase ROS production. A recent paper describes how the relative levels of ROS in malignant and benign cells can be exploited to design therapies that can preferentially target the increased stress response in cancer cells.

*N*-acetylcysteine (NAC) is a precursor of L-cysteine that can enter the glutathione synthesis pathway to augment physiological glutathione reserves. NAC binds directly to toxic metabolites together with glutathione and is a scavenger of free radicals via interaction of reactive thiol groups with ROS. The antioxidant activity of NAC has been investigated in clinical studies to prevent contrast-induced nephropathy with variable success. NAC may also directly bind to and inactivate platinum-based chemotherapy agents including cisplatin. NAC is able to form a complex with platinum that modulates the metabolism of cisplatin and enables a reduction in cisplatin adverse side effects with concomitant administration of NAC. Published studies have demonstrated that NAC reduces bone marrow toxicity and prevents ototoxicity associated with chemotherapy in
murine models as well as reducing the cytotoxicity of alkylating chemotherapeutic agents in vitro\textsuperscript{904-907}. The toxicity of chemotherapeutic agents has been shown to be prevented or reduced by antioxidant treatment without compromising their anti-cancer effects\textsuperscript{906 908}.

Cellular resistance to cisplatin is often associated with elevated levels of intracellular glutathione. The role of glutathione activity and metabolism in the mechanism of cellular resistance to cisplatin is a subject of some debate\textsuperscript{909 910}.

Studies for this thesis demonstrate consistent effects of a significant cancer-specific reduction in OE-33 cell proliferation with NAC antioxidant treatment without adverse effects in benign cells. The combination of 10mM NAC with cisplatin and 5-FU enabled a significant reduction in the IC\textsubscript{10} and IC\textsubscript{50} doses for both reagents (0.1 μgmL\textsuperscript{-1} and 2.875 μgmL\textsuperscript{-1} respectively for cisplatin and 0.07 μgmL\textsuperscript{-1} and 6.5 μgmL\textsuperscript{-1} respectively for 5-FU, p < 0.05). These findings appear to be somewhat at odds with existing studies of NAC and cisplatin activity. Recent published data describe a reduction in cisplatin-induced apoptosis in vitro if NAC was present in the culture medium. Washout of NAC prior to cisplatin addition prevented the NAC chemoprotective effects, suggesting a direct effect on cisplatin by NAC binding as opposed to a downstream metabolite or increased glutathione. Concurrent administration of NAC prevented apoptosis and impaired cisplatin cytotoxicity but these effects were diminished if NAC was added more than 2 hours after cisplatin treatment and minimal by 8 hours post-chemotherapy treatment\textsuperscript{911}. Timing appears to be a crucial factor and the effects of adding NAC after the chemotherapy agents may well contribute to the continued activity of cisplatin observed in our thesis studies. Irreversible protein changes may already be set in motion within a short space of time after cisplatin administration. Cisplatin is able to rapidly enter target cells and interact irreversibly with nuclear DNA\textsuperscript{911}.

Furthermore, an important finding is the selective inhibition of heme oxygenase enzymatic activity demonstrated with NAC administration. The reduction in HO activity may be solely responsible for the cell proliferation effects observed without influence from cisplatin activity. In addition there may be effects on downstream cell signalling pathways mediated by HO activity that demand further investigation. It has been
suggested that NAC chemoprotection observed in vitro may be dependent upon p53 tumour suppressor activity, and this requires clarification in the oesophagogastric cancer cell model studied here.

Resistance to cisplatin has been shown to be associated with high levels of basal p38 mitogen activated protein kinase (MAPK) activity in cancer cell line experiments. Recent studies attempting to clarify the influence of p38 MAPK signalling on cisplatin sensitivity or resistance demonstrate an enhanced sensitivity to cisplatin chemotherapy associated with p38 MAPK inhibition in human cancer cells. Investigators found that inhibition of p38 MAPK resulted in upregulation of ROS and activation of the JNK pathway to increase the sensitivity of malignant cells to cisplatin-induced apoptosis. Published evidence suggests that NAC causes inhibition of p38 MAPK signalling. NAC interacts with cell signalling molecules to inhibit the activation of p38 MAP kinase and JNK pathways and reduce nuclear factor κB transcription factor activities. This suggests a potential mechanism by which NAC treatment is able to maintain or enhance cisplatin activity.

The mechanisms that control the effect of NAC on the anticancer activity of agents such as cisplatin have not been fully established. The interaction between NAC and p53, p21 and apoptotic signalling are incompletely understood. The additional influence of HO activity needs further investigation with particular interest in p38 MAPK signalling pathways. Further studies are required in animal models to investigate whether the cancer-specific anti-proliferative effects of NAC described here can be reproduced in vivo and whether NAC treatment can enable administration of lower doses of chemotherapeutic agents whilst maintaining their anti-tumour activity. NAC will only be a useful adjunct for the treatment of oesophagogastric cancer if a rational delivery regimen can be determined to maximise chemoprotective effects while minimising any impact on chemotherapeutic efficacy. The timing and route of NAC administration may provide a mechanism by which the toxic side effect profile of cisplatin is reduced without any therapeutic compromise in vivo.
5.3 Pharmacological modulation of heme oxygenase activity

5.3.1 The effect of pharmacological induction of HO by Hemin

Treating AGS cells with increasing concentrations of hemin for 24 – 72 hours produced no significant difference in cell proliferation measured by MTT assay at any time point observed. Identical experiments in HGC-27 cells caused significant reductions in MTT assay only after 24 or 48 hour treatment with higher doses of hemin at 100 and 150 µM.

Experiments with oesophageal cell lines demonstrated no significant changes in OE-33 cell growth at any time point but significantly reduced HET-1A proliferation at hemin concentrations of 50 µM and above after 24 hours and at 100 µM over 24 – 72 hours. These findings reflect the relative toxicity of hemin at higher concentrations. Comparing the normal oesophageal squamous cells to the adenocarcinoma cells suggests an increased resistance to the effects of hemin on cell proliferation in malignant cell lines.

5.3.2 The effect of competitive inhibition of HO by Zinc Protoporphyrin

Treatment of AGS and HGC-27 gastric adenocarcinoma cells with zinc protoporphyrin at concentrations of 10, 20 and 40 µM caused significant reductions in cell proliferation at 24, 48 and 72 hours as measured by MTT assay.

Experiments in oesophageal cell lines demonstrate a differential response to zinc treatment in cell proliferation between malignant and benign cells. MTT assay was significantly reduced for OE-33 cells after 24 hours of treatment with zinc at concentrations of 10, 20 and 40 µM whereas significant reductions in MTT assay for HET-1A cells were only seen at a zinc concentration of 40 µM. These effects were replicated in time-course experiments with a significant reduction in OE-33 cell growth at all observed time points with all experimental concentrations of zinc. Cell proliferation measured by MTT assay was significantly reduced in HET-1A cells after 24 – 72 hours only with a zinc concentration of 40 µM.

Extending the growth curve experiments to 7 days showed consistent effects of significantly reduced cell proliferation in AGS and OE-33 cells with zinc treatment at 20 and 40 µM at all time points beyond 24 hours.
5.3.3 The effect of Hemin and Zinc Protoporphyrin on HO protein expression

Expression of constitutive HO-2 protein was equal in all cell lines and essentially unchanged by treatment with either hemin or zinc protoporphyrin. Exposure of OE-33 cells to zinc protoporphyrin at 20 and 40µM concentration caused a mild decrease in HO-2 isoform expression after 24 hours. There was a demonstrable up-regulation of inducible HO-1 protein in response to both hemin treatment and zinc treatment in all cell lines investigated. In OE-33 cells HO-1 protein expression was less marked and only in response to the highest dose of zinc protoporphyrin at 100 µM.

Western blot analysis demonstrates absent basal expression of HO-1 protein in the entire panel of cell lines investigated with readily inducible HO-1 expression in response to pharmacological agents. In an attempt to gain a broader picture of HO-1 expression in oesophagogastric cancer a further study was performed to assess HO-1 expression in ex-vivo samples. This study was part of a separate study comparing the histopathological expression of various cell proteins before neoadjuvant chemotherapy to the pathological response after chemotherapy according to Mandard criteria. Samples were identified from patients with oesophagogastric cancer who had undergone neoadjuvant chemotherapy as part of their treatment pathway. A total of 72 samples taken at index diagnostic endoscopy were retrieved from paraffin-embedded blocks. The tissue samples were then cut and slides prepared for a panel of immunohistochemistry including staining for HO-1. Samples from human spleen were used as a positive control to ensure an adequate staining protocol for HO-1 protein. Review of the slides with an independent consultant revealed that none of the retrieved ex-vivo samples stained positive for HO-1 protein. However, review of the cut slides also highlighted inconsistencies with the quality of prepared tissue. In some of the slides there was no malignant tissue visible. Due to these inconsistencies the findings of the study were deemed to be highly questionable and essentially unreliable and were therefore discarded. The inherent difficulties of the study relate to the robust retrieval and preparation of retrospectively identified tissue samples from paraffin blocks. A relatively small amount of tissue is routinely taken at index endoscopy for histological diagnosis and the amount of tissue available in paraffin blocks for future studies is limited. After processing the tissue for the panel study of cellular proteins any tissue left over proved to be inadequate for any
meaningful subsequent analysis of HO-1 protein expression. Therefore this study is planned to be repeated with prospective collection, preparation and staining of oesophagogastric cancer tissue samples.

5.3.4 Heme Oxygenase Enzyme Activity Assay

Enzyme assay results demonstrate an increase in total HO enzyme activity after treatment with hemin consistent with up-regulation of HO-1 protein. There was a significant decrease in total HO enzyme activity after treatment with zinc 40 µM compared to untreated control cells despite expression of inducible HO-1 protein. These findings are consistent with competitive inhibition of heme oxygenase enzyme by zinc protoporphyrin.

The collective evidence indicates that the observed reductions in cell proliferation after zinc treatment are accompanied by a reduction in total HO enzyme activity despite induction of HO-1 protein. It cannot be deduced that basal HO-2 enzyme activity is therefore more important to maintain malignant cell proliferation as the zinc protoporphyrin inhibits both constitutively expressed and induced HO enzyme isoforms. The up-regulation of HO-1 protein is not sufficient to overcome the inhibition by zinc and cannot rectify the reduction in total HO enzyme activity.

5.3.5 Apoptosis Studies: Caspase 3/7 activity assay

Caspase-3/7 assay demonstrates a significant increase in relative caspase activity after 12 hours treatment with zinc protoporphyrin 20 µM and no increase in fold caspase-3/7 induction after 12 hours exposure to hemin 20 µM. These data suggest a process of apoptosis induced by the reduction in HO enzyme activity caused by zinc treatment.
5.3.6 Pharmacological modulation of heme oxygenase activity: Conclusions

The collective data indicate that inhibition of heme oxygenase enzyme activity causes a reduction in cell growth of all cultured cell lines under investigation. HO-1 isoform is not expressed under basal conditions, but is inducible by treatment with hemin and zinc protoporphyrin. Hemin treatment and consequent increased HO-1 expression does not cause an increase in cell growth, with results showing either no significant effect or a decrease in MTT assay.

Treatment with hemin caused up-regulation of inducible HO-1 protein and a consequent increase in total cellular HO enzymatic activity without adverse effects on cell proliferation, notwithstanding the relative toxicity of heme at higher concentrations. Therefore an increase in HO activity is not detrimental to cancer cell proliferation. This means that cellular antioxidant or anti-inflammatory mechanisms mediated by heme oxygenase activity do not prevent or hinder exponential proliferation in malignant cells. Heme oxygenase products may confer anti-apoptotic properties to cells with increased HO activity, or these effects may be mediated by alternative pathways activated by HO-1 protein.

Pharmacological competitive inhibition of HO enzyme by zinc protoporphyrin causes a significant reduction of cell proliferation in all malignant cell lines at all time points studied. Treatment with zinc protoporphyrin 10 µM or 20 µM produced consistent reductions in cell proliferation with statistical significance only in malignant gastric and oesophageal adenocarcinoma cells and not in control oesophageal squamous epithelium. Zinc treatments at doses of 10 – 20 µM provide a cancer-specific effect to cause reduced cell growth. Growth curves up to seven days were significantly reduced in studies using AGS and OE-33 cells. There was a clear reduction in total HO activity mediated by zinc treatment despite induction of HO-1 enzyme. Results indicate that inhibition of HO-2 enzyme under basal conditions and inhibition of the up-regulated HO-1 enzyme causes a reduction in overall enzymatic activity that is detrimental to cancer cell proliferation. Treatment with zinc protoporphyrin caused a concentration-dependent increase in relative caspase-3/7 activity following 12 hours of incubation compared to untreated
controls. The observed effects on cell growth can be observed to be mediated by an increase in apoptosis when HO activity is inhibited.

Inhibition of the HO-2 enzyme present will cause intracellular heme substrate accumulation and consequent up-regulation of HO-1 isoform. This is clearly demonstrated for zinc concentrations of 5 and 10 µM in gastric cancer cells. In oesophageal cancer cells there is less obvious HO-1 expression, and only then at higher zinc doses of 40 and 100 µM. Despite up-regulation of HO-1 to augment basal HO-2 activity, it is likely that enzyme levels do not overcome the potent competitive inhibition of the zinc protoporphyrin. In gastric cancer cell lines the anti-proliferative outcomes of both HO-1 induction by hemin at higher doses and HO activity inhibition by zinc may seem to be contradictory. However this may correspond to cellular responses to enzyme products or substrate accumulation causing cellular toxicity. The effects of CO, biliverdin, Fe²⁺ and intracellular heme are not mutually exclusive, and the overall proliferative response in cancer cells is difficult to predict. There may be a fine balance between oxidative and antioxidant and inflammatory and anti-inflammatory cellular states that govern cell phenotype and cell growth.

Collectively these data suggest a novel role for the constitutive (HO-2) rather than inducible (HO-1) isoform in the tonic regulation of gastro-oesophageal adenocarcinoma cell growth. The zinc protoporphyrin inhibited proliferation by acting on the constitutively expressed HO-2 enzyme as the cells did not express HO-1 under basal conditions.

The potential anti-cancer activity of pharmacological heme oxygenase enzyme inhibition by metalloporphyrins has been previously investigated. Zinc protoporphyrin has been demonstrated to inhibit lung cancer tumour growth in a mouse model in a dose-dependent fashion. Additional rat models have indicated a significant reduction of tumour growth with inhibition of HO activity by zinc PP. Potent anti-tumour effects in vivo have been reported with a water-soluble conjugated zinc protoporphyrin used in a murine model with demonstrable induction of apoptosis in solid sarcoma tumours. Pegylated zinc PP treatment also significantly reduced the growth of sarcoma 180 tumours in a mouse model.
One recently published report investigated the ability of zinc protoporphyrin to potentiate chemotherapeutic activity. This study found that zinc PP caused significant accumulation of reactive oxygen species in tumour cells and exhibited potent cytotoxic effects against human and murine tumour cell lines with evidence for the induction of apoptosis. In a murine model, however, transplanted melanoma cells over-expressing HO-1 did not respond to zinc treatment. Zinc PP was unable to sensitise tumour cells to the chemotherapeutic activity of 5-fluorouracil, cisplatin or doxorubicin in three different tumour models.

Treatment with pegylated zinc protoporphyrin has been demonstrated to reduce the required lethal doses of doxorubicin and hydrogen peroxide in cultured human colonic carcinoma cells. More recent studies have focused on the potential for selective tumour targeting by heme oxygenase inhibitors. Fang and colleagues report studies using a water-soluble micelle containing zinc protoporphyrin for tumour-targeted inhibition of HO activity. They report a profound in vitro cytotoxic effect of the water-soluble zinc micelle specific to various tumour cells from studies using eleven cultured tumour cell lines and six normal cell lines. A potent anti-tumour effect was also observed in murine melanoma and fibrosarcoma xenograft in vivo models. The zinc micelle was found to predominantly accumulate in the liver tissue after intravenous injection facilitating an impressive anti-cancer effect seen in a rabbit liver tumour model.

The findings from the current studies demonstrate an important role of heme oxygenase enzyme activity in maintaining cell growth. The inhibition of heme oxygenase enzyme produces anti-cancer effects even though over-expression of HO-1 enzyme is not a feature of these cancer cells investigated. The results provide evidence for a crucial role of the HO-2 isoform in this context, with inhibition of the constitutively expressed protein causing anti-proliferative and pro-apoptotic sequelae that cannot be prevented by up-regulation of HO-1 protein. Reduction of the total cellular heme oxygenase enzymatic activity by pharmacological inhibition provokes a reduction in cancer cell growth that is consistent with published reports.
The limitations of these studies relate to potential non-specific pharmacological effects of zinc protoporphyrin. It is possible that inhibition of cell growth is achieved through non-specific zinc effects as opposed to inhibition of HO-2 isoform enzyme. Zinc protoporphyrin (zinc PP) is a potent selective inhibitor of heme oxygenase enzyme that inhibits the oxidative catalytic cleavage of the α-meso carbon bridge of the heme porphyrin ring by heme oxygenase. Zinc PP however also inhibits other intracellular enzymes and signalling molecules including interleukin-1 and nitric oxide synthase. It exhibits inactivation of all three isoforms of nitric oxide synthase in a time-dependent and concentration-dependent manner. The diverse effects of zinc protoporphyrin may be responsible for the proliferation effects observed in these cell lines investigated. The effect of zinc PP may be cell-specific as it has been demonstrated to promote the proliferation of growth factor-stimulated vascular smooth muscle cells but inhibit endothelial cell proliferation during angiogenesis. The problem arises when considering possible non-specific and HO-independent effects of zinc PP as a pharmacological agent.

Metalloporphyrins (MP) have been used to inhibit heme oxygenase activity both in vitro and in vivo for several decades. It has recently been suggested that not all commonly-used MPs are specific to HO enzyme. A recent study comparing metalloporphyrins reported that zinc protoporphyrin was neither the most selective nor the most effective inhibitor of HO. The majority of tested MPs (four out of five) also inhibited the activity of other enzymes including nitric oxide synthase (NOS). This raises doubt about whether the observed zinc PP-mediated effects on cell proliferation are indeed related to the HO pathway, particularly as the NOS system is closely related to the cellular oxidative stress response. The use of a more specific and effective agent would be beneficial but every pharmacological agent comes with its own profile of adverse and off-target effects.

Subsequent experiments were therefore designed to investigate a specific role of HO-2 enzyme in cell proliferation using siRNA-mediated knockdown of the HO-2 gene. This methodological approach attempts to minimise the potential diverse effects that are a common concern with pharmacological agents.
5.4 Investigating a Specific Role for HO-2 Enzyme in Cell Growth

5.4.1 Establishing protocols for adequate transfection of siRNA complex

Short interfering RNA-mediated HO-2 gene knockdown experiments were carefully designed with the aim of investigating a specific role for the HO-2 isoform protein. Experiment protocols were developed to minimise adverse effects of various reagents used on the proliferation of cells investigated. The transfection reagent Lipofectamine did not significantly affect MTT assay for AGS cells up to 72 hours at concentration between 0.5 and 1.5 µL. The efficacy of oligonucleotide transfection was assessed with experiments using a fluorescently tagged oligonucleotide (AlexaFluor) that demonstrated improved oligo uptake into cell cytoplasm on fluorescent microscopy at 10 nM concentration with no significant effects on MTT proliferation assay. These results demonstrate effective transfection of the labelled siRNA using Lipofectamine reagent at 1 µL per well and oligonucleotide at 10nM concentration. Using a negative universal control oligonucleotide at concentrations of 5, 10 or 20 nM resulted in no significant differences in MTT assay outcomes for AGS and HGC-27 cells up to 72 hours.

Therefore the experiment protocol derived from the experiment described above included final concentration of Lipofectamine reagent at 1 µL and siRNA oligonucleotide at 5 – 20 nM in experiment wells to facilitate efficient siRNA transfection with minimal adverse effects. Given the findings from the labelled RNAi duplex and NUC experiments it was decided to start siRNA experiments using oligonucleotide at 10 – 20 nM concentration. Although this does not rule out the possibility of mild cellular phenotype effects as a direct result of oligonucleotide transfection in HGC-27 cell lines, it does ensure uptake of the siRNA into the cell. Experiments were designed to include the negative universal control siRNA duplex at the same concentrations as the gene-targeted siRNA duplex to allow for direct comparison between the two. This would give further confidence in any observed results being due to effects mediated by knockdown of the targeted gene as opposed to the transfection process.
5.4.2 Efficacy of supplied siRNA and HO-2 mRNA knockdown

Agarose gel analysis of RT-PCR products demonstrated effective knockdown of HO-2 mRNA after transfecting cells with targeted siRNA for 24, 48 and 72 hours. The most effective knockdown of HO-2 RNA was achieved after transfection with supplied siRNA duplex 02. Experiments were repeated in an attempt to further validate effective HMOX2 mRNA knockdown but inconsistent results were obtained with PCR products using GAPDH primer controls. These findings suggested a compromise in RNA quality such that every stage of the PCR process had to be interrogated for possible contamination of samples. All eluted RNA samples were therefore treated repeatedly with DNase enzyme and examined for DNA contamination with agarose gel electrophoresis. All samples with visible evidence of DNA contamination were discarded along with any corresponding complementary DNA stored. Repeat steps of RNA elution and RT-PCR were performed and the PCR products analysed on agarose gels to demonstrate effective knockdown of HMOX2 mRNA after transfection with the selected 02 siRNA duplex.

Real-time quantitative PCR analysis was performed to further clarify siRNA-mediated knockdown of the heme oxygenase target mRNA. The quality of experimental RNA samples was confirmed with an accepted experimental standard level of purity obtained. Quantitative RT-PCR results indicate a significant reduction in relative HO-2 mRNA expression in both AGS and OE-33 cells after transfection with the selected siRNA duplex 02 oligonucleotide. For all subsequent siRNA experiments the siRNA 02 duplex was therefore used for effective HO-2 gene knockdown. There was a significant corresponding increase in the relative expression of HO-1 mRNA in both cell lines after transfection with HMOX2-targeted siRNA.

Real-time quantitative PCR results confirm efficient knockdown of HO-2 mRNA from the siRNA experiment protocols used and indicate a concurrent up regulation of HO-1 mRNA. These findings are in keeping with previous published studies of the interaction between HO-1 and HO-2 proteins.399

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5.4.3 Effect of siRNA-mediated HO-2 knockdown on heme oxygenase expression & total heme oxygenase enzyme activity

Western blot analyses of protein expression demonstrate effective reduction of HO-2 protein 24, 48 and 72 hours after transfection of AGS cells using siRNA duplex 02 for HO-2 gene knockdown. Probing the blots for HO-1 protein indicates HO-1 up-regulation in cells treated with HMOX2-targeted siRNA with a time-dependent increase in HO-1 expression over 24 – 72 hours demonstrated in AGS cells. Experiments using OE-33 cells demonstrate similar patterns of reduced HO-2 protein expression and increased HO-1 protein expression up to 48 hours after transfection with HMOX2-targeted siRNA duplex. Induction of HO-1 protein may represent a cellular response to various elements of siRNA-mediated HO-2 mRNA knockdown as well as the potential build-up of heme substrate as a consequence of reduced functional HO-2 protein.

Specific colorimetric HO enzyme assays demonstrate a significant reduction in total heme oxygenase activity 24 and 48 hours after treatment with siRNA targeted toward HO-2 protein. The combined activity of HO-2 and HO-1 enzyme proteins was however significantly increased after 12 hours. This represents a short-lived increase in total enzyme activity that may be due to an increased HO-1 expression in response to siRNA-mediated HO-2 knockdown. Although HO-1 protein expression increases over 24 – 72 hours after HO-2 knockdown the effect on overall HO enzyme activity is only seen within the initial 12 hours. This suggests that basal activity of constitutively expressed HO-2 enzyme provides the major contribution to overall HO enzyme activity. It is possible that the induced HO-1 protein is sensitive to cellular feedback mechanisms or that activity of the HO-1 enzyme is specifically regulated by downstream enzyme products. The collective data indicate that in siRNA-treated cells measurable total HO enzyme activity is not maintained despite up-regulation of HO-1 protein in response to knockdown of HO-2 mRNA. This has important implications for the cell proliferation effects observed with HO-2 protein knockdown.
5.4.4 The effect of siRNA-mediated HO-2 knockdown on cell growth

Experiments to assess cell proliferation demonstrate a significantly reduced MTT assay at 48 and 72 hours in cells transfected with HMOX2 siRNA at 10 nM concentration as compared to an equivalent treatment with negative universal control siRNA. Consistently significant results were obtained in repeated experiments using AGS, HGC-27 and OE-33 cell lines. There were no significant effects on cell growth observed at the 24 hour time point.

Results from cell counts using trypan blue exclusion suggest that the observed reductions in MTT assay are not due to increased numbers of necrotic cells. The very limited number of dead cells counted in all experiment wells suggests an anti-proliferative or pro-apoptotic response to reduced HO-2 activity. HO-2 knockdown in AGS, HGC-27 and OE-33 cells produced effects on cell counts that are more dramatic than those seen with MTT. The discrepancy between cell count numbers and the equivalent MTT results is likely to be explained by the technical aspects and relatively cumbersome nature of cell counting with the major factor being fewer experiments performed leading to greater variability and an increased likelihood of observer error.

Further experiments were designed to assess cell cycle kinetics with flow cytometry and to investigate apoptosis after specific HO-2 knockdown.

5.4.5 The effect of siRNA-mediated HO-2 knockdown on cellular apoptosis

Cells treated with HMOX2 siRNA at 10 nM concentration demonstrated a significantly increased affinity for annexin V binding as evidence for early apoptosis after 24 hours as compared to cells transfected with NUC 10 nM over the same time period. At 72 hours there were significantly fewer viable cells and significantly increased late apoptotic or necrotic cells in samples transfected with HMOX2 siRNA compared to NUC. The observed movement of siRNA-treated cells over time through annexin V positive and PI negative to annexin V and PI positive suggests a process of early apoptosis progressing to late apoptosis and eventual cell death.

Cell cycle studies demonstrated cells undergoing a process of apoptosis with a sub-G₀ peak population of cells visible 24 and 48 hours after transfection with HMOX2 siRNA.
duplex at 10 nM concentration. After 48 hours the siRNA-treated sample showed a further reduction in the proportion of cells in G₀/G₁ or G₂/M phase with an increase in the proportion of cellular debris or apoptotic cells.

The measured coefficient of variation of the G₀/G₁ peak and the ratio of G₀/G₁ to G₂/M cells indicated reduced accuracy of DNA measurement and interference of doublet discrimination by increased cellular debris. The ratio between G₁ and G₂ peaks should be between 1.95 and 2.05 and the cell debris measured should be up to a maximum of 30%. The linearity of the plot should produce a tight and short distance G₁ peak with a CV of less than 8% to indicate good quality DNA measurement.

Cytometry studies show a significant increase in DNA fragmentation 24 hours after transfecting cells with HMOX2 siRNA at 10 nM concentration and a significant reduction in the proportion of non-apoptotic cells present. After 48 hours there is a further significant increase in DNA fragmentation and fewer non-apoptotic cells in samples treated with HMOX2 siRNA as compared to cells transfected with negative universal control siRNA. These results indicate that one of the hallmarks of apoptosis is detectable in AGS cell lines 24 hours after siRNA-mediated knockdown of HO-2 mRNA and that this process progresses up to 48 hours after transfection.

Caspase-3/7 assays performed in AGS cells demonstrate a statistically significant increase in relative caspase activity 24 hours after siRNA-mediated HO-2 knockdown when compared to appropriate controls and provide additional supporting evidence for a process of apoptosis.
5.4.6 Investigating a Specific Role for HO-2 Enzyme in Cell Growth: Conclusions

There are several limiting technical factors in the siRNA experiments. In oesophageal cell lines transfection with the negative universal control caused non-significant reductions in cell growth when compared to non-transfected control cells. Despite the careful design of control oligonucleotides from specialist manufacturers, there must be off-target effects either related to RNA interference or non-specific cellular stress responses. However subtle these effects are, there is a detectable change in cell phenotype with the outcome under investigation. Comparison between the negative universal control and the HO-2 targeted siRNA remains a valid approach to elicit phenotypic changes that are a consequence of specific gene knockdown.

These preliminary experiments demonstrated cellular uptake of transfected siRNA and efficient knock down of targeted HO-2 mRNA. A reduction in HO-2 protein expression and reduction in total HO enzyme activity has been achieved by this approach. Cells with reduced HO activity despite increased HO-1 expression demonstrate an altered phenotype with a reduction in cell proliferation and increase in apoptosis. Together with the experiments using zinc protoporphyrin, the different approaches of modulation of HO-2 expression by mRNA knockdown and HO-2 activity by competitive pharmacological inhibition result in the same biological outcome, i.e. inhibition of cancer cell growth.

Targeted knockdown of HO-2 protein had a clear effect on the proliferation of AGS and HGC-27 gastric cancer cells and OE-33 oesophageal cancer cells with significant growth inhibition at 48 and 72 hours. The time-dependent effect does not appear to be correlated to the presence of HO-1 protein, as HO-1 protein is induced within 48 hours of HO-2 knockdown. As demonstrated with assays of HO enzyme activity the contribution of induced HO-1 enzyme is short-lived. It is noteworthy that the cellular phenotype effects of reduced proliferation are observed after a measurable reduction in total HO enzyme activity. Furthermore the continued expression of HO-1 protein cannot compensate for the lack of HO-2 protein. Residual activity of HO-2 enzyme following mRNA knockdown or short-lived activity of up-regulated HO-1 isoform does not restore total HO enzyme activity to the same level as untreated control cells. The reduction in overall HO enzyme activity appears to be sufficient to cause an anti-proliferative and pro-apoptotic effect.
These findings support the idea of tonic HO-2 activity having a vital role in oesophagogastric cancer cell biology. Tonic HO activity provided by the HO-2 isoform may be required to maintain redox balance in the cell, and reduction of this activity could lead to intracellular oxidative stress which may affect proliferative pathways. The cellular biology of HO-1 and HO-2 enzyme isoforms is clearly closely linked: HO-1 induction was clearly demonstrated in response to HO-2 mRNA knockdown. Although HO-1 protein expression increases over 24 – 72 hours after HO-2 knockdown the effect on overall HO enzyme activity is only seen within the initial 12 hours. This suggests that basal activity of constitutively expressed HO-2 enzyme provides the major contribution to overall HO enzyme activity. Induced HO-1 protein may be sensitive to specific cellular feedback mechanisms or regulated by downstream enzyme products. The relative contribution of HO-1 and HO-2 isoforms toward overall HO enzyme activity require further investigation. The specific interaction of HO-1 and HO-2 proteins with cell signalling pathways may influence the resulting phenotype. The experiments detailed above demonstrate that the specific phenotypic effects of increased apoptosis caused by HO-2 knockdown are not prevented by induction of HO-1 expression. This may be due to either a reduction in enzyme activity or a differential interaction of HO-1 and HO-2 isoforms with cell signalling and apoptotic pathways. Total enzyme activity is not maintained beyond the initial significant increase at 12 hours, so the activity of the up-regulated HO-1 protein is either compromised or not relevant to the cell outcomes measured. It is possible that tonic or residual HO activity from specifically the HO-2 isoform is responsible for maintenance of exponential proliferation in oesophagogastric cancer cells. It is possible that the constitutive HO-2 isoform and not the inducible HO-1 isoform is responsible for interaction with pathways to apoptosis, or indeed that the balance between the relative expression of HO isoforms maintains cellular homeostasis.

Published studies provide supporting evidence for the role of HO-2 protein in cellular homeostasis. Recent reports have established the important antioxidative functions of HO-2 protein. The HO-2 protein molecule contains heme-binding heme regulatory motifs (HRMs) that function as heme – oxygen sensors in bacteria, yeast and mammals. The presence of HRMs in the HO-2 enzyme suggests the further unique function of
binding and regulating the heme gaseous ligands nitric oxide (NO), carbon monoxide (CO) and oxygen (O$_2$)$^{116\ 224}$. 

Recent studies have shown that the expression levels of HO-1 and HO-2 proteins are reduced under hypoxia. Certain features suggest separate physiologic roles of HO-1 and HO-2 and support the proposed function of HO-2 as a potential oxygen sensor$^{206}$. HO-2 has recently been demonstrated to interact with nitric oxide synthase enzyme, binding the vasodilator NO with high affinity and the potential vasodilator CO with less affinity$^{214}$. Calcium-sensitive potassium channels are stimulated by the interaction of HO-2 with CO to mediate the excitatory responses of the carotid body to hypoxia$^{215}$. 

Recent studies provide evidence for a critical role of HO-2 protein in acute inflammation with specific roles in repair responses of the cornea to injury$^{927\ 928}$. Halilovic and co-workers demonstrate that the highly-expressed HO-2 protein in the corneal epithelium is critical for corneal wound healing. Specific knockdown of HO-2 expression significantly impaired wound healing whereas knockdown of HO-1 protein had no significant effect$^{928}$. Specific HO-2 knockdown has been shown to impair the acute inflammatory and reparative response after epithelial injury and recent in vivo studies demonstrate an exaggerated inflammatory response in HO-2 -/- models of antigen-induced peritonitis$^{174}$. Cerebral vascular endothelial cells from HO-2 knockout mice have been reported to show a greater sensitivity to oxidative stress and apoptosis caused by serum deprivation and TNF-α treatment than cells from wild-type mice. Studies suggest that HO-2 plays an important anti-apoptotic role in the protection of cerebrovascular endothelium against oxidative stress and acute cytokine-mediated inflammation$^{209}$. 

Published studies suggest a role for HO-1 enzyme activity in cancers with constitutive over-expression of HO-1 protein. Berberat and colleagues found a 6-fold up-regulation of HO-1 mRNA in human pancreatic cancer cells when compared to normal human pancreas. Significant inhibition of cancer cell proliferation was effected by siRNA-mediated knockdown of HO-1 protein with enhanced cancer cell sensitivity to gemcitabine chemotherapy and radiotherapy$^{334}$. The current study demonstrates an interaction between constitutive HO-2 and inducible HO-1 proteins. Specific siRNA-mediated HMOX2 knockdown caused up-regulation of
inducible HO-1 protein. This finding is consistent with published studies from Ding and colleagues who demonstrated induction of HO-1 expression at both mRNA and protein levels with siRNA-mediated HO-2 knockdown in cultured cell lines\textsuperscript{399}. It is noteworthy that in cells that over-expressed HO-1 protein, specific knockdown of HO-1 expression did not affect HO-2 expression. Reduction of HO-2 protein expression caused heme accumulation only when the affected cells were exposed to exogenous hemin. This means that the induction of HO-1 protein in response to HO-2 knockdown is not due to a reduced total HO enzymatic activity causing heme substrate build-up. These results suggest that HO-2 protein may directly down-regulate the expression of HO-1 to facilitate the co-ordinated expression of both cellular isoforms. Alternative findings suggest that HO-2 protein is critical for HO-1 expression with deletion of HO-2 associated with impaired induction of HO-1 and subsequent development of chronic inflammation\textsuperscript{174}.

The current study provides further supportive evidence for interaction between HO-1 and HO-2 isoforms and presents a novel finding of the importance of tonic HO-2 activity for the regulation of cancer cell growth.

Products of enzyme activity such as bilirubin or carbon monoxide may directly reduce HO-1 enzyme activity by negative feedback. Inhibition of heme oxygenase activity or specific knockdown of HO-2 enzyme might be detrimental to cells both due to the accumulation of pro-oxidant substrate heme and due to reduced production of beneficial anti-oxidant downstream products.

Further studies were designed to assess the influence of downstream enzyme products bilirubin and carbon monoxide on HO enzyme activity and on cellular proliferation outcomes.
5.5 Downstream Effectors of Heme Oxygenase Activity: Bilirubin

5.5.1 The effect of Bilirubin on cell proliferation

Experiments to investigate the effects of bilirubin on cell growth yielded some surprising results. There was no significant effect on cell proliferation as measured by MTT assay performed after 24 hours of treatment with bilirubin at various doses. However, extending cell exposure to bilirubin at doses of 10 µM and 20 µM produced a significant reduction in MTT assay after 48 hours and at 72 hours. Interestingly these effects were reproduced when the bilirubin reagent medium was replaced with fresh serum-complete medium after 24 hours. Treatment of cells with bilirubin at 10 or 20 µM for 24 hours produced significant reductions in cell proliferation measured at 48 or 72 hours respectively. Cell count data demonstrated a similar trend with increases in viable cell counts at 24 hours and reductions in viable cell counts 72 hours after bilirubin treatment.

These findings indicate a direct effect of bilirubin treatment on cell proliferation that is not apparent within the first 24 hours of bilirubin exposure. A possible explanation for the increase in cell numbers at 24 hours lies in the well-established antioxidant properties of bilirubin\textsuperscript{210 847 929-931}. Bilirubin may exert a cytoprotective effect on the cells by providing protection against oxidative damage and thus facilitating survival and exponential proliferation within the first 24 hours. In addition, bilirubin has recently been found to possess anti-nitrosative properties. Peroxynitrite is a highly reactive molecule formed when the gaseous mediator nitric oxide reacts with superoxide anions. Bilirubin has been shown to effectively scavenge peroxynitrite and thus reduce nitrosative damage to cells\textsuperscript{932 933}.

The current experiments consistently demonstrate a decrease in viable cell number and reduced cell proliferation 48 – 72 hours after addition of bilirubin. These findings are consistent with previous findings of cytotoxic activity of bilirubin via pro-oxidant mechanisms\textsuperscript{884}.

The cytotoxicity of bilirubin at high concentrations has been established in physiological studies. When serum bilirubin concentrations reach 300 µM neurotoxicity becomes a problem causing cell death. In the infantile condition of kernicterus brain damage ensues
as a result of high serum bilirubin levels\(^9\). One proposed mechanism of bilirubin cytotoxicity is damage to mitochondrial membranes with consequent impairment of cellular metabolism\(^9\). Anti-proliferative effects of bilirubin may be mediated by apoptotic pathways. Bilirubin has been shown to cause cell cycle arrest at the G0/G1 phase\(^9\). The effects of bilirubin have been proposed to be dependent upon various factors including the differential susceptibility of cells to bilirubin toxicity, variation in the expression of membrane transporter proteins, the extent of conjugation and oxidation of the bilirubin pigment and the prevailing milieu of cytosolic binding proteins\(^9\).

Replacement of the bilirubin with fresh media does not prevent the anti-proliferative effects observed in the current experiments. This suggests possible interaction with intracellular signalling pathways that once activated no longer require continued stimulus from the presence of bilirubin.

### 5.5.2 The effect of Bilirubin after HO enzyme inhibition or HO-2 knockdown

Experiment results demonstrate that although cell proliferation is maintained with bilirubin treatment alone for 24 hours the effects mediated by heme oxygenase inhibition or HO-2 gene knockdown cannot be reversed or prevented by treatment with bilirubin at 5 or 10 \(\mu\)M concentrations for 24 hours. There was a significant reduction in cell proliferation after treatment with zinc protoporphyrin 10 \(\mu\)M for 48 hours observed up to 72 hours and significant reductions in cell proliferation observed at 72 hours after siRNA transfection that bilirubin treatment did not affect. One possible explanation for these observations is the inhibition of HO enzyme activity via a bilirubin negative feedback mechanism. It could be that the anti-oxidant and cytoprotective mechanisms of bilirubin are mediated by or dependent upon activity of heme oxygenase and more specifically the HO-2 isoform. This may explain the apparent lack of cell protection with bilirubin treatment that is observed in the setting of HO-2 gene knockdown or HO-2 enzyme inhibition.
5.5.3 RNA and Protein expression after siRNA HO-2 knockdown and Zinc PP

Agarose gel analysis of PCR products demonstrate a clear reduction in HMOX2 mRNA from cells treated with HMOX2 siRNA that is not mitigated by treatment with bilirubin 5 µM for 24 hours. Western blot analyses show a clear reduction in HO-2 protein expression in cells transfected with HMOX2 siRNA after 24, 48 and 72 hours. HO-2 protein expression is not affected when cells are incubated with bilirubin at 5 µM for 24 hours. There is a reduction in control protein β-actin expression at 48 hours that is not proportional to the reduction in HO-2 protein expression at the same time point and represents unequal protein loading. The return of HO-2 protein expression beyond 72 hours was not investigated in these studies. The transient nature of siRNA-mediated knockdown of HO-2 mRNA may allow restoration of protein expression without preventing the phenotypic cellular effects of reduced proliferation. A possible strategy to avoid the transient action of transfected siRNA oligonucleotides would be to use viral vectors engineered to continuously produce the required siRNA.

Expression of HO-2 protein is reduced by treatment with zinc protoporphyrin (zinc PP) for 24 – 72 hours as described above. Zinc protoporphyrin is a potent competitive inhibitor of heme oxygenase enzyme. Treating cells with zinc PP is demonstrated to reduce total HO enzyme activity in enzyme assays detailed above. This will cause heme substrate build-up and thus provide a direct stimulus for induction of HO-1 protein. The up-regulation of HO-1 protein may lead to a compensatory down-regulation in HO-2 expression. This idea is supported by published reports suggesting that the interaction between HO-1 and HO-2 isoforms serves to limit total HO activity and promote non-enzymatic functions of heme oxygenase.

5.5.4 The effect of Bilirubin on cellular apoptosis

Cell populations treated with bilirubin 10 µM demonstrated a significantly increased affinity for annexin V binding as evidence for early apoptosis after 36 hours when compared to vehicle-treated control cells over the same time period. At 48 hours there were significantly fewer viable cells and significantly increased late apoptotic or necrotic cells in samples treated with bilirubin as compared to vehicle-treated cells. The demonstrable shift in the proportion of cells staining annexin V positive 36 hours after
bilirubin exposure to staining both annexin V and PI after 48 hours suggests a process of apoptosis in cells treated with bilirubin.

Cell cycle studies demonstrate a reduction in the proportion of cells in G₀/G₁ phase or G₂/M phase with a sub-G₀ peak population of cells visible 36 hours after treatment with bilirubin at 10 µM concentration. The measured coefficient of variation of the G₀/G₁ peak was 5.65 and the ratio of G₀/G₁ to G₂/M cells 2.19 indicating good quality of DNA measurement and accurate doublet discrimination.

Studies of DNA fragmentation show no observable difference between cells treated with vehicle control for 36 hours and untreated control cells. Cells treated with bilirubin 10 µM demonstrate a significant increase in DNA fragmentation with a significant reduction in the proportion of non-apoptotic cells when compared to control cells after 36 hours. There is a further significant increase in DNA fragmentation observed 48 hours after bilirubin treatment. Results indicate that a hallmark feature of apoptosis is detectable in AGS cell lines 36 and 48 hours after treatment with bilirubin 10 µM.

Collectively the studies of apoptosis contribute persuasive evidence for a process of apoptosis that occurs in response to treatment with bilirubin. This process is demonstrable 36 hours after treatment with bilirubin. The effects on cell proliferation are measurable at 48 hours and at 72 hours by MTT assay. The timing of apoptosis assays was informed by the observed effects on cell growth, given that no reductions in cell proliferation were recorded within 24 hours of bilirubin treatment. Initial apoptosis studies were able to identify a process of early apoptosis as demonstrated by increased affinity for annexin V at 36 hours. This supports the idea that the process of apoptosis is not significantly increased in bilirubin-treated cells until at least 24 hours of reagent exposure, further promoting the suggested interaction of bilirubin with intracellular signalling pathways.

An important feature of these bilirubin experiments relates to the duration of bilirubin exposure and the observed effects at various time points. No difference in cell proliferative effects was observed between cells exposed to bilirubin for 24 hours and cells treated with bilirubin for up to 72 hours when these effects were measured at the same time points of 24, 48 and 72 hours. Replacing the reagent medium with fresh
serum-complete media after 24 hours made no difference to the cell phenotype. Studies of apoptosis were therefore designed to elucidate a process of apoptosis in cells treated with bilirubin for only 24 hours: reagent media was replaced with serum-complete media at 24 hours as described above and the apoptosis assays performed at 36 or 48 hours. These data indicate that the bilirubin-mediated effects on apoptosis and cell growth are not dependent on continued exposure to bilirubin beyond 24 hours. This provides additional supporting evidence for the activation of downstream signalling pathways by cellular interaction with bilirubin.

5.5.5 The effect of treatment with Bilirubin: Conclusions

The heme oxygenase pathway and its downstream products are widely regarded to possess cytoprotective and antioxidant properties, both in physiological and pathological states. However little is known about the mechanisms by which these effects are conferred, particularly in the field of cancer research. Previous studies suggest that the actions of the HO pathway may depend on the activity of its downstream products. Administration of bilirubin and carbon monoxide to cells has been demonstrated to produce the same effects mediated by induction of the HO-2 pathway. The observed neuroprotective activity of HO-2 enzyme has been reported to be due to accumulation of bilirubin. Bilirubin can no longer be considered simply a toxic waste product of heme catabolism. There is persuasive evidence for an important role of heme oxygenase as an endogenous cytoprotective compound at physiological concentrations.

The activity of biliverdin reductase enzyme is recognised as a critical component in the role of heme oxygenase in protecting cells from the toxic effects of heme. The antioxidative and anti-inflammatory effects of bile pigments and their regulatory functions can be mediated by the activity of biliverdin reductase. Published evidence supports the diverse functions of biliverdin reductase and its peptide fragments in the regulation of gene expression and activation of cell signalling pathways.

Bilirubin can exhibit either cytotoxic or cytoprotective activity depending on the tissue concentration of its unbound fraction and the cellular redox state of the target tissue. Bilirubin has been established to exert antioxidant effects at low to moderate concentrations while possessing pro-oxidant activity at high concentrations. In vitro
studies demonstrate that both free and albumin-bound bilirubins are able to scavenge peroxylradicals and protect low-density lipoprotein against peroxidation. Subsequent studies have indicated a cytoprotective role for bilirubin in response to various oxidative insults. Bilirubin reacts with various reactive oxygen and nitrogen species including singlet oxygen, hydroxyl radical, nitric oxide and peroxynitrite to reduce oxidative stress.

The antioxidant, anti-mutagenic and anti-carcinogenic activities of bilirubin are well established in published reports of in vitro experiments. Bilirubin has demonstrable in vitro effects against inflammation and fibrosis with published reports of activity in immune-mediated inflammatory diseases and pathophysiological processes including atherosclerosis. Bilirubin exerts critical anti-inflammatory actions in the vascular system. The protective effects of bilirubin in the vasculature include inhibition of neointima formation and the reduction of vascular smooth muscle cell proliferation and migration. Bilirubin treatment inhibits the expression of leucocyte adhesion molecules in endothelial cells and prevents the adhesion and infiltration of leucocytes into the vessel wall. Low bilirubin concentrations induce apoptosis in microvascular endothelial cells that is more pronounced under conditions of hyperglycaemia and particularly relevant in the endothelial cells of the blood-brain barrier.

Bilirubin has beneficial effects in pulmonary and vascular diseases, improves renal blood flow and renal haemodynamics in an animal model of hypertension and displays antiviral activity. A mildly elevated level of circulating bilirubin has been shown to prevent disease in human subjects by antioxidant mechanisms. Unconjugated bilirubin may have an important protective physiological role in the prevention of gastrointestinal and colorectal cancers.

The current thesis studies indicate that bilirubin plays a role in reducing oesophagogastric cancer cell growth. This role involves activation of cellular apoptosis most likely by time-dependent intracellular pathways that are effective after removal of the bilirubin reagent. Addition of bilirubin after HO inhibition or HO-2 gene knockdown does not ameliorate the anti-proliferative effects of reduced HO enzymatic activity.
The effects of bilirubin on heme oxygenase protein expression and enzymatic activity are less clear. There is no change in HO-2 protein expression 24 hours after treatment with bilirubin. There is a slight reduction in HO-2 protein demonstrated after 48 hour treatment with bilirubin at 20 µM concentration. The HO-1 isoform is not up-regulated in response to bilirubin treatment despite changes in the cellular phenotype observed at 48 hours and beyond. Bilirubin may well exert a negative-feedback effect on HO-2 protein expression and activity and indeed on the induction of HO-1 enzyme. A limitation of these experiments is the lack of data explaining the effects of bilirubin on overall HO enzymatic activity. The methodology of the specific HO enzyme activity assay involves colorimetry to measure the production of bilirubin pigment. The problem comes when treating cells with bilirubin pigment for 24 hours. Despite best attempts to washout the reagent with fresh media there are inevitably bilirubin salts remaining in the adherent cell monolayer. It was decided that there was sufficient residual bilirubin pigment in bilirubin-treated cells to render the colorimetric HO activity assay invalid for these experiments.

Two published studies to date have demonstrated a direct effect of bilirubin on cancer cell growth in vitro. Bilirubin treatment inhibited cell proliferation in multiple cell lines with induction of apoptosis and cell cycle arrest. Activation of cell signalling pathways was demonstrated with induction of p53 and p27. Bilirubin has been shown to decrease the viability of colonic adenocarcinoma cell monolayers in a dose-dependent manner with induction of apoptosis and caspase activation. Bilirubin was capable of activating the intrinsic apoptotic pathway by inducing loss of mitochondrial membrane potential in cultured cell lines. In addition, the presence of bilirubin has been associated with inhibition of tumour cell growth in a murine colon cancer xenograft model. A more recent study demonstrated critical damage to human colorectal adenocarcinoma (Caco2) and hepatocellular carcinoma (HepG2) cells with DNA strand breaks caused by treatment with unconjugated bilirubin and other structurally related tetrapyrroles.

The concentration of free unbound bilirubin depends upon the ratio of bilirubin to albumin. This ratio dictates the degree to which unbound bilirubin can diffuse across plasma cell membranes and interact with cell mitochondrial membrane. In the methodology of the current studies bilirubin was added to cell culture medium at a final
concentration of 10 – 20 µM per experiment well. The culture medium included 10% foetal bovine serum that would reduce the free unbound fraction of bilirubin. A recent meta-analysis of in vitro neurotoxicity mediated by unconjugated bilirubin found evidence of adverse cellular effects when the free unbound bilirubin concentration was close to the aqueous saturation limit of 70 nM\textsuperscript{937}.

The anti-proliferative in vitro activity of relatively higher levels of bilirubin demonstrated in the current study is supported by at least 2 previous studies as described above, and is the first demonstration in oesophagogastric cancer cells.

One of the most important practical problems encountered in conducting these studies was the accurate preparation of bilirubin solutions. The lack of an accepted standard protocol meant that bilirubin preparation became largely a trial and error exercise. Bilirubin is not readily soluble, is sensitive to light and is particularly susceptible to oxidization that can change its chemical and biological properties. Careful design of a reproducible and reliable bilirubin preparation protocol was of paramount importance.

A further limitation of these studies is the relative lack of experimental data for cell proliferation outcomes and apoptosis assays measured at further time points. Further studies are indicated to assess the effects of bilirubin treatment at 6, 12 and 36 hours and extended growth curves to seven days.
5.6 Downstream Effectors of Heme Oxygenase Activity: Carbon Monoxide

5.6.1 The effect of CORMS on cell proliferation

Cell treatment with carbon monoxide-releasing molecules (CORM3) and their inactive controls (iCORM) produced consistent cancer-specific results. There were no significant effects on cell proliferation for normal squamous epithelial HET-1A cells treated with iCORM controls or CORM3 at doses of 10 – 60 µM for 24 – 72 hours. Cell proliferation measured by MTT assay was significantly reduced in malignant adenocarcinoma OE-33 cells treated with CORM3 at doses of 10 – 60 µM at each time point measured over 24, 48 and 72 hours as compared to equivalent treatment with iCORM. Gastric adenocarcinoma AGS cell growth was significantly reduced 24, 48 and 72 hours after treatment with CORM3 at doses of 20 and 40 µM when compared to equivalent treatment with iCORM. Morphological changes are demonstrated after 48 hours of CORM3 treatment with cells becoming more rounded and an increase in cellular debris. These findings represent a novel cancer-specific in vitro anti-proliferative effect of carbon monoxide on oesophagogastric cancer cells.

5.6.2 Protein expression after CORM and bilirubin treatment

Western blot analyses of protein expression in AGS cells show a marked up-regulation of HO-1 protein after 24 and 48 hours of treatment with active CORM3 but no HO-1 induction with equivalent iCORM treatment. There is a small reduction of HO-2 protein after 48h treatment with active CORM3 molecule.

There are no marked changes in HO-2 protein expression after 24 hours treatment with bilirubin and a slight reduction in HO-2 protein demonstrated after 48 hour treatment with bilirubin 20 µM. Expression of HO-1 protein was not induced by up to 48 hours of bilirubin treatment.
5.6.3 The effect of carbon monoxide on cellular apoptosis

Experiment cell samples treated with CORM3 molecule at 20 µM demonstrated a significantly increased affinity for annexin V binding after 24 hours as compared to those treated with equivalent inactive iCORM to indicate a process of early apoptosis. Over 48 – 72 hours there was further progression of CORM3-treated cells with significantly fewer viable cells and significantly increased late apoptotic or necrotic cells measurable over time as compared to iCORM-treated cells.

DNA fragmentation was detectable 48 hours after treatment with CORM3 with a significant increase in the proportion of apoptotic cells measured by Apo-BrdU assay as compared to both untreated control cells and those treated with inactive iCORM. Studies using JC-1 labelling demonstrate a significant loss of mitochondrial membrane potential after 48 hours in cells treated with CORM3 20 µM compared to equivalent treatment with iCORM. There is a significantly reduced proportion of viable cells measured at 48 hours in CORM3-treated cells compared to iCORM controls. These findings indicate that two hallmark features of apoptosis occur in AGS cell lines specifically after treatment with CORM3 20 µM for 48 hours.

Caspase activity assay results demonstrate a significant increase in fold caspase-3/7 induction after 48 hours exposure to active CORM3 as compared to inactive iCORM. Treatment with bilirubin produced a significant increase in caspase-3/7 activity after 48 hours as compared to vehicle control.

These findings provide collective supportive evidence for the concept of a process of apoptosis. Results indicate that the observed cell proliferation effects of both bilirubin and carbon monoxide are mediated by a process of apoptosis.

5.6.4 Heme Oxygenase Enzyme Activity Assay

Specific colorimetric assay demonstrates a decrease in total cellular HO enzyme activity after treatment with CORM3 molecule for 24 and 48 hours as compared to untreated control cells and cells treated with inactive iCORM. These results were consistent despite the induced expression of HO-1 protein in response to CORM3 treatment. This finding is important because it implies the inhibition of heme oxygenase activity by carbon
monoxide via direct or indirect mechanisms. Both bilirubin and CORM3 treatment reduce HO-2 protein expression at 48 hours but only CORM3 causes induction of HO-1. This suggests that carbon monoxide and bilirubin interact with specific and distinctive intracellular signalling pathways. Carbon monoxide inhibits the activity of HO enzyme: it may achieve this via a direct negative feedback mechanism and this action may apply to both constitutive HO-2 and inducible HO-1 isoforms. Bilirubin meanwhile also inhibits the HO system by causing reduced expression of HO-2 protein. The specific effects of bilirubin on HO-1 protein and on HO enzymatic activity require further investigation. In the studies performed within the scope of this thesis, induction of HO-1 enzyme did not occur in response to bilirubin treatment. The fact that HO-1 protein was induced by carbon monoxide suggests a more pivotal role played by CO in the regulation of the HO pathway. This regulation includes up-regulation of HO-1 protein and inhibition of total HO enzyme activity. One interesting question is whether or not the induction of HO-1 isoform occurs simply as a response to inhibition of HO enzymatic activity by carbon monoxide and the consequent build-up of heme substrate. Alternatively HO-1 induction could occur as a direct response to CO exposure.

The mechanisms and signalling pathways involved in the CO-mediated regulation of HO activity demand further study and are beyond the scope of this thesis.

5.6.5 The effect of treatment with carbon monoxide: Conclusions

The protective effects of HO activity may largely depend upon the activity of endogenously produced carbon monoxide (CO). Treatment with CO has been demonstrated to reverse or reduce the adverse effects of treatment with inhibitors of heme oxygenase enzyme. Induction of HO-1 enzyme and administration of CO have been shown to interfere with liver ischaemia and reperfusion injury and to improve transplant recipient and graft survival\textsuperscript{695 955}.

As discussed previously constitutive over-expression of HO-1 is a feature of various tumours including hepatocellular carcinoma (HCC)\textsuperscript{955}. The anti-apoptotic properties of HO enzyme are predominantly mediated by CO and confer a risk of tumour growth promotion when activity is increased in relevant tumours. Whereas the biliverdin and bilirubin products have direct capability to scavenge oxidants and prevent damage, the
CO product has additional unique activity in cell signalling pathways. The mechanisms and signal transduction pathways implicated in the anti-apoptotic and anti-inflammatory activity mediated by carbon monoxide include the mitogen-activated protein kinase cascade (MAPK), particularly the p38 protein MAP kinases, phosphatidylinositol 3-kinases (PI3-K) and NF-κB signalling pathways.\textsuperscript{201, 237, 249}

One of the most important effects of CO is its ability to influence cellular proliferation. CO blocks the proliferation of smooth muscle cells of hyperplastic intima following vessel trauma, as well as chronic vascular remodelling in transplant vascular stenosis or pulmonary hypertension.\textsuperscript{271, 732} CO has been demonstrated to inhibit apoptosis of endothelial and epithelial cells and to reduce proliferation of smooth muscle cells and fibroblasts via cell cycle arrest.\textsuperscript{775, 955} CO inhibits the proliferation of pancreatic stellate cells by activating p38 MAPK\textsuperscript{714} whereas CO produces a similar reduction in proliferation of human airway smooth muscle cells by inhibiting ERK activation.\textsuperscript{715}

CO-releasing compounds have been shown to be effective in delivering CO and to cause induction of HO-1 protein.\textsuperscript{670, 695} There is the distinct possibility that CO delivery can be achieved with negligible elevations in HbCO, as has been shown with CORM3.\textsuperscript{956} Compounds such as CORMA1 or CORM3 are pharmacologically stable, water soluble and exhibit protective effects. CORMs allow for the design of tissue-selective agents for disease-specific targeting; a clear advantage over inhaled CO. For example, control of CO in the stomach may well be modulated by a CORM sensitive to pH.\textsuperscript{672} CORMs have been demonstrated to mediate cytoprotection in cultured lung cells via activation of p38 MAPK.\textsuperscript{957}

Recent in vitro studies have observed that CORMs are capable of potent anti-proliferative effects in a model of pancreatic fibrosis with inhibition of pancreatic stellate cell proliferation. In vivo, CO confers tissue protection in animal models of pancreatic disease, including those with hyperglycaemia and inflammatory injury of the gland.\textsuperscript{816, 958} Recent studies with CORMS have demonstrated the ability of CO delivery to attenuate paracetamol-induced liver failure in mice.\textsuperscript{820} Studies of the therapeutic potential of carbon monoxide published to date have focused on the cytoprotective and antioxidant
properties of CO in the context of inflammatory conditions or acute bacterial infection.

The current study provides the first evidence for the benefit of CORM treatment that relates to a specific anti-cancer effect. This is an important novel finding that warrants further investigation to evaluate the possible benefits of CO administration in therapy regimens for oesophagogastric cancer.

The presence of transition metals may affect the overall activity of CO released by CORMs. However, it is also true that in most cases the effect of CORMs have been corroborated to be essentially mediated by CO through the use of proper negative controls (i.e. the inactive prepared CORM) such as those employed in the current studies.

CORM3 exhibits a rapid CO release (half-life < 1 min) and the anti-proliferative and HO enzyme effects observed after CORM3 treatment are likely to involve activation of downstream intracellular pathways that are activated by a relatively brief exposure to CO.

Cell treatment with carbon monoxide-releasing molecules (CORM3) produced consistent cancer-specific results with a significant reduction in the proliferation of malignant gastric AGS and oesophageal OE-33 adenocarcinoma cells and morphological changes after 48 hours of CORM3 treatment. These findings represent a novel cancer-specific in vitro anti-proliferative effect of carbon monoxide on oesophagogastric cancer cells. Results indicate that the observed cell proliferation effects of both bilirubin and carbon monoxide are mediated by a process of apoptosis with two hallmark features of apoptosis and increased caspase activity demonstrated in AGS cell lines after treatment with CORM3 20 µM for 48 hours.

CORM3 treatment caused a reduction in HO-2 protein expression after 48 hours with up-regulation of inducible HO-1 protein. Carbon monoxide exposure inhibited total HO enzymatic activity despite induced expression of HO-1 protein in response to CORM3 treatment. Both bilirubin and CORM3 treatment reduce HO-2 protein expression at 48 hours but only CORM3 causes induction of HO-1 suggesting that carbon monoxide and bilirubin interact with specific and distinctive signalling pathways to regulate HO enzyme.
activity. The specific mechanisms and signalling pathways involved in the CO-mediated regulation of HO activity require further investigation.

The cancer-specific effect of apoptosis induction by CORM3 treatment has important implications for potential clinical applications that demand further in vivo studies. The potential anti-cancer activity of CO therapy adds another aspect to the diverse therapeutic possibilities of CO described above. The development of CORMS and specific tissue delivery mechanisms are particularly relevant to the current findings. The environment of the stomach is characterized by a distinctive pH range and this fact could be exploited by design of a CORM agent whose release of CO is governed by a specific pH. In this way tissue-specific delivery of therapeutic CO could be achieved with the release of CO from orally-administered water-soluble CORMS only occurring in the acidic pH environment of the stomach.
5.7 The role of the heme oxygenase pathway: Summary

5.7.1 Thesis conclusions

The main conclusions from the studies performed for this thesis include the finding that the prevailing cellular redox state has an important influence on the in vitro cell biology of oesophagogastric cancer cells. Oesophageal adenocarcinoma cells appeared to thrive under conditions of oxidative stress and the exponential growth of gastric adenocarcinoma cells was not affected by oxidative stress simulated by treatment with hydrogen peroxide. Treatment with N-acetyl cysteine antioxidant, by contrast, had a cancer-specific effect with a significant reduction in cell proliferation and increased apoptosis in oesophageal and gastric cancer cells.

Constitutive over-expression of heme oxygenase-1 protein was not a feature of the oesophagogastric adenocarcinoma cell lines studied. Tonic activity of HO-2 enzyme was essential for the maintenance of in vitro exponential cell growth in the cell lines studied. Up-regulation of HO-1 isoform protein was readily induced by treatment with HO substrate hemin. Hemin caused a demonstrable increase in enzymatic activity of total HO enzyme that was not accompanied by any observed effects on cell proliferation. This means that any antioxidant activity mediated by an increase in HO enzymatic activity may not affect cell proliferation and may not be detrimental to continued exponential growth of cancer cells. Treatment with NAC antioxidant however caused a cancer-specific reduction in cell proliferation in these studies that was associated with a significant reduction in overall HO enzymatic activity. These findings suggest that N-acetyl cysteine antioxidant and HO-1 enzyme activate distinct and diverse signalling pathways involved in the oxidative stress response.

Although increased HO enzymatic activity mediated by HO-1 induction did not adversely affect cell growth there was a significant reduction in the proliferation of cells with an increase in apoptosis caused by a reduction in total HO enzymatic activity. Treatment with zinc protoporphyrin caused a clear reduction in total cellular HO activity despite up-regulation of inducible HO-1 protein. The findings indicate that heme oxygenase activity from either HO-1 or HO-2 isoforms is essential for the maintenance of growth in the oesophageal and gastric cell lines studied and that increasing the activity of HO-1 enzyme
in cancer cell phenotypes that do not constitutively over-express the protein is not unfavourable for continued tumour cell growth.

Notable findings from specific siRNA-mediated knockdown of HO-2 protein include a significant reduction in measurable total HO enzyme activity despite the up-regulation of HO-1 protein in response to knockdown of HO-2 mRNA. These experiments confirm published reports of the interaction between HO-1 and HO-2 isoforms and suggest an intrinsic regulation pathway to maintain a co-ordinated expression of heme oxygenase proteins and resulting enzymatic activity. These experiments represent a novel role for the HO-2 protein and its tonic activity in sustaining the growth of cancer cells in vitro. Furthermore the findings suggest that induced HO-1 protein may be sensitive to cellular feedback mechanisms or regulated by downstream enzyme products in the cell lines investigated.

Studies of possible downstream effectors of HO activity demonstrate that neither bilirubin nor carbon monoxide are beneficial to oesophagogastric cancer cells with reduced cell growth effects that are not reproduced by an increase in HO activity in the same cells. An increase in the activity of HO-1 enzyme does not lead to comparable reductions in cell proliferation and apoptosis induction as those caused by bilirubin or carbon monoxide treatment.

The current studies indicate that bilirubin plays a role in reducing oesophagogastric cancer cell growth. This role involves activation of cellular apoptosis most likely by time-dependent intracellular pathways that maintain an effect after washout of the bilirubin reagent. Addition of bilirubin after HO inhibition or HO-2 gene knockdown does not ameliorate the anti-proliferative effects of reduced HO enzymatic activity. Bilirubin appears to exert a negative-feedback activity on HO protein expression pathways with a demonstrable reduction in HO-2 protein expression without HO-1 induction 48 hours after bilirubin treatment. The effects of bilirubin treatment on overall total HO enzymatic activity are unknown. The anti-proliferative in vitro activity of relatively higher levels of bilirubin demonstrated in the current study is the first demonstration of these effects in oesophagogastric cancer cells.
Cell treatment with carbon monoxide-releasing molecules (CORM3) produced consistent and profound cancer-specific reductions in cell proliferation with increased apoptotic activity. CORM3 treatment inhibited total HO enzymatic activity with a reduction in HO-2 protein expression and despite up-regulation of HO-1 protein. The findings support future potential clinical applications of CO therapy that demand further experimental animal model and clinical studies wherever appropriate.

These studies of HO enzyme products suggest that carbon monoxide and bilirubin interact with specific and distinctive signalling pathways to regulate HO enzyme activity given that CO but not bilirubin caused up-regulation of HO-1 protein.

The findings challenge any assumptions that the recognised cellular consequences of HO activity such as cytoprotective and antioxidant effects are as a result of the downstream effectors bilirubin and CO. Both CO and bilirubin have clear anti-proliferative effects in cells with disordered growth, i.e. malignant oesophagogastric cancer cells.

These conclusions do not preclude the possibility that CO and bilirubin have potent and significant antioxidant and cytoprotective roles in the context of damaged healthy cells. Only in a situation of altered cell biology and disordered exponential growth do the effects of bilirubin and carbon monoxide result in induction of apoptosis and reduced proliferation. These effects could still be mediated via the same cell signalling pathways that in healthy cells have cytoprotective and antioxidant effects. This idea is supported by studies of N-acetyl cysteine treatment that also produce reduced proliferation in malignant cells that may be related to reduced HO activity and involve MAP kinase signalling pathways. The specific mechanisms and signalling pathways involved with NAC, bilirubin and CO treatment warrant further investigation that is beyond the scope of this thesis.

5.7.2 Limitations of the cultured cell line model

A widely accepted limitation of in vitro cell culture work is the fact that the cell phenotype may not accurately reflect that of the cellular phenotype in vivo. By the time cells are obtained from in vivo tumours, immortalised and made ready for commercial use, the phenotype of the cell lines may change to the extent that it is no longer an accurate
representation of the primary tumour from which it is derived. In addition, in vivo tumour cell growth is often influenced by surrounding tissue particularly the extracellular matrix that is not usually accounted for in cell culture models. Consequently, trends and effects demonstrable from in vitro studies require further validation from in vivo studies.

A further consideration is whether or not normal epithelial cell lines can reliably be used for comparative purposes. The normal cells are not genuinely resting cells but proliferate in response to serum stimulation when grown under recommended culture medium conditions.

These limitations notwithstanding, exponential growth was demonstrated in the malignant cell lines under basal conditions and not in the comparative normal epithelial cell line. The experiments produced statistically significant and reproducible proliferation effects that were specific to cancer cell lines in certain cases as described above. The cell culture experiments provide further important information about the mechanisms of growth effects with assays of apoptosis and the possible regulatory pathways involved. The cell line model is also a very useful methodology for investigating the effects of siRNA-mediated knockdown of HO-2 mRNA and novel information about the importance of HO-2 protein in cancer cell growth was gained from these studies.

HO-1 gene expression is readily induced by a variety of stimuli and it is recognised that simply describing a process of increased HO protein expression or demonstrating an increased level of HO protein does not directly equate to overall HO enzymatic activity. Previous studies have reported that increased HO-1 protein expression in Zucker rats did not result in increased total HO activity.

Specific colorimetric assays indicate the effects of variations in cellular heme oxygenase activity that provides additional valuable information about the role of the enzyme system in cancer cell proliferation.

5.7.3 Future work

The main findings that warrant further specific investigation include the cell signalling pathways involved in the proliferation effects observed after treatment with N-acetyl cysteine, bilirubin and carbon monoxide-releasing molecules. Further work is also
required to determine whether the cancer-specific cell proliferation effects observed in vitro can be reproduced in vivo.

The pathways of interest include the p21-activated kinases as part of the p53/p21 DNA damage-signalling pathway and the mitogen-activated protein kinase pathways, specifically the extracellular signal-related kinases (ERK1/2 MAP kinase pathway) and the p38 MAP kinase pathway. Further in vitro experiments are planned to elicit expression of p38 and ERK1/2 protein using western blotting and probing of blots with anti-phosphorylated and anti-total p38 and ERK1/2 antibodies (Cell Signaling Technology, Danvers, MA) as well as p21 expression using monoclonal mouse anti-p21 antibody (Santa Cruz, CA). Studies will aim to elucidate how activation of these pathways is associated with NAC activity and heme oxygenase enzyme activity with its downstream products. Co-treatment of cells with the specific p38 MAPK inhibitor SB203580 (Calbiochem, Bad Soden, Germany) is planned to determine whether the observed proliferation effects are mediated via p38 MAPK activity.

As mentioned above the novel findings in preliminary in vitro studies require further in vivo clarification. The experiments with the most relevant therapeutic potential are the cancer-specific effects observed with N-acetyl cysteine treatment and CORM3 treatment. In collaboration with colleagues at the Northwick Park Institute for Medical Research (NPIMR) further work is planned to investigate the therapeutic and adverse effects of CORM treatment in a xenograft rodent model of oesophagogastric cancer. This work will aim to answer outstanding questions about the optimal delivery methods of carbon monoxide from CORM constructs to facilitate therapeutic levels of CO to target tissues as well as to clarify whether cancer-specific anti-proliferative effects of CO can be reproduced in an animal model.
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APPENDICES

Appendix I: Cell seeding protocol

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<tr>
<th>PLATES</th>
<th>Wells</th>
<th>Growth Area (cm$^2$)</th>
<th>volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>SARSTEDT</td>
<td>24W</td>
<td>2.0</td>
<td>max 3.6mL</td>
</tr>
<tr>
<td></td>
<td>6W</td>
<td>9.5</td>
<td>max 17.2mL</td>
</tr>
<tr>
<td>NUNC</td>
<td>24W</td>
<td>1.9</td>
<td>1mL recommended</td>
</tr>
<tr>
<td></td>
<td>12W</td>
<td>3.5</td>
<td>2mL recommended</td>
</tr>
<tr>
<td></td>
<td>6W</td>
<td>9.6</td>
<td>3mL recommended</td>
</tr>
</tbody>
</table>

24W plates seed at 500,000 – 600,000 cells per plate = 25,000 cells per well AGS

6W plates seed at 750,000 cells per plate = 125,000 cells per well AGS

NUMBERS REFLECT THE DIFFERENTIAL SEEDING SUCCESS OF CELL LINES

OE-33 & HET-1A seeding for Flow Cytometry

<table>
<thead>
<tr>
<th>Wells</th>
<th>Growth Area (cm$^2$)</th>
<th>volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>6W</td>
<td>165,000 cells per well in 2.5mls</td>
<td>1 x 10$^6$ cells in 15mls</td>
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<tr>
<td>24W</td>
<td>33,000 cells per well in 0.5mls</td>
<td>800,000 cells in 12mls</td>
</tr>
<tr>
<td>96W</td>
<td>7000 cells per well in 100μl</td>
<td>672,000 cells in 9.6mls</td>
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</table>

OE-33 & HET-1A seeding for siRNA transfection

<table>
<thead>
<tr>
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<th>Growth Area (cm$^2$)</th>
<th>volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>6W</td>
<td>125,000 cells per well in 2.5mls</td>
<td>750,000 cells per plate in 15mls</td>
</tr>
<tr>
<td>24W</td>
<td>25,000 cells per well in 0.5mls</td>
<td>600,000 cells per plate in 12mls</td>
</tr>
<tr>
<td>96W</td>
<td>5,000 cells per well in 100μl</td>
<td>480,000 cells per plate in 9.6mls</td>
</tr>
</tbody>
</table>

AGS & HGC-27 seeding for Flow Cytometry

<table>
<thead>
<tr>
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<th>Growth Area (cm$^2$)</th>
<th>volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>6W</td>
<td>125,000 cells per well in 2.5mls</td>
<td>750,000 cells per plate in 15mls</td>
</tr>
<tr>
<td>24W</td>
<td>25,000 cells per well in 0.5mls</td>
<td>600,000 cells per plate in 12mls</td>
</tr>
<tr>
<td>96W</td>
<td>5000 cells per well in 100μl</td>
<td>480,000 cells per plate in 9.6mls</td>
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</table>

AGS & HGC-27 seeding for siRNA transfection

<table>
<thead>
<tr>
<th>Wells</th>
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</thead>
<tbody>
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<tr>
<td>24W</td>
<td>15,000 cells per well in 0.5mls</td>
<td>360,000 cells per plate in 12mls</td>
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<tr>
<td>96W</td>
<td>3,000 cells per well in 100μl</td>
<td>288,000 cells per plate in 9.6mls</td>
</tr>
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</table>
Appendix II: Assessment of cell proliferation

Figure II.1a: 24-hour cell counts from HGC-27 cell seeding

![24-hour cell counts HGC-27 cells](image)

Pearson correlation: 0.992145
Sig. (2-tailed): 1.2 x 10^{-6}

Figure II.1b: MTT assay for HGC-27 cells seeded in 24-well plate

![HGC-27 MTT Assay 24 hrs](image)

Pearson correlation: 0.966893
Sig. (2-tailed): 8.85 x 10^{-5}

Figure II.1: HGC-27 cells were seeded into 24-well plates at known density per well. Cell counts & MTT were performed at 24h. Results demonstrate a close correlation between cell counting using Trypan blue exclusion and MTT assay to assess cell proliferation.
Figure II.2a: 24-hour cell counts from AGS cell seeding

Pearson correlation 0.947167
Sig. (2-tailed) 0.00354

Figure II.2b: MTT assay for AGS cells seeded in 24-well plate

Pearson correlation 0.988064
Sig. (2-tailed) 4.21 x10^{-6}

Figure II.2c: MTT assay for AGS cells seeded in 96-well plate

Pearson correlation 0.969
Sig. (2-tailed) 7.0 x10^{-5}
Figure II.2: AGS cells were seeded into 24 and 96-well plates at known density per well. Cell counts & MTT were performed at 24h. Results demonstrate a close correlation between cell counting using Trypan blue exclusion and MTT assay to assess cell proliferation. Standard curves were constructed to enable estimation of cell number using MTT data.

\[ y = -2E-10x^2 + 2E-05x + 0.0372 \]
Appendix III: Growth curves for all cell lines studied

Figure III.1a: Viable cell counts time course AGS cells

Figure III.1b: MTT assay time course AGS cells: Growth curve

Figure III.1c: AGS MTT assay: Growth curve

Figure III.1: AGS growth curves using MTT and cell counts to assess cell proliferation. Cells were seeded into 24-well plates and maintained in serum-complete Ham F12 medium under humidified 5% CO₂ at 37°C. Results demonstrate exponential growth of AGS cells under recommended culture conditions.
Figure III.2: HGC-27 growth curves using MTT and cell counts to assess cell proliferation. Cells were seeded into 24-well plates and maintained in serum-complete DMEM medium under humidified 5% CO₂ at 37°C. Results demonstrate exponential growth of HGC-27 cells under recommended culture conditions.
Figure III.3a: MTT standard curve OE-33 cells 24 hours

![MTT Standard Curve 24 hrs](image)

\[ y = -6 \times 10^{-11}x^2 + 1 \times 10^{-05}x + 0.0354 \]

Figure III.3b: MTT assay time course OE-33 cells: Growth Curve

![OE-33 cell proliferation MTT assay](image)

Figure III.3c: OE-33 MTT assay: Growth Curve

![OE-33 cells growth curve](image)

Figure III.3: OE-33 growth curves using MTT to assess cell proliferation. Cells were seeded into 24-well plates and maintained in serum-complete RPMI medium under humidified 5% CO\(_2\) at 37°C. Results demonstrate exponential growth of OE-33 cells under recommended culture conditions.
Figure III.4a: MTT assay time course HET-1A cells

Figure III.4b: Growth Curve HET-1A cells

Figure III.4: HET-1A growth curves using MTT to assess cell proliferation. Cells were seeded into 24-well plates and maintained in supplemented BEGM medium under humidified 5% CO₂ at 37°C. Results demonstrate exponential growth of HET-1A cells under recommended culture conditions.
Figure III.5: All cells different media Growth at 36 hours

![MTT Absorbance vs Cell Line](image)

Figure III.5: Cell growth using MTT to assess cell proliferation at 36h. Cells were seeded into 24-well plates and maintained in various culture media under humidified 5% CO₂ at 37°C. Results demonstrate significantly increased exponential growth of malignant cells under recommended culture conditions compared to HET-1A cell growth. Significantly increased* cell growth in malignant cell lines compared to benign oesophageal epithelial cell line HET-1A in all media studied: HET-1A compared to OE-33 $p = 0.000174$; HET-1A compared to AGS cells $p = 3.23 \times 10^{-5}$; HET-1A compared to HGC-27 cells $p = 3.23 \times 10^{-5}$.

*Mann Whitney U test

Assessment of cell proliferation

To evaluate the relationship between MTT optical density and number of viable cells, cell seeding experiments were performed with all cell lines seeded in 24-well and 96-well plates at a specific number. Cell counts using trypan blue exclusion and MTT assay were performed at 24, 48 and 72 hours. Experiment results demonstrate that both the cell counts and the MTT assay results correlated well with the original cell seeding number. The results support the MTT and cell counting technique as a valid measure of cell proliferation in cell lines studied for cells seeded in a 24-well or a 96-well plate.

To investigate cell proliferation of the four available cell lines, cells were seeded at the same number and grown under conditions recommended by the cell suppliers. Growth curves obtained for each cell line provide a comparison of growth patterns for each of the cell lines under investigation. Exponential cell growth was demonstrated up to 72 hours with significantly higher proliferation in malignant cell lines as compared to benign. Cell seeding protocols for all experiments were devised according to the differential growth pattern and the success of cell types in adhering to the growth surface in monolayer at a particular seeding density. This approach provided the optimal seeding density for each individual experiment, e.g. seeding OE-33 and HET-1A cells at 33,000 cells per well in a 24-well plate and seeding AGS and HGC-27 cells at 25,000 cells per well in a 24-well plate produced the same degree of cell confluence after 24 hours for the addition of experimental reagents.
Appendix IV: WESTERN BLOTS: Additional representative protein blots

Figure IV.1 Representative Blots: AGS cells anti-HO-1 blots
Figure IV.2 Representative Blots: AGS cells anti-HO-2 blots
Figure IV.3 Representative Blots: AGS cells anti-Tubulin blots

Figure IV.4 WESTERN BLOTS: Additional Protein Blots

OE-33 H$_2$O$_2$ and NAC protein expression

M   Con   2     10    20    50    1     5     10     20

H$_2$O$_2$ µM   NAC mM
Appendix V: Annexin V & Propidium Iodide staining – controls to set gating and quadrant regions

Figure V.1: Unstained cells control – to set gating and quadrant regions

![Graph showing unstained cells control]

<table>
<thead>
<tr>
<th>Region</th>
<th>R3</th>
<th>R4</th>
<th>R5</th>
<th>R6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Count</td>
<td>403</td>
<td>14</td>
<td>24233</td>
<td>4</td>
</tr>
<tr>
<td>% Histogram</td>
<td>1.63</td>
<td>0.06</td>
<td>98.29</td>
<td>0.02</td>
</tr>
<tr>
<td>Median X,Y</td>
<td>1.07, 16.73</td>
<td>27.74, 20.04</td>
<td>2.38, 1.29</td>
<td>26.76, 10.46</td>
</tr>
</tbody>
</table>

Figure V.2: Control cells Ethanol treated – PI positive control staining

![Graph showing control cells Ethanol treated]

<table>
<thead>
<tr>
<th>Region</th>
<th>R3</th>
<th>R4</th>
<th>R5</th>
<th>R6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Count</td>
<td>10201</td>
<td>3</td>
<td>1913</td>
<td>2</td>
</tr>
<tr>
<td>% Histogram</td>
<td>84.17</td>
<td>0.02</td>
<td>15.79</td>
<td>0.02</td>
</tr>
<tr>
<td>Median X,Y</td>
<td>1.49, 35.72</td>
<td>42.79, 17.35</td>
<td>1, 5.66</td>
<td>24.89, 10.09</td>
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</table>
Figure V.3: Control cells Staurosporine treated – Annexin V positive control

<table>
<thead>
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<th>R4</th>
<th>R5</th>
<th>R6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Count</td>
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<td>21</td>
<td>2419</td>
<td>1470</td>
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<tr>
<td>% Histogram</td>
<td>0</td>
<td>0.54</td>
<td>61.87</td>
<td>37.6</td>
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<tr>
<td>Median X,Y</td>
<td>0, 0</td>
<td>85, 16.14</td>
<td>9.39, 1</td>
<td>53.15, 3.06</td>
</tr>
</tbody>
</table>

Figure V.1 – V.3: AGS control cells were seeded into 6-well plates, samples harvested at 24 – 72h and prepared for staining with Annexin V-FITC and PI for flow cytometry analysis. Controls were prepared to set gating parameters with unstained cells, ethanol-treated cells to permeabilise the cell membrane for PI staining and with staurosporine-treated cells to induce apoptosis for annexin V positive control staining. Staurosporine-treated cells demonstrate a significant increase in the proportion of cells counted in the apoptotic phase (R6) as compared to untreated control cells.
Appendix VI: ApoBrdU DNA Fragmentation assay – controls to set gating and quadrant regions

Figure VI.1a: Positive control cells Apo-BrdU assay NAC experiments

<table>
<thead>
<tr>
<th>Region</th>
<th>Cell Count</th>
<th>% Histogram</th>
<th>Median X</th>
<th>Median Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>R8</td>
<td>3612</td>
<td>33.84</td>
<td>81</td>
<td>98.21</td>
</tr>
<tr>
<td>R9</td>
<td>6858</td>
<td>64.25</td>
<td>52</td>
<td>7.56</td>
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<tr>
<td>Total</td>
<td>10674</td>
<td>100</td>
<td>55</td>
<td>8.73</td>
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</table>

Figure VI.1b: Negative control cells Apo-BrdU assay NAC experiments

<table>
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<tr>
<th>Region</th>
<th>Cell Count</th>
<th>% Histogram</th>
<th>Median X</th>
<th>Median Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>R8</td>
<td>103</td>
<td>0.82</td>
<td>64</td>
<td>101.82</td>
</tr>
<tr>
<td>R9</td>
<td>12419</td>
<td>98.85</td>
<td>58</td>
<td>7.29</td>
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<tr>
<td>Total</td>
<td>12563</td>
<td>100</td>
<td>58</td>
<td>7.29</td>
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</tbody>
</table>

445
Figure VI.2a: Positive control cells Apo-BrdU assay siRNA experiments

<table>
<thead>
<tr>
<th>Region</th>
<th>Cell Count</th>
<th>% Histogram</th>
<th>Median X</th>
<th>Median Y</th>
</tr>
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<tbody>
<tr>
<td>R8</td>
<td>2860</td>
<td>34.06</td>
<td>176</td>
<td>172.83</td>
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<tr>
<td>R9</td>
<td>5329</td>
<td>63.46</td>
<td>136</td>
<td>19.33</td>
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<tr>
<td>Total</td>
<td>8398</td>
<td>100</td>
<td>142</td>
<td>21.16</td>
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Figure VI.2b: Negative control cells Apo-BrdU assay siRNA experiments

<table>
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<th>% Histogram</th>
<th>Median X</th>
<th>Median Y</th>
</tr>
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<tbody>
<tr>
<td>R8</td>
<td>112</td>
<td>1.03</td>
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<tr>
<td>R9</td>
<td>10353</td>
<td>95.49</td>
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<td>8.22</td>
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<td>Total</td>
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<td>8.22</td>
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Figure VI.3a: Negative control cells Apo-BrdU assay Bilirubin & CORM experiments

<table>
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<th>Region</th>
<th>Cell Count</th>
<th>% Histogram</th>
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<th>Median Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>R8</td>
<td>98</td>
<td>0.84</td>
<td>64</td>
<td>101.82</td>
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<tr>
<td>R9</td>
<td>11617</td>
<td>99</td>
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<td>7.29</td>
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<td>Total</td>
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<td>100</td>
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<td>7.29</td>
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Figure VI.3b: Positive control cells Apo-BrdU assay Bilirubin & CORM experiments

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<th>Cell Count</th>
<th>% Histogram</th>
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<th>Median Y</th>
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</thead>
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<tr>
<td>R8</td>
<td>2225</td>
<td>35.81</td>
<td>79</td>
<td>101.82</td>
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<tr>
<td>R9</td>
<td>3903</td>
<td>62.81</td>
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<td>7.56</td>
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<td>Total</td>
<td>6214</td>
<td>100</td>
<td>55</td>
<td>8.73</td>
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Figure VI.3c: Untreated control cells Apo-BrdU assay 36 hours Bilirubin experiments

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<th>Median Y</th>
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</thead>
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<tr>
<td>R8</td>
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<tr>
<td>R9</td>
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<tr>
<td>Total</td>
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<td>82</td>
<td>21.54</td>
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Figure VI.3d: Untreated control cells sample Apo-BrdU assay 48 hours CORM experiments

<table>
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<tr>
<td>R9</td>
<td>4903</td>
<td>97.55</td>
<td>103</td>
<td>8.12</td>
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<tr>
<td>Total</td>
<td>5026</td>
<td>100</td>
<td>103</td>
<td>8.12</td>
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</tbody>
</table>

Figures VI.1 – VI.3: Incorporation of brominated deoxyuridine triphosphate nucleotides into DNA strand breaks was measured by the Apo-BrdU assay to assess DNA fragmentation. Positive and negative control cells provided with the Apo-BrdU assay kit were used to set gating regions and reliably identify positive and negative-stained cells for each assay performed as described above. Positive control sample cells demonstrated DNA fragmentation with increased FITC fluorescence compared to negative control cells and untreated controls.
Appendix VII: PCR Products – additional results

Figure VII.1: PCR products siRNA knockdown AGS cells additional samples

Experiments were repeated and PCR products evaluated using agarose gel electrophoresis for both AGS and HGC-27 cell lines. Although analysis demonstrated effective knockdown of HO-2 mRNA after transfecting cells with siRNA duplexes there was inconsistent findings with PCR products using GAPDH primers (Figures VII.1 & VII.2). These results suggested a compromise in RNA quality with possible DNA contamination in the eluted RNA samples. Treatment with Turbo DNase enzyme was repeated and any samples with visible DNA contamination were discarded along with any corresponding complementary DNA stored (Figures VII.3 & VII.4).

Representative blots of ethidium bromide gel electrophoresis for PCR products are shown in Figures VII.5 – VII.6.
Figure VII.2: PCR products siRNA knockdown HMOX2 and GAPDH HGC-27 cells

Lanes
1 = NUC 10nM 24hrs
2 = NUC 10nM 48hrs
3 = NUC 10nM 72hrs
4 = siRNA 02 10nM 24hrs
5 = siRNA 02 10nM 48hrs
6 = siRNA 02 10nM 72hrs
7 = Lipofectamine 24hrs
8 = cDNA negative control
9 = PCR negative control
Figure VII.3: RNA samples AGS cell experiments DNase treated

Lanes
1 = NUC 10nM 24hrs 4 = NUC 10nM 48hrs 7 = NUC 10nM 72hrs
2 = siRNA 01 10nM 24hrs 5 = siRNA 01 10nM 48hrs 8 = siRNA 01 10nM 72hrs
3 = siRNA 02 10nM 24hrs 6 = siRNA 02 10nM 48hrs 9 = siRNA 02 10nM 72hrs
10 = siRNA 03 10nM 24hrs 11 = siRNA 03 10nM 48hrs 12 = siRNA 03 10nM 72hrs
13 = NUC 10nM 72hrs 14 = Lipofectamine 24hrs 15 = Lipofectamine 72hrs

Figure VII.4: RNA samples HGC-27 and AGS cell experiments DNase treated

Lanes
1 = NUC 10nM 24hrs 2 = NUC 10nM 48hrs 3 = NUC 10nM 72hrs
4 = siRNA 02 10nM 24hrs 5 = siRNA 02 10nM 48hrs 6 = siRNA 02 10nM 72hrs
7 = Lipofectamine 24hrs 8 = NUC 10nM 24hrs 9 = NUC 10nM 48hrs
10 = NUC 10nM 72hrs 11 = siRNA 02 10nM 24hrs 12 = siRNA 02 10nM 48hrs
13 = siRNA 02 10nM 72hrs 14 = AGS cells Lipofectamine 48hrs 15 = AGS cells NUC 10nM 48hrs
Representative Gels PCR Products

Figure VII.5: RNA samples AGS cell experiments HO-2 knockdown 01
Figure VII.5: RNA samples AGS cell experiments HO-2 knockdown 02
Appendix VIII: Cell Growth after siRNA-mediated HO-2 knockdown – cell counts

Figure VIII.1: AGS cell counts after HO-2 knockdown

![AGS cell counts HO-2 siRNA](image)

†p < 0.05 Independent Samples Kruskal-Wallis test cf. NUC

Figure VIII.2: HGC-27 cell counts after HO-2 knockdown

![HGC-27 cell counts HO-2 siRNA](image)

†p < 0.004 Independent Samples Kruskal-Wallis test cf. NUC

Summary of 3 experiments both AGS and HGC-27 cell lines
$p = 0.406$ Independent Samples Kruskal-Wallis test

Summary of 2 experiments OE-33 cell line

Cell counts performed for siRNA experiments demonstrated significantly fewer live AGS cells counted at 24, 48 and 72 hours from wells treated with siRNA 10 nM as compared to those transfected with NUC 10 nM ($p < 0.05$). In HGC-27 experiments there were significant reductions in cell counts with HO-2 gene knockdown at 48 and 72 hours when compared to negative universal control ($p < 0.004$). In two cell counts performed from OE-33 cell experiments, there was a marked reduction in live cells treated with siRNA at 48 hours and 72 hours. OE-33 siRNA experiment cell count data are insufficient for statistical significance. HO-2 knockdown in AGS, HGC-27 and OE-33 cells produced effects on cell counts that are more dramatic than those seen with MTT. The discrepancy between cell count numbers and the equivalent MTT results is likely to be explained by the technical aspects and relatively cumbersome nature of cell counting with the major factor being fewer experiments performed leading to greater variability and an increased likelihood of observer error.