Pharmacological Characterisation of Novel Furoxans and Furoxan-Aspirin Hybrid Drugs in Blood Vessels, Inflammatory Cells and Platelets

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Declaration

I hereby declare that the data published in this thesis are the result of my own work, carried out under the supervision of Profs Ian L. Megson and Adriano G. Rossi at The University of Edinburgh, and that this thesis has been completed entirely by myself and has not been submitted for any other degree or qualification.

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Abstract

Aspirin has long been used as a prophylactic against thrombotic coronary events, acting through inhibition of cyclooxygenase (COX)-1 mediated platelet aggregation. Unfortunately, gastrointestinal disorders are a common side-effect of aspirin that limit its use.

A novel range of hybrid drugs in which a furoxan nitric oxide (NO)-donating moiety is joined by ester linkage to the aspirin molecule were developed in the hope that the cytoprotective actions of drug-derived NO would overcome the gastric side-effects of aspirin. In addition, the presence of the NO group instils the potential for them to have additional cardiovascular benefit over the parent compound. These effects are investigated in this thesis and compared to that of an established nitrooxy-ester derivative of aspirin (NCX4016).

A novel electron paramagnetic resonance assay was developed in order to investigate whether the esterification had impacted on the ability of the compounds to inhibit COX-1. Results indicated that the furoxan-aspirin hybrids and their NO-free furazan counterparts all retained an aspirin-like action *in vitro*.

The NO-mediated effects in the vasculature were investigated via their capability to cause relaxation of phenylephrine-constricted isolated rat aortic rings. The furoxan hybrids were found to be powerful vasodilators and the effect was shown to be NO-mediated on account of the lack of effect with their NO-free equivalents and a susceptibility to the guanylate cyclase inhibitor, ODQ. Myography also revealed the phenomenal potency of the stand-alone furoxan compound B13, which was

demonstrated to cause vasorelaxation in the picomolar range – approximate 300-fold more potent than an established powerful NO donor, DEA/NO.

Antiplatelet studies in both platelet rich plasma (PRP) and washed platelets (WP) revealed the furoxan-aspirin hybrids to inhibit platelet aggregation in both WP and PRP. The effects were attenuated in PRP, likely due to the acetyl instability in plasma. The comparatively weak effect of the NO-free counterparts, along with the susceptibility to ODQ, is indicative of the dominant role of NO over aspirin in this arena. However, in contrast to NCX4016, an aspirin-like action is retained, especially in WP.

Electrochemical detection of NO release revealed the compounds to be stable in solution and to only release significant amounts of NO in response to intracellular concentrations of antioxidant elements glutathione and ascorbate, raising a potential for primarily intracellular delivery of NO.

Finally, the capability of the compounds to inhibit release of pro-inflammatory cytokines from monocytes and monocyte-derived macrophages was investigated. Following LPS stimulation, cells treated with furoxan-aspirin hybrid, B8, displayed reduced TNFα release. Immunofluorescence studies suggested that the mechanism behind the reduction in TNFα release involved inhibiting the translocation of its transcription factor, NF-κB, to the nucleus.

Taken together these studies reveal that both elements of the furoxan-aspirin hybrids are active *in vitro*. Whilst chemical modification is likely required to improve

balance of action between the two hybrid elements, drugs of this class may become promising antithrombotic therapies.

Publications and Presentations

Turnbull, C.M., Cena, C., Fruttero, R., Gasco, A., Rossi, A.G. Megson, I.L.

'Mechanism of action of novel NO-releasing furoxan derivatives of aspirin in human platelets' (2006). Brit J Pharmacol, Jun;148(4):517-26.

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'A Novel Electron Paramagnetic Resonance-Based Assay for Prostaglandin H Synthase-1 Activity' (2006). J. Inflamm. 3:12 (28 September).

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'A novel hybrid aspirin-nitric oxide donor drug inhibits TNFα-release from LPS-activated human macrophages *in vitro*' (2005) Proceedings of the British Pharmacological Society (pA_2 Online). Published Abstract.

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6th UK NO Forum, British Pharmacological Society 2005 Winter Meeting (19th December 2005), Institute of Education, UCL, London, UK. Poster presentation.

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'Mechanism of Action and Potential Benefits of Novel Nitroaspirin Drugs in Platelets and Inflammatory cells.'

Abbreviations and Drug Names

ABBREVIATION	DEFINITION
AA	Arachidonic acid
ACh	Acetylcholine
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
AUC	Area under curve
ASA	Aspirin
ATL	Aspirin-triggered lipoxin
ADP	Adenosine triphosphate
B7	3-carbamoylfuroxan-4-yl)methyl 2-acetoxybenzoate
B8	3-cyanofuroxan-4-yl)methyl 2-acetoxybenzoate
B12	4-(ethanoyloxymethyl)-3-isocyano-1,2,5-oxadiazole 2-oxide
B13	3-carbamoyl-4-(ethanoyloxymethyl)-1,2,5-oxadiazole 2-oxide
B15	4-carbamoylfurazan-3-yl) methyl 2-acetoxybenzoate
B16	4-cyanofurazan-3-yl)methyl 2-acetoxybenzoate
BH_4	Tetrahydrobiopterin
CAM	Cell adhesion molecule
cAMP	Cyclic adenosine monophosphate
CBA	Cytometric bead array
cGMP	3', 5'- cyclic guanosine monophoshate
COX (-1, -2)	Cyclooxygenase (-1, -2)
CP	3-carboxy-proxyl
CPH	1-Hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine .HCl
cPTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
DEA/NO	1,1-diethyl-2-hydroxy-2-nitrosohydrazine
DMSO	Dimethyl sulfoxide
dH2O	Deionised water
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
EC_{50}	Drug concentration required for obtaining 50 % of the maximum effect
EC_{80}	Drug concentration required for obtaining 80 % of the maximum effect
ECM	Extracellular matrix
EDHF	Endothelium-derived hyperpolarisation factor
EDRF	Endothelium-derived relaxation factor
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
EPR	Electron paramagnetic resonance
ERK	Extracellular signal-regulated kinases
FAD	Flavin adenine dinucleotide
FITC	Fluorescein isothiocyanate
FMN	Flavin mononucleotide
GEA 3162	1,2,3,4-oxatriazolium, 5-amino-3-(3,4-dichlorophenyl)-chloride
GSH	Glutathione
GTN	Glyceryl trinitrate
GTP	Guanosine-5'-triphosphate
HBSS	Hank's buffered salt solution
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
5-HPETE	5-hydroperoxyicosatetraenoic acid
IC_{50}	The half maximal inhibitory concentration
ICAM	Intercellular adhesion molecule
IKK	IκB-kinase
IgG	Immunoglobulin G
IL-(1,6,8,10,12,12p70)	Interleukin-(1,6,8,10,12,12p70)

iNOS	Inducible nitric oxide synthase
ISCOVES DNEM	Iscove's Dulbecco´s Modified Eagle´s Medium
ISDN	Isosorbide dinitrate
LDH	Lactate dehydrogenase
L-NAME	N^G-nitro-L-arginine methyl ester
L-NMMA	L-arginine; N^G-methyl-L-arginine
L-NNA	L-nitroarginine
(5, 12, 15)-LO	(5, 12, 15)-Lipoxygenase
LPS	Lipopolysaccharide
LT(A_4,B_4,C_4,D_4,E_4)	Leukotriene (A_4,B_4,C_4,D_4,E_4)
LX	Lipoxin
MI	Myocardial infarction
NADPH	nicotinamide adenine dinucleotide phosphate
NANC	Non-adrenergic non-cholinergic (neurone)
NCX4016	2 acetoxy-benzoate 2-(2-nitroxymethyl)-phenyl ester
NCX4215	2-acetoxybenzoate 2-(2-nitroxy)-butyl ester
NF-κB	Nuclear factor kappa B
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NS398	N-[2-(cyclohexyloxy)- 4-nitrophenyl]-methanesulfonamide
NSAID	Non-steroidal antiinflammatory drug
ODQ	1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one
ONOO-	Peroxynitrite
oxyHb	Oxy-haemoglobin
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PGHS	Prostaglandin H synthase (COX)
PGI_2	Prostaglandin I_2 / prostacyclin
PG (G_2, H_2)	Prostaglandin (G_2, H_2)
PPP	Platelet poor plasma
PRP	Platelet rich plasma
RT	Room temperature
SA	Salicylic acid
SC-560	5-(4- chlorophenyl)-1-(4-methoxyphenyl)-3- trifluoromethylpyrazole
s.e.m	Standard error of mean
SERCA	Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase
SIN-1	3-morpholinosydnonomine
sGC	Soluble guanylate cyclase
SOD	Superoxide dismutase
TNFα	Tumour necrosis factor α
TX (A_2, B_2)	Thromboxane (A_2, B_2)
VCAM	Vascular cell adhesion molecule
vWF	von Willebrand factor
WP	Washed platelets

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CHAPTER ONE

General Introduction

1. General Introduction

1.1 Introduction

Cardiovascular disease is the leading cause of death in the world (Murray & Lopez, 1997). The most common clinical manifestations of the disease are myocardial infarction (MI) and angina. Common prophylactic treatments for MI and angina include the antithrombotic drug, aspirin, and the nitric oxide (NO)-yielding vasodilator compounds, glyceryl trinitrate (GTN) and isosorbide dinitrate (ISDN).

This study investigates a set of compounds that combine a novel NO-containing moiety with aspirin and, therefore, have the potential to provide a multi-faceted approach to treating heart disease.

1.2 Platelet Function

1.2.1 Platelets

Platelets are small, discoid cell fragments that are heavily involved in haemostasis. They originate from megakaryocytes in the bone marrow, but circulate in the blood. Platelets are anuclear and lack replicative machinery. They do, however, possess ribosomes and mRNA and so can synthesise some proteins (Bugert *et al.*, 2003; Lindemann *et al.*, 2001). In the resting state, platelets are approximately 2 μm in size, however, once activated they rapidly adopt a larger irregular shape characterized by protruding filopodia (Winokur & Hartwig, 1995).

1.2.2 Activation Mechanisms and Consequences

Platelets become activated in response to factors such as thrombin or adenosine diphosphate (ADP), but also by exposure to collagen and von Willebrand factor (vWF) following damage to the blood vessel endothelium. Once activated, platelets release a number of factors involved in further activation and recruitment of platelets, as well as activation of the coagulation cascade. Platelets adhere to the vessel wall and to each other to form a haemostatic plug.

Initial weak interaction of platelets with collagen of the vessel wall occurs via vWF. It binds collagen to platelet GPIb-V-IX complex (Alevriadou *et al.*, 1993; Ruggeri, 1997; Savage *et al.*, 1996). A more stable interaction follows, binding the platelets directly to collagen via GPVI collagen receptors (Moroi *et al.*, 1996; Saelman *et al.*, 1994). Thrombus growth is associated with binding of platelet fibrinogen to glycoprotein (GP) IIb/IIIa receptors which are expressed by activated platelets. These receptors bind fibrinogen and thus link adjacent platelets, generating an aggregate. At this stage, NO and prostacyclin (PGI_2) are also released by the endothelial cells and by activated platelets in order to act as negative feedback mechanisms for the thrombus formation (Coughlin *et al.*, 1980; Freedman & Loscalzo, 2003).

1.2.3 Endogenous Inhibitory Mechanisms

A number of endogenous inhibitory mechanisms modulate platelet function and can act in a synergistic manner to prevent pathologic thrombus formation. These endogenous factors are produced by the endothelium and include NO and PGI_2.

NO, acting via its second messenger, cyclic guanosine monophosphate (cGMP), inhibits both platelet adhesion and aggregation, through mechanisms discussed further in section 1.4.4.1. Depression of NO in a dog model of endothelial injury was demonstrated to increase thrombus formation, whereas an infusion of L-arginine delayed it (Yao *et al.*, 1992). NO also controls thrombus formation in the cerebrovascular and renal systems (Shultz & Raij, 1992; Stagliano *et al.*, 1997).

PGI_2 acts to prevent platelet cytosolic calcium increase, platelet ATP secretion and aggregation, but, unlike NO, PGI_2 is poor at preventing adhesion (Doni *et al.*, 1988; Krishnamurthi *et al.*, 1984; Radomski *et al.*, 1987d). PGI_2 is formed by the action of the enzymes cycloooxygenase (COX, section 1.5.2) and prostacyclin synthase in endothelial cells. Shear stress increases PGI_2 production and upregulates COX mRNA transcription (Frangos *et al.*, 1985; Okahara *et al.*, 1998). PGI_2 acts locally (on platelets and smooth muscle cells), but is rapidly converted to the inactive metabolite, $PGF_{1\alpha}$. PGI_2 is a powerful inhibitor of platelet aggregation and the antiaggregatory effect of PGI_2, together with the antiplatelet effects of NO, is important in the regulation of platelet-vessel wall interactions (Radomski *et al.*, 1987a; Radomski *et al.*, 1987c). Further evidence for the involvement of PGI_2 in protection against thrombosis came with the development of IP-receptor knockout mice. Inhibition of platelet aggregation is not seen in these animals when a stable PGI_2 analogue is administered (Murata *et al.*, 1997). PGI_2 is utilised experimentally in the preparation of washed platelets, where it transiently prevents activation and prolongs viability of the platelets (Blackwell *et al.*, 1982; Read *et al.*, 1985).

1.2.4 Current Pharmacological Interventions

Several antithrombotic treatments are available for use in patients at risk of stroke or MI. Of these, the most commonly used today is aspirin (discussed further in section 1.5.2.2). Unfortunately, the use of aspirin is limited by its gastrotoxic side effects and consequently alternative antithrombotic therapies are still being developed.

Clopidogrel acts to irreversibly block the adenosine diphosphate (ADP) receptor (P_2Y_{12}) on platelet cell membranes. The P_2Y_{12} receptor is responsible for the completion and amplification of the response to ADP and to all platelet agonists, including thromboxane A_2 (TXA_2), thrombin, and collagen (Storey, 2001). Clopidogrel is a powerful antithrombotic agent (Uchiyama *et al.*, 1992). The CAPRIE study (1996) demonstrated that clopidogrel had superior clinical benefit over aspirin for secondary prevention of atherothrombotic disease, with reduced risk of MI and stroke (CAPRIE, 1996). Furthermore, its use in combination with aspirin gives synergistic inhibitory effects (Moshfegh *et al.*, 2000). However, a recent study showed evidence of a relationship between the use of clopidogrel and surgical bleeding among patients undergoing coronary artery surgery (Kapetanakis *et al.*, 2006). There is also a recently ignited debate over whether or not clopidogrel resistance can develop, a factor which may limit its future use (Morel *et al.*, 2005).

Glycoprotein (GP)IIb/IIIa inhibitors (e.g. abciximab and tirofiban) bind to GPIIb/IIIa receptors exposed on the surface of activated platelets, thus preventing aggregate formation. Drugs of this class administered percutaneously have been shown to be safe and beneficial in preventing acute coronary syndromes (Bhatt & Topol, 2000; Kong *et al.*, 1998; White, 1999). However, studies have demonstrated increased

mortality following oral delivery of GPIIb/IIIa antagonists (Chew *et al.*, 2001). There is also a major risk of bleeding associated with use of GPIIb/IIIa receptor inhibitors in some patients (Sitges & Villa, 1997; SoRelle, 2001; Trivedi *et al.*, 2002).

1.3. Inflammation

Atherosclerosis is a major cardiovascular disease of the western world, characterised by plaque build-up in arterial walls. It is now generally considered to be a chronic inflammatory disease and so drugs with anti-inflammatory action may bring about beneficial effects (Shaw *et al.*, 2006; Shaw *et al.*, 2005; Stoll & Bendszus, 2006).

1.3.1 Inflammatory Cells in Atherosclerosis

The first evidence for the involvement of inflammatory cells in the process of atherosclerosis came from the observation of infiltrating T-cells in atherosclerotic plaque tissue (Stemme *et al.*, 1995; Zhou *et al.*, 1996). The T-cells are thought to play a role in the activation of inflammatory cells such as monocytes and macrophages within the plaque (Hansson *et al.*, 1989a; Hansson *et al.*, 1989b). Further evidence for the stimulation of inflammatory cells via an immune response was obtained from experiments using transgenic immunodeficient mice crossed with atherosclerotic-prone mice. This cross led to a dramatic reduction in development of the earliest lesion, the fatty streak. Subsequent reintroduction of T-cells led to increased circulating levels of the pro-inflammatory cytokine interferon-γ and accelerated atherosclerosis (Whitman *et al.*, 2000; Zhou *et al.*, 2000).

Atherosclerosis is characterised by formation of lipid-rich plaques in the sub-endothelial space of arterial walls. Atherosclerosis progresses from a fatty streak,

which, in itself, is not clinically significant. The fatty streak occurs due to the movement of lipoprotein into the arterial wall via the damaged endothelium (Celermajer, 1997). The lipoprotein likely becomes trapped in the intima by matrix components (Fry, 1987; Schwenke & Carew, 1989a; Schwenke & Carew, 1989b) and then modified by oxidation (Steinberg & Witztum, 1990). At this stage, inflammatory cells such as monocytes also migrate into the fatty streak (Fig. 1.3c). Monocytes are mononuclear leukocytes that demonstrate migratory, chemotactic, pinocytic and phagocytic activities. They typically circulate in the blood for a few days before migrating into other tissue types and maturing into macrophages. The monocytes are recruited to lesion sites via endothelial cell-cell adhesion molecules (CAMs, fig. 1.3c). CAMs, such as VCAM and ICAM are upregulated at atherosclerotic-prone sites during endothelial dysfunction (section 1.4.4.2) and also in patients with hypercholesteraemia (Celermajer, 1997; Iiyama *et al.*, 1999; Shi *et al.*, 2005; van de Stolpe & van der Saag, 1996; Walpola *et al.*, 1995). Monocytes differentiate into macrophages, which are phagocytic leukocytes whose function is to protect against infection and noxious substances. Macrophages also act to release cytokines in order to attract further inflammatory cells, such as the highly motile phagocytes, neutrophils. Macrophages subsequently take up the oxidised lipid via specific scavenger receptors (Yamada *et al.*, 1998) to become foam cells, which have characteristic cytoplasmic droplets of cholesterol esters (Steinberg, 1987).

Further complexities arise on the migration of smooth muscle cells into the subendothelial space. Here they contribute to formation of the fibrous cap via synthesis of extracellular matrix (ECM) proteins. The advanced lesion is formed following deposition of collagen and elastin in the fibrous cap region. This advanced

lesion also displays a characteristic necrotic core, comprising an acellular region of cell debris, lipid and calcium deposits.

It is from here that clinical events are manifest, either when increases in lesion size impact on the lumen size, resulting in ischaemia or angina, or in the event of plaque erosion or rupture. Plaque stability is a balance between the actions of inflammatory cells (such as macrophages) and smooth muscle cells. Macrophages release proteolytic enzymes named matrix metalloproteases that degrade the ECM laid down by smooth muscle cells (Jones *et al.*, 2003; Shah *et al.*, 1995). Plaque rupture most commonly occurs at the site of greatest haemodynamic stress; erosion or rupture exposes lipid contents of the plaque to the lumen. This in turn may initiate the coagulation cascade, leading to formation of a thrombus and predisposing to complications such as MI or stroke (Fuster *et al.*, 1990).

1.3.2 Inhibitory Mechanisms & Current Interventions

NO is an endogenous inhibitor of atherosclerosis. The various NO-mediated anti-inflammatory mechanisms that control atherosclerosis are discussed in section 1.4.4.3.

Pharmacological interventions for patients at risk of atherosclerosis-induced cardiovascular disease are also available. Statins (or 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)–reductase inhibitors) were first used clinically in patients with hypercholesterolemia to lower cholesterol in those at risk of cardiovascular disease (Blauw *et al.*, 1997). However, as patients with normal serum levels of cholesterol also benefit from statin treatment, it is likely that they possess alternative therapeutic mechanisms. Several studies provide evidence that this further effect of

statins is likely to be an anti-inflammatory action; statins have been demonstrated to significantly lower levels of the inflammatory marker C-reactive protein (Chan *et al.*, 2004; Milionis *et al.*, 2005). Intensive treatment with atorvastatin decreased inflammatory activity in carotid atherosclerotic plaques - possibly through inhibition of inflammatory activity of macrophages (Martin-Ventura *et al.*, 2005). Statins downregulate the activation of the transcription factor, NF-κB, involved in the inflammatory response (Dichtl *et al.*, 2003; Martin-Ventura *et al.*, 2005). Other reported effects of statins include inhibition of platelet activation (Colli *et al.*, 2004) and antioxidant activity (Kowalski *et al.*, 2005; Mason *et al.*, 2006). Finally, statins also enhance endothelial NO production by directly upregulating expression and activity of its synthase, thus providing increased NO, which itself has anti-inflammatory actions, such as attenuating endothelium-leukocyte interactions (section 1.4.4.3; Laufs *et al.*, 1998).

The anti-inflammatory drug, aspirin, has therapeutic benefit in atherosclerosis through its anti-inflammatory actions. It decreases the adherence of monocytes and T cells to human coronary artery endothelial cells (Khan & Mehta, 2005). It can also reduce expression of proinflammatory cytokines such as TNFα by inhibiting the function of its transcription factor, NF-κB. NF-κB is normally kept in an inactive state in the cytoplasm by being bound to an inhibitory subunit named IκB. Phosphorylation of IκB, leads to its degradation, releasing NF-κB, which translocates to the nucleus and stimulates expression of TNFα. Aspirin impacts upon this process by inhibiting NF-κB mobilisation (Hachicha *et al.*, 1999; Khan & Mehta, 2005). Furthermore, aspirin prevents IκB degradation by inhibiting function of the IκB kinase (IKK; Kopp & Ghosh, 1994; Tegeder *et al.*, 2001; Yin *et al.*, 1998).

The antiplatelet action of aspirin (discussed in detail in section 1.5) also indirectly contributes to its anti-inflammatory action. Platelets can be considered inflammatory cells due to their release of pro-inflammatory agents. The release of IL-1ß and CD40L by platelets is thought to play a role in triggering atherogenesis (Gawaz *et al.*, 2005).

1.4 Nitric Oxide

NO is a ubiquitous signalling messenger molecule involved in diverse physiological and pathophysiological processes. These include various effects in cardiovascular and inflammatory processes including inhibition of platelet and inflammatory cell adhesion, inhibition of platelet activation and also control of vascular tone (Ahluwalia *et al.*, 2004; De Caterina *et al.*, 1995; Gruetter *et al.*, 1981; Ignarro *et al.*, 1987; Radomski *et al.*, 1987c; Radomski *et al.*, 1987d). Furthermore, NO also functions as a neurotransmitter at non-adrenergic, non-cholinergic (NANC) neurones (Rand, 1992).

NO is composed of a single atom each of nitrogen and oxygen, is uncharged and has an unpaired electron. Due to its free radical nature, it can rapidly react with reactive oxygen species (ROS) and molecular oxygen at high NO concentrations, forming a variety of biologically active species, which may go some way to explaining paradoxical effects of NO observed in some systems (Shaw, 2006).

1.4.1 Historical Overview

During the early 1980s, the vasodilator endothelium-derived relaxation factor (EDRF) was discovered to be released in response to agonists such as acetylcholine (ACh), bradykinin and thrombin (Busse *et al.*, 1985; Furchgott, 1984; Furchgott &

Zawadzki, 1980). It was several years later that independent studies revealed that NO accounted for at least some of the actions of EDRF (Furchgott, 1988; Ignarro *et al.*, 1988; Moncada *et al.*, 1988; Palmer *et al.*, 1987). Whilst some of those studies merely suggested that NO and EDRF had similar physical, chemical and pharmacological properties, Palmer *et al*'s work was definitive in that they directly measured NO release from cultured endothelial cells using chemiluminescence (Palmer *et al.*, 1987).

1.4.2 Synthesis of NO

NO is produced by a group of enzymes known as NO synthases (NOS), of which there are three recognized isoforms: endothelial (Pollock *et al.*, 1991), neuronal (Bredt *et al.*, 1991) and inducible (Yui *et al.*, 1991). NOS converts arginine and oxygen to citrulline and NO in a process that requires tetrahydrobiopterin (BH_4) in addition to other co-factors; flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and nicotinamide adenine dinucleotide phosphate (NADPH; Mayer *et al.*, 1989; Siddhanta *et al.*, 1996). NO synthases can be separated into two types: the constitutive isoforms (neuronal and endothelial) and the inducible isoform. The constitutive isoforms are calcium-dependent, whilst the inducible isoform is not due to calcium being permanently bound (Alderton *et al.*, 2001). The three NOS isozymes are fairly homologous: they are all homodimers, with each monomer containing a reductase domain linked to a haem oxygenase (Griffith & Stuehr, 1995). The former contains binding sites for FAD, FMN and NADPH, and the latter binds the substrates L-arginine and oxygen. The two monomers are linked by a calmodulin recognition site.

1.4.2.1 Constitutive NOS

The constitutive forms of NOS are always present in neuronal or endothelial cells and remain inactive until a rise in intracellular calcium is stimulated. Calcium binds calmodulin, forming a complex that forms an electron bridge between the reductase and oxygenase domains and thus, activates NOS. The production of NO will continue until calcium levels subsequently decrease. Calmodulin then dissociates and the enzyme reforms its complex with caveolin (Forstermann *et al.*, 1991). More recently, a calcium-independent phosphorylation of the synthase has been described as a mechanism that stimulates the activity of endothelial NOS (eNOS; Ming *et al.*, 2002).

eNOS is membrane-bound and associated with membrane structures called caveolae (Forstermann *et al.*, 1991). On encountering an increase in Ca^{2+} mobilization caused by agonists such as bradykinin or during shear stress, eNOS is activated to generate NO, which subsequently diffuses in three dimensions away from its source (Govers & Rabelink, 2001). The position of the endothelium at the interface between flowing blood and the vessel wall means that the NO generated will encounter a number of different cell types, depending on the direction in which it diffuses: NO that diffuses abluminally passes into vascular smooth muscle cells where it causes vascular relaxation; that which diffuses into the vessel lumen encounters platelets and leukocytes, whereupon it inhibits their aggregation and adhesion to the vessel wall. It will also encounter red blood cells where it is scavenged by haemoglobin (section 1.4.3.3).

NO synthesized by neuronal NOS (nNOS) in the neurones of the central nervous system acts as a neuromodulator involved in processes such as memory formation, pain modulation and neuronal control of blood flow (Moore & Handy, 1997).

1.4.2.2 Inducible NOS

Unlike the constitutive isoforms, which only bind calmodulin when the intracellular calcium concentration increases, inducible NOS (iNOS) tightly binds calmodulin even at resting calcium concentrations, and so is active once synthesized (Cho *et al.*, 1992). Instead, regulation of iNOS occurs at the transcriptional level; iNOS is normally absent in most cells but is stimulated in cells such as macrophages during inflammation, immunological defence, or infection. Substances such as cytokines (e.g. TNF-α, IL-1β or IFN-γ) and bacterial products (lipopolysaccharides; LPS) are known to regulate iNOS by stimulating binding of transcription factors such as NF-κB to promoter regions of the iNOS gene (Aktan, 2004; Galea *et al.*, 1992; Hibbs *et al.*, 1992; Lee *et al.*, 1993; Nathan, 1992; Xie & Nathan, 1994). A mechanism of post-transcriptional regulation of iNOS has also been reported whereby LPS and IFN-γ act to stabilise iNOS mRNA (Xie & Nathan, 1994).

Once expressed, the enzyme begins to synthesise large quantities (μM) of NO which can result in host tissue damage (Nathan & Xie, 1994). The deleterious effects of iNOS-derived NO persist and can last for days because the enzyme does not rely on calcium to function (Nathan & Xie, 1994).

The large quantities of NO produced by iNOS result in inflammation, cell damage and apoptosis (Lowenstein & Snyder, 1992; Moncada & Higgs, 1995; Nathan, 1992). Increased NO has been demonstrated in patients with chronic inflammatory

conditions such as asthma (Persson *et al.*, 1994), arthritis (Farrell *et al.*, 1992) and ulcerative colitis (Boughton-Smith *et al.*, 1993), thought to be due to the induction of iNOS (Aktan, 2004; Moncada & Higgs, 1995). iNOS has been localised in human atherosclerotic lesions to macrophages and smooth muscle cells, suggesting it plays a role in oxidative damage during the disease progression (Luoma *et al.*, 1998). With the increased presence of ROS, as is common with endothelial dysfunction, it is likely that the reaction of iNOS-derived NO with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$) may occur. The deleterious effects of peroxynitrite are likely to play a role in atherosclerotic disease progression (Shaw, 2006).

Despite commonly being thought to be involved in pathological roles, there is some evidence of favourable effects of iNOS-derived NO. It has been demonstrated to be an anti-bacterial agent (Nozaki *et al.*, 1997), to be involved in wound healing (Yamasaki *et al.*, 1998), to have a protective response to intestinal injury (McCafferty *et al.*, 1997) and to have possible roles in cardioprotection (Muller *et al.*, 2000; Parratt & Szekeres, 1995).

1.4.2.3 NOS Inhibitors

There are many NOS inhibitors available for use as experimental tools to help investigate the contribution of NOS in vasodilator responses. These compounds are mainly analogues of the endogenous substrate, L-arginine. The most commonly used non-selective NOS inhibitors include: N^G-monomethyl-L-arginine (L-NMMA), L-nitroarginine (L-NNA), N^G-nitro-L-arginine methyl ester (L-NAME) and the endogenous asymmetric dimethylarginine (ADMA; Leiper & Vallance, 1999; Moore & Handy, 1997; Palmer *et al.*, 1988; Vallance *et al.*, 1992).

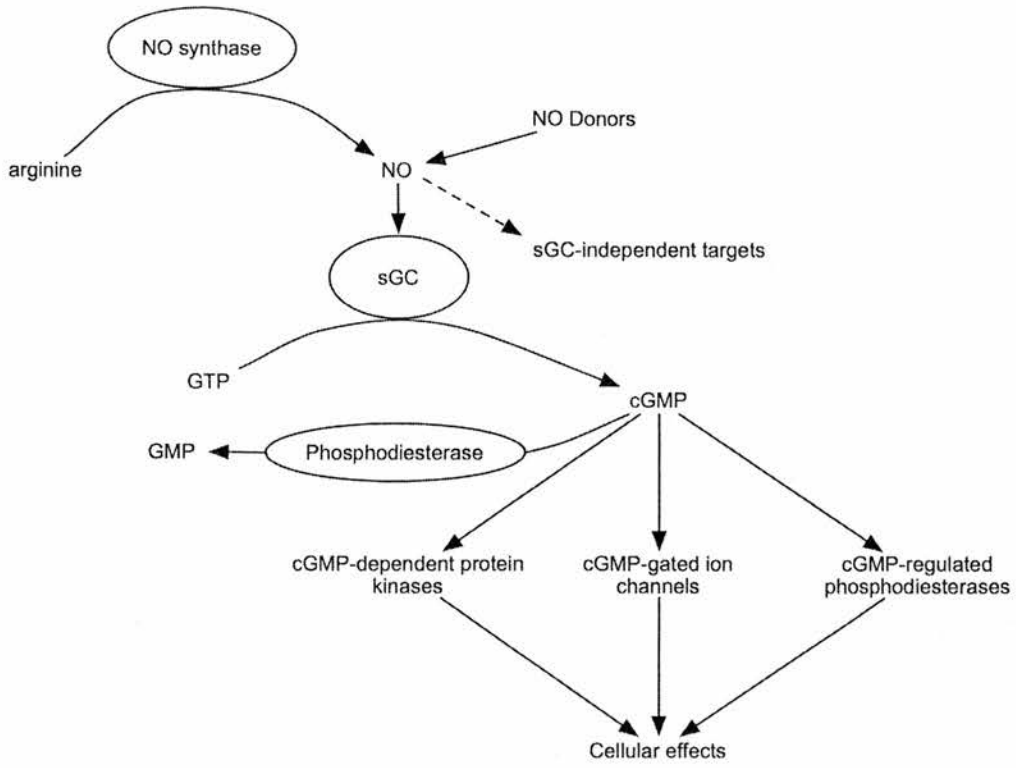


Fig. 1.1. Synthesis and targets of NO.

1.4.3 Targets of NO

1.4.3.1 Soluble Guanylate Cyclase

Soluble guanylate cyclase (sGC. Fig. 1.1) is the primary target for NOS-derived NO. sGC is a heterodimeric enzyme consisting of two subunits (α and β), in addition to a prosthetic haem group (Kamisaki *et al.*, 1986; Karow *et al.*, 2005). Each subunit can be divided into three domains: the N-terminal haem-binding domain, a central dimerisation domain and a C-terminal catalytic domain. NO has a high affinity for the haem domain and so binds to it forming a NO-haem complex which undergoes a subsequent conformational change resulting in a 400-fold increase in the rate of enzyme catalytic activity (Stone & Marletta, 1996).

Activated sGC converts guanosine-5'-triphosphate (GTP) to 3', 5'- cyclic guanosine monophosphate (cGMP; Ishii *et al.*, 1991; Moro *et al.*, 1996; Wolin *et al.*, 1982). There are a number of cGMP-dependent effector pathways that rely on stimulation of cGMP-dependent protein kinases, cGMP-regulated phosphodiesterases or cGMP-gated ion channels. For instance, NO-induced vasorelaxation is brought about by cGMP-dependent protein kinases activating Ca^{2+}-activated potassium channels leading to membrane hyperpolarisation. This then closes voltage-dependent Ca^{2+} channels resulting in a decrease in intracellular calcium which brings about the vasorelaxation (Fukao *et al.*, 1999; Lucas *et al.*, 2000; Robertson *et al.*, 1993).

In addition to reducing intracellular calcium, NO-donors also induce relaxation by an unknown mechanism which uncouples 'stress' from the phosphorylated myosin light chain of the smooth muscle (McDaniel *et al.*, 1992). The stress is defined as 'force/cell cross-sectional area' (Hai & Murphy, 1989) and refers to the shortening of the filament. Furthermore, it has been demonstrated that the relaxation of vascular smooth muscle caused by NO involves the activation of myosin light chain phosphatase (MLCP), correlated with a decline in light chain phosphorylation and force (Etter *et al.*, 2001).

1.4.3.1.1 sGC Deactivation

The first suggestion of a quick reactivation of sGC came from the observation that NO-induced relaxation of aortic rings could be repeated within two minutes of the first stimulus (Palmer *et al.*, 1987). NO dissociation from the sGC haem triggers deactivation.

Studies have shown the half-life of NO-sGC to vary between five seconds and a few minutes (Brandish *et al.*, 1998; Kharitonov *et al.*, 1997a; Kharitonov *et al.*, 1997b; Margulis & Sitaramayya, 2000; Russwurm *et al.*, 2002), depending on the presence of factors such as GTP, oxy-haemoglobin and glutathione (GSH). Furthermore, in intact cells, the half-life has been estimated to be 190 ms (Bellamy & Garthwaite, 2001).

1.4.3.1.2 sGC Inhibitors

1H-[1,2,4]oxadiazolo[4,3-a]-quinoxalin-1-one (ODQ) inhibits sGC (Garthwaite *et al.*, 1995) and is therefore a useful experimental tool in determining the cGMP-dependent and -independent effects of NO. ODQ is the preferred alternative to the non-specific inhibitors methylene blue and LY-83583, which have been shown to interfere with NO synthesis (Mayer *et al.*, 1993). ODQ binds in a competitive manner and inhibits NO-stimulated activity leaving basal enzymatic activity. The mechanism of ODQ–mediated inhibition involves sGC haem oxidation (Schrammel *et al.*, 1996; Zhao *et al.*, 2000).

1.4.3.2 cGMP-Independent Effects of NO

A rise in intracellular cGMP has been shown not to be necessary for several NO-dependent effects. Such cGMP-independent effects show an interesting correlation with extracellular NO generation (Crane *et al.*, 2005; Miller *et al.*, 2004; Sogo *et al.*, 2000a) and appear to be particularly relevant in the inhibition of platelet aggregation (Beghetti *et al.*, 2003; Crane *et al.*, 2005; Gordge *et al.*, 1998; Homer & Wanstall, 2002; Trepakova *et al.*, 1999; Tsikas *et al.*, 1999). In platelets, the evidence is fairly strong in support of NO-mediated activation of the sarcoplasmic/endoplasmic

reticulum Ca^{2+}-ATPase (SERCA), leading to refilling of Ca^{2+} stores and thus a decrease in intracellular calcium (Trepakova *et al.*, 1999).

It has also been reported that S-nitrosocysteine inhibits collagen-induced thromboxane A_2 (TXA_2) synthesis in platelets by a cGMP-independent mechanism (Tsikas *et al.*, 1999). This effect is likely due to NO-mediated inhibition of COX-1 as has been previously reported (Kanner *et al.*, 1992; Tsai *et al.*, 1994). Alternatively it could occur via inhibition of TXA_2 synthase (Wade & Fitzpatrick, 1997).

1.4.3.3 NO Scavengers

NO can be scavenged by various agents which are used experimentally. Of these scavengers, two are commonly used: haemoglobin and carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (cPTIO).

The stable radical compound cPTIO reacts with NO to form nitrite. It has been demonstrated to antagonise effectively the effects of NO in biological systems; it significantly inhibits vasorelaxation (Akaike *et al.*, 1993), inhibits endothelial cell mitogenesis and proliferation (Sarkar *et al.*, 1995), reverses inhibition of neutrophil adherence to the coronary artery endothelium (Sato *et al.*, 1996) and significantly inhibits NO-induced accumulation of cGMP in endothelial cells (Pfeiffer *et al.*, 1997).

NO also reacts with haemoglobin in its oxygenated state (oxyHb) to form methaemoglobin and nitrate. The rapid reaction of NO with oxyHb is a confounding factor for *in vivo* NO delivery. However, this issue is considerably complicated by the possibility of NO transport on haemoglobin as S-nitrosohaemoglobin, resulting

from S-nitrosation of Cys 93 residue (McMahon *et al.*, 2002; Pawloski *et al.*, 2001; Stamler *et al.*, 1992). The existence of such a pathway and its relevance to human physiology is the topic of heated debate (Hobbs *et al.*, 2002), and is disputed by the counter-argument that the role of haemoglobin is to release NO from NO_2 (Gladwin *et al.*, 2004; Gladwin & Schechter, 2004).

1.4.4 Physiological Effects of NO

1.4.4.1 NO and Platelets

NO displays antithrombotic actions through its ability to inhibit platelet adherence (Radomski *et al.*, 1987b; Radomski *et al.*, 1987d) and aggregation (Pasqui *et al.*, 1991; Radomski *et al.*, 1990; Radomski *et al.*, 1987b; Radomski *et al.*, 1987c). Radomski *et al.* first demonstrated that when activated, a constitutive NO synthase in platelets is stimulated to produce NO (Radomski *et al.*, 1990). This gives a subsequent increase in cGMP which is responsible for the antiaggregatory effect (Fig. 1.2; Mellion *et al.*, 1981).

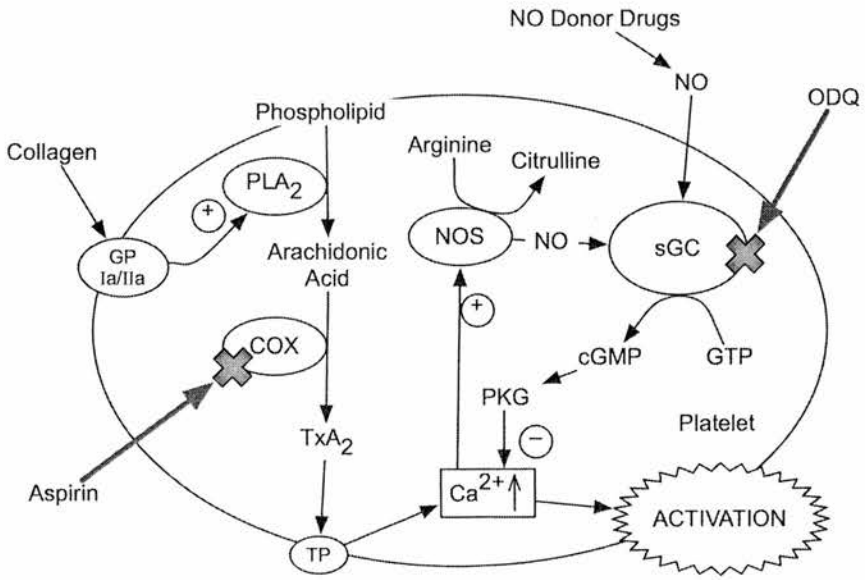


Fig. 1.2. Anti-platelet actions of NO and aspirin

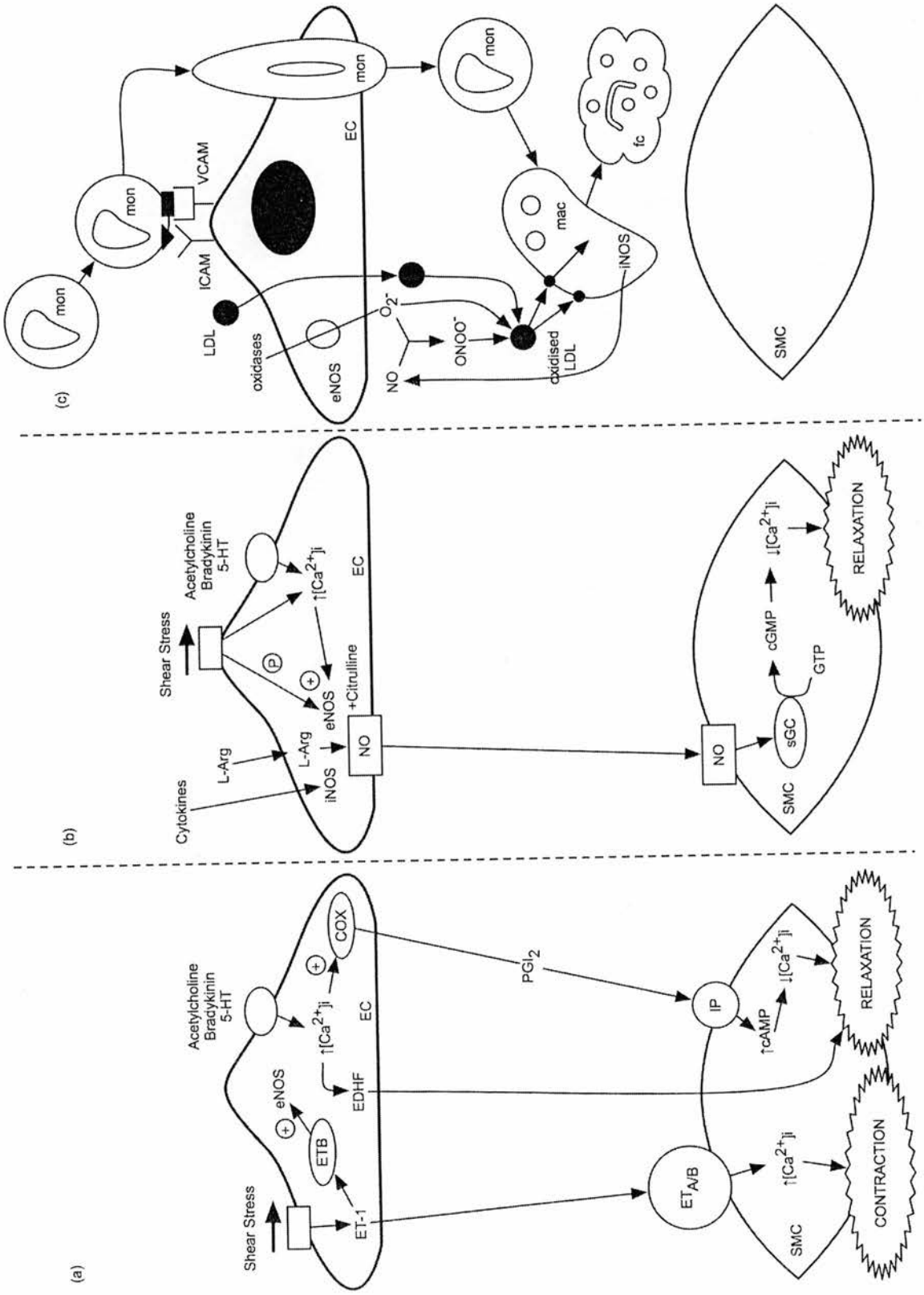
1.4.4.2 NO and the Endothelium

The endothelium (Fig. 1.3) is a single layer of cells that lies between the smooth muscle cell layer and the lumen of blood vessels that was, until recently, regarded to be inert. Within the past two decades the endothelium has been shown to be an antithrombogenic surface involved in diverse roles related to metabolic, synthetic and regulatory pathways (Rubanyi, 1993).

The normal function of the endothelium is to help control vascular homeostasis. The important role of the endothelium to release a substance (later identified as NO) that brings about vasodilatation was first reported in 1980 (Furchgott & Zawadzki, 1980). However, in addition, the endothelium produces a powerful vasoconstrictor peptide, endothelin (ET-1; Fig 1.3a). The substances released by the endothelium act directly on the adjacent smooth muscle cells to bring about relaxation or contraction.

Endothelial dysfunction or the loss of normal function of the endothelium, is a consequence of the decreased activity or bioavailability of endothelium-derived NO. Endothelial dysfunction is known to precede the development of atherosclerosis (section 1.3.1; Celermajer, 1997; Suwaidi *et al.*, 2000) and has been associated with the following risk factors: hypercholesterolemia (Drexler & Zeiher, 1991), obesity (Shankar & Steinberg, 2005) and hypertension (Taddei *et al.*, 2001; Zhu *et al.*, 2002).

Fig. 1.3. Function of the endothelium. (a) shows function of agonist and shear stress action on the endothelium and the consequences on the adjacent smooth muscle cells. (b) shows the role that NO plays in endothelium-dependent relaxation of smooth muscle cells. (c) shows a dysfunctional endothelial cell expressing CAMs such as ICAM and VCAM and the resulting recruitment of monocytes. Monocytes migrate into the subendothelial space where they differentiate into macrophages and later take up LDL (lipoprotein) that has been oxidised by free radical species, to become foam cells, an important constituent of the atherosclerotic plaque. EC = endothelial cell, SMC = smooth muscle cell, FC = foam cell, mon = monocyte, mac = macrophage (P)= phosphorylation.


(a)
Shear Stress
ET-1
ETB
eNOS
EDHF
Acetylcholine
Bradykinin
5-HT
↑[Ca2+]i
COX
EC
PGI2
IP
↑cAMP
↓[Ca2+]i
RELAXATION
ETA/B
↑[Ca2+]i
SMC
CONTRACTION
(b)
Shear Stress
Acetylcholine
Bradykinin
5-HT
↑[Ca2+]i
EC
P
eNOS
+Citrulline
L-Arg
L-Arg
iNOS
Cytokines
NO
NO
sGC
GTP
cGMP
↓[Ca2+]i
SMC
RELAXATION
(c)
mon
mon
VCAM
ICAM
LDL
oxidases
eNOS
EC
mon
O2-
NO
ONOO-
oxidised
LDL
mac
iNOS
fc
SMC

NO brings about smooth muscle cell relaxation in a process relying upon cytosolic guanylate cyclase to increase cGMP. (Fig 1.3b; Gruetter *et al.*, 1981; Ignarro *et al.*, 1987; Liu *et al.*, 1992; Rapoport *et al.*, 1983). NO also stimulates synthesis of PGI_2, a potent vasorelaxant and inhibitor of platelet aggregation. NO has been demonstrated to increase the production of eicosanoids such as PGI_2 through activation of COX in endothelial cells by a cGMP-independent mechanism (Davidge *et al.*, 1995). Interestingly, the Davidge results are in contrast to other studies where NO has been demonstrated to inhibit COX activity (discussed in section 1.4.3.2). It is plausible that these opposing effects occur due to differing intermediary pathways and not as a result of a direct interaction of NO with COX (Tsai *et al.*, 1994). A further possible explanation is that the NO-related species, peroxynitrite, is responsible for the effect, an occurrence that would not be observed in the studies with purified enzyme (Davidge *et al.*, 1995).

Prostaglandins, such as PGI_2, are also released by endothelial cells (Fitzgerald, 2004). PGI_2, along with PGE_2, brings about endothelium-dependent vasorelaxation by action on specific receptors, named IP receptors (Coleman *et al.*, 1994). These receptors are G-protein-coupled receptors that act to increase levels of cyclic adenosine monophosphate (cAMP; Coleman *et al.*, 1994; Halushka *et al.*, 1989) via the Gs subunit (Jaschonek *et al.*, 1988) coupling to adenylate cyclase (Gorman *et al.*, 1977). IP receptors have been localised to platelets (Oliva & Nicosia, 1987), where they bring about their antiaggregatory effects, and also to vascular smooth muscle cells.

After inhibition of the synthesis of NO and prostaglandins, an endothelium-dependent, hyperpolarisation-mediated vasodilatory mechanism can still be observed following stimulation by ACh or bradykinin. The mediator of this action has been named endothelium-derived hyperpolarizing factor (EDHF).

Several studies have shown *in vitro* evidence for the existence of EDHF in human arteries (Bussemaker *et al.*, 2003; Kemp & Cocks, 1997; Miura *et al.*, 1999; Nakashima *et al.*, 1993). The action of EDHF relies upon the release of intracellular calcium as occurs under shear stress or following receptor activation. Calcium-activated potassium channels are then stimulated, resulting in an endothelium-derived hyperpolarisation, or release of an 'EDHF'(Ding & Triggle, 2003; Griffith, 2004). EDHF may be a purely electrical contact-mediated event, or it may rely upon a diffusible mediator, of which several candidates have been suggested, including potassium ions themselves (Edwards *et al.*, 1998; Griffith, 2004), C-type natriuretic peptide (Chauhan *et al.*, 2003b), derivatives of the cytochrome-P450-monooxygenase, the epoxyeicosatrienoic acids (Fulton *et al.*, 1998) or even L-NAME insensitive NO (Chauhan *et al.*, 2003a; Cohen *et al.*, 1997).

1.4.4.3 NO and Inflammatory Cells

NO has various anti-inflammatory actions in the vasculature. It plays a role in endothelial cell-mediated inhibition of leukocyte recruitment to a site of injury (i.e. during atherosclerosis) or infection. P-selectins are contained within Weibel Palade bodies in the endothelium (Merten & Thiagarajan, 2004; Smith, 1993). The expression of selectins on the surface of the endothelial cell is upregulated following stimulation, for example, by histamine during an infection, or by the presence of

activated platelets (Dole *et al.*, 2005; Smith, 1993). Here they can bind with complementary L-selectins on the leukocyte surface (Kansas *et al.*, 1993). Leukocytes are loosely bound and so are free to 'roll' along the endothelium. Firmer bonds between leukocytes and endothelial cells are soon formed via endothelial molecules such as ICAM and VCAM expressed by the endothelial cells (Fig. 1.3c), but also by integrins expressed by the leukocytes. The integrins are heterodimeric glycoproteins such as CD11 or CD18 (Tonnesen, 1989). NO impacts on this process in a number of ways. It reduces expression of P-selectin and thus leukocyte recruitment in a cGMP-dependent process (Ahluwalia *et al.*, 2004). The administration of NOS inhibitors, L-NMMA and L-NAME, both increased adherent leukocytes by 15-fold in a process abolished by administration of the CD18 antibody, IB_4 (Kubes *et al.*, 1991). NO has also been demonstrated to inhibit ICAM and VCAM expression (Berendji-Grun *et al.*, 2001; De Caterina *et al.*, 1995; Spiecker *et al.*, 1997).

In addition, NO has anti-inflammatory effects in macrophages. Immunological or inflammatory stimuli (such as activation by cytokines and bacterial products) stimulate macrophages to express iNOS and produce NO (Moncada & Higgs, 1995). NO inhibits production of inflammatory cytokines such as the interleukins (IL-1 and IL-12; Huang *et al.*, 1998; Obermeier *et al.*, 1999; Thomassen *et al.*, 1997). NO also downregulates the proinflammatory cytokine TNFα in macrophages (Sinha *et al.*, 1998; Thomassen *et al.*, 1997). It has been further shown *in vitro* and *in vivo* that L-NMMA treatment increases TNFα production, whereas L-arginine treatment reduces it (Iuvone *et al.*, 1996). As discussed in section 1.3.2, phosphorylation of IκB is required for NF-κB to become activated and induce TNFα production. It has been

shown that NO can impact on this process by inhibiting IκB-phosphorylation, thus inhibiting the activation of NF-κB (Hattori *et al.*, 2004). Furthermore, NO-donors can directly inhibit the DNA binding activity of NF-κB preventing it from activating genes (Matthews *et al.*, 1996; Sekkai *et al.*, 1998).

Interestingly, under certain conditions, NO can have pro-inflammatory actions (Grisham *et al.*, 1999). For example, in asthma and during pulmonary disease, the high concentrations of iNOS-derived NO may produce various deleterious effects such as increased vascular permeability, damage to the airway epithelium, and promotion of inflammatory cell infiltration (Flak & Goldman, 1996; Laskin *et al.*, 2001; Mulrennan & Redington, 2004). Pro-inflammatory effects of excessive NO production have also been implicated in neurodegenerative diseases such as Parkinson's, Alzheimer's and multiple sclerosis (Liu *et al.*, 2002; Santiago *et al.*, 1998). It is possible that the inflammatory effects of NO are actually due to the generation of peroxynitrite which is causing these inflammatory actions (Shaw, 2006).

NO has been reported to have other paradoxical actions (such as being pro- and anti-oxidant, pro- and anti-apoptotic and also cytotoxic and cytoprotective) and it is suggested that the opposing effects of NO are due to the method of synthesis, the NO-related species involved, its concentration and the cell type involved (Grisham *et al.*, 1999; Shaw, 2006; Taylor *et al.*, 2003).

1.4.5 Current NO Donors and their Limitations

NO-donors are used as experimental tools in the quest to understand the complex physiological processes in which NO plays a role. In addition, some NO-donor drugs

are used clinically to treat the symptoms of vascular disease and thrombotic disorders.

1.4.5.1 Organic Nitrates

Organic nitrate compounds are still the most widely used therapeutic NO donors, despite having been used for over 125 years (Murell, 1879). These include compounds such as GTN and ISDN, commonly used in the symptomatic treatment of angina. They rapidly bring about their effect by dilating veins and coronary arteries, thus reducing cardiac work and improving blood supply to the heart (Abrams, 1885). However, the use of compounds such as GTN is limited by the development of tolerance to the haemodynamic effects with long-term use or high dosage (Stewart, 1888). Nitrate tolerance is characterized by a loss of nitrate-induced, cGMP-mediated vasodilation and tolerance has been demonstrated to lead to the loss of antiplatelet actions of nitrates, even at low doses (Chirkov *et al.*, 1997; Schwemmer & Bassenge, 2003). The mechanism behind tolerance is highly controversial, but is likely due to the interruption of the bioactivation pathways that are required to release NO from these compounds. Nitrate-mediated inhibition of vascular mitochondrial aldehyde dehydrogenase, the enzyme that accomplishes bioactivation of GTN has been suggested as a mechanism of tolerance (Chen *et al.*, 2002; Sydow *et al.*, 2004). Possible additional mechanisms for the tolerance may be oxidative stress (Munzel *et al.*, 1995), desensitization of sGC (Artz *et al.*, 2002), or an induction of reflex sodium retention, and thus plasma volume (Parker *et al.*, 1991). Another potential drawback for the clinical use of nitrates is their poor antiplatelet effects. This is explained by the poor capability of platelets to release NO from organic nitrates (Weber *et al.*, 1996).

1.4.5.2 Diazeniumdiolates

Diazeniumdiolates (NONOates) are compounds with the general formula X-[N(O)NO]$^-$ which are formed by the process of exposing nucleophiles to pressurised NO gas (Morley & Keefer, 1993). Diazeniumdiolates do not require bioactivation, are generally stable when in solid form, but will spontaneously release NO when in solution at a rate that is dependent on pH, temperature and the nature of the nucleophile (Horstmann *et al.*, 2002; Keefer *et al.*, 2001; Ramamurthi & Lewis, 1997). Unlike the organic nitrates, diazeniumdiolates do not induce tolerance (Homer & Wanstall, 1998).

Diethylamine diazeniumdiolate (DEA/NO) is a commonly used experimental diazeniumdiolate. It has a short half-life (around 2 min) at physiological temperature and pH (Homer & Wanstall, 1998) and inhibits platelet activation by a cGMP-independent mechanism (Sogo *et al.*, 2000a).

1.4.5.3 Sydnonomines

Molsidomine is converted in the liver to its active metabolite, 3-morpholinosydnonomine (SIN-1; Reden, 1990). SIN-1 releases NO spontaneously in a two-step process in the blood (Feelisch *et al.*, 1989). It was originally thought that SIN-1 was an NO-donor drug, but as SIN-1 is known to simultaneously generate O_2^-, it is now more correctly regarded as a peroxynitrite generator (Feelisch *et al.*, 1989; Hogg *et al.*, 1992).

It was originally hoped that sydnonomines would provide tolerance-free alternatives to the nitrates, but despite being considerably more potent than nitrates at inhibiting

platelet activation (Bult *et al.*, 1995; Gerzer *et al.*, 1988), in a large-scale clinical trial molsidomine failed to show any benefit in MI (ESPRIM, 1994).

1.4.5.4 S-Nitrosothiols

S-Nitrosothiols have the general formula RSNO. They are synthesised by S-nitrosation of reduced thiols (Williams, 1985), but are also produced endogenously (e.g. S-nitrosoalbumin and S-nitrosoglutathione).

Release of NO from S-nitrosothiols *in vivo* likely takes place by utilising Cu^{2+}-containing enzymes (Al-Sa'doni *et al.*, 1997; Dicks *et al.*, 1996), although other enzymes can also achieve the release of NO (Freedman *et al.*, 1995; Jourd'heuil *et al.*, 1999; Zai *et al.*, 1999). S-Nitrosothiols have been demonstrated to be more selective for platelets over blood vessels (Crane *et al.*, 2002; de Belder *et al.*, 1994; Ramsay *et al.*, 1995) and are known to mediate their effects via both cGMP-dependent (Mellion *et al.*, 1983; Radomski *et al.*, 1992) and cGMP-independent effects (Crane *et al.*, 2005; Gordge *et al.*, 1998; Sogo *et al.*, 2000a; Tsikas *et al.*, 1999).

The stability of older S-nitrosothiols can be unpredictable and their half-life can vary depending on the R-group present, the solvent and the pH. However, more recently developed S-nitrosothiols may offer improvement (Crane *et al.*, 2005; Megson *et al.*, 1997; Miller *et al.*, 2003; Sogo *et al.*, 2000b).

1.5 Aspirin

1.5.1 Historical Overview

Aspirin (acetylsalicylic acid) was first synthesized in 1899 by Felix Hoffman of Bayer Corp. It was the first drug of the family of nonsteroidal anti-inflammatory drugs (NSAIDs) on the market and it is used today in the treatment of headache, rheumatic pain, inflammation, and as a prophylactic against thrombotic events in the cardiovascular system. Over 40 billion aspirins are taken per year in the United States (Schiffmann *et al.*, 2005). Despite being used for many years, it took until 1971 for Vane to discover that inhibition of prostaglandin synthesis was the mechanism for the therapeutic effect of aspirin (Vane, 1971).

1.5.2 Pharmacological Mechanism of Action

Prostaglandins are derived from arachidonic acid (AA) in a pathway dependent on the COX family of enzymes, where aspirin has its effect. There are two structurally similar COX isoforms named COX-1 and COX-2 (discussed in sections 1.5.2.2 and 1.5.2.3 respectively), which are encoded by different genes. The expression of the COX isozymes varies between tissues.

In contrast to other NSAIDs, such as naproxen and ibuprofen, which reversibly bind at the active site of COX (Garcia Rodriguez *et al.*, 2004), aspirin causes an irreversible inhibition. It selectively and rapidly (within minutes) acetylates the hydroxyl group of a serine residue (Ser 530) near the C-terminus of COX, forming an impediment to the binding of AA (DeWitt & Smith, 1988; Roth & Majerus, 1975;

Roth *et al.*, 1975). Acetylation causes irreversible COX inhibition and a requirement for new COX to be synthesised for subsequent production of prostaglandins. As platelets are widely thought to lack the necessary cellular machinery to synthesise new proteins, the effect of aspirin will last for the lifetime of the platelet (approximately 10 days); indeed a recovery of COX activity of 10 % per day has been observed, in-line with platelet turnover (Altman *et al.*, 2004).

1.5.2.1 Arachidonic Acid Metabolism

AA is an essential fatty acid found amongst membrane phospholipids. Activation of the enzyme, phospholipase A_2, liberates AA from the membrane, making it available for two further pathways. AA can either be utilized by COX-1 or COX-2 to form prostaglandins and thromboxanes, or it can be utilised by another enzyme called 5-lipoxygenase (5-LO) to form leukotrienes (Fig. 1.4). The lipoxygenases, which comprise three major types (5-, 15-, and 12-LO), are compartmentalized within different cell types of peripheral blood and act to transform AA to biologically active compounds (Serhan *et al.*, 1990).

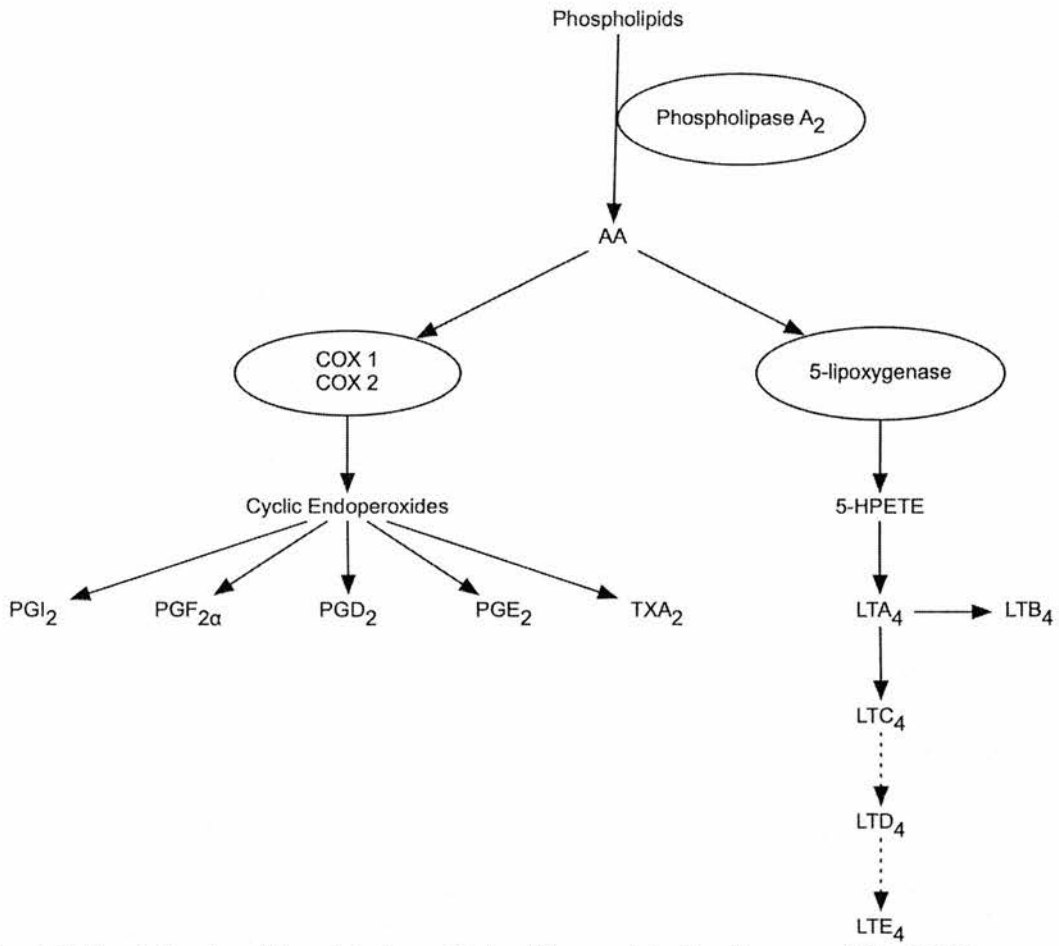


Fig. 1.4. Arachidonic acid metabolism. AA is either metabolized by one of the COX enzymes to form prostaglandins and thromboxanes or by 5-LO to form the leukotrienes.

5-LO is a calcium- and ATP-dependent enzyme which catalyses the conversion of AA to (5S)-trans-5,6-oxido-7,9-trans-11,14-cis-eicosatetraenoic acid (5-HPETE) and also the subsequent conversion to the fairly unstable leukotriene A_4 (LTA_4), the first of a series of leukotrienes formed. LTA_4 is the substrate for two enzymes; leukotriene A_4 hydrolase and leukotriene C_4 synthase, which form LTB_4 and LTC_4 respectively (Bertolini *et al.*, 2001). LTB_4 is a potent chemotaxin and LTC_4, along with the subsequent leukotrienes, LTD_4 and LTE_4, formed from it, is involved in bronchoconstriction and increased vascular permeability (Parente, 2001).

1.5.2.1.1 Lipoxins

Lipoxins (LXs) and aspirin-triggered lipoxins (ATL) are endogenously produced anti-inflammatory agents. Lipoxins are short-lived eicosanoids whose appearance in inflammation signals the resolution of inflammation. LXs were first identified in human leukocytes by Serhan *et al.* in the early 1980s, who proposed that LXs were formed via interactions of the 5- and 15-lipoxygenase pathways in leukocytes (Serhan *et al.*, 1984). The common mammalian forms of LXs are LXA_4 and its positional isomer LXB_4 (McMahon & Godson, 2004).

LXs are typically formed through cell-cell interactions. The most common pathway identified within the vasculature is via platelet-polymorphonuclear neutrophil (PMN) interaction. This pathway involves the activities of 5-LO, present in cells such as neutrophils, and 12-LO, present in platelets. 5-LO generates LTA_4 from AA that is rapidly taken up by platelets. Platelets then convert LTA_4 to LXA_4 through the oxygenase activity of their 12-LO (Serhan *et al.*, 1990).

LXs are also formed in a process triggered by aspirin, known as the 15-epi-LX pathway. In environments rich in cytokines, aspirin will acetylate COX-2, prompting it to act as a lipoxygenase rather than an endoperoxide and thus, converting AA to 15-HETE which is subsequently transformed by 5-LO to 15-epi-LXA_4 or 15-epi-LXB_4 (Claria & Serhan, 1995).

ATLs are thought to bring about beneficial effects of aspirin further to the antithrombotic action, particularly in the resolution of inflammation (McMahon *et al.*, 2001). Recent evidence has shown that LXA_4 regulates the production of the

inflammatory mediator IL-12 (Machado *et al.*, 2006). In the absence of LXA_4 and the consequent cessation of IL-12 production, inflammation would persist, resulting in host damage. Machado *et al.* demonstrated that LXs activate two receptors (AhR and LXAR) in dendritic cells, causing expression of a suppressor of cytokine signalling (SOCS-2), which in turn, regulates the inflammatory response. They further demonstrated that SOCS-2-deficient mice display uncontrolled inflammation (Machado *et al.*, 2006). A further LX-mediated mechanism has been proposed which helps explain some of the actions of aspirin that cannot be attributed to the inhibition of prostanoids - such as how aspirin inhibits cell accumulation during inflammation. In two separate studies aspirin has been demonstrated to trigger production of NO through eNOS and iNOS (Paul-Clark *et al.*, 2004; Taubert *et al.*, 2004). The NO acts to provide anti-inflammatory effects such as inhibition of leukocyte-endothelial interactions (Paul-Clark *et al.*, 2004) and preservation of endothelial function (Taubert *et al.*, 2004).

1.5.2.2 COX-1 and its Inhibition

COX-1 is often referred to as the 'house-keeping' isoform due to its functions in many tissues. It has high-profile regulatory roles in the gastric mucosa, the kidneys and platelets.

In platelets, the effect of aspirin is irreversible, due to the inability of platelets to generate new COX. This means that there is a cumulative inhibition of platelet TxA_2 production (Patrignani *et al.*, 1982), leading to a prolonged antithrombotic effect. As depicted in figure 1.2, the antithrombotic action of aspirin comes about through its ability to inhibit platelet COX-1. The normal coagulation pathway relies on the

production of TxA_2 as described above (Fig. 1.4). TxA_2 was first identified in the guinea pig lung during anaphylaxis. It was known to cause vasoconstriction of rabbit aortic smooth muscle and consequently, was known as rabbit aorta-contracting substance (Piper & Vane, 1969).

A few years later, endoperoxides (PGG_2 and PGH_2), which induced platelet aggregation, were found to be generated when platelets were incubated with AA (Hamberg & Samuelsson, 1974; Hamberg *et al.*, 1974). It was soon noted that these agents alone could not account for the activity of rabbit aorta-contracting substance and it was later realised that incubation of PGG_2 or PGH_2 with platelets resulted in the formation of a very unstable compound that induced irreversible platelet aggregation and was degraded to the inactive, but stable, TxB_2 (Hamberg *et al.*, 1975). The unstable compound was named TxA_2.

TxA_2 exerts its effect by action on G-protein coupled receptors called TP receptors, of which different types exist in platelets and vascular smooth muscle (Masuda *et al.*, 1991). It has been reported that two different effector systems are involved in TxA_2-induced platelet signalling. Phospholipase C activation is primarily involved in mediating platelet aggregation and Ca^{2+} mobilisation controls platelet shape change (Brass *et al.*, 1987). TP receptor mutations, and defective signal transduction by the TP receptor have both been linked to bleeding disorders (Hirata *et al.*, 1994; Mitsui *et al.*, 1997).

Unstable TxA_2 has a half-life of ~30 seconds (Bhagwat *et al.*, 1985) so it is not surprising that most experimental studies of TxA_2 activity are carried out with the longer-lasting TP receptor agonist, U46619. U46619 studies have revealed that the

TxA_2 receptor is located on the platelet membrane (Hung *et al.*, 1983), that U46619 directly stimulates platelet shape change (Parise *et al.*, 1982), that it induces platelet aggregation in humans but not in sheep (Leach & Thorburn, 1982) and that sickle-cell anaemia patients have decreased aggregation in response to U46619, but not thrombin (Foulon *et al.*, 1993).

As aspirin is nearly 170-fold more selective for inhibiting COX-1 than COX-2 (Vane *et al.*, 1998), low dose aspirin (75 mg) can be taken daily and is effective at inhibiting platelet thromboxane without affecting vascular PGI_2 or having an anti-inflammatory effect (Forster & Parratt, 1997; Vane *et al.*, 1998).

1.5.2.3 COX-2 and its Inhibition

In contrast to COX-1, COX-2 is virtually undetectable under normal conditions in most tissues. It is often referred to as the 'inducible' isoform because of its tendency to be expressed in response to inflammatory stimuli.

The COX-2 gene is commonly stimulated by both growth factors and mediators of inflammation such as IL-1, TNFα and LPS. Furthermore, its expression is suppressed by anti-inflammatory cytokines and glucocorticoids. With the development of COX-2 selective inhibitors, it was shown that COX-2 plays a critical role in inflammation, pain, and fever (DeWitt, 1999).

The regulation by growth factors suggests that COX-2 has a role in mitogenesis, or perhaps wound repair. Indeed, colon cancers have been demonstrated to produce high levels of the COX-2-derived prostaglandins that act to enhance tumour growth (Karim & Rao, 1976; Lim *et al.*, 2001; Lupulescu, 1996; Sano *et al.*, 1995;

Sinicrope, 2006; Yona & Arber, 2006) The implication of COX-2 in the development of cancers further suggests a role for COX-2 in control of cell growth. COX-2 also performs various specific functions in specialised cell types. For example, it is involved in reproduction. In granulosa cells it is stimulated by follicle-stimulating hormone and luteinizing hormone (Sirois & Richards, 1992; Sirois *et al.*, 1992) where it impacts on ovulation, fertilisation, implantation, and decidualisation (Lim *et al.*, 1999; Lim *et al.*, 1997). COX-2 is also stimulated in the kidney macula densa by lumenal salt concentrations (Harris *et al.*, 1994) to play a role in renal physiology, where it mediates renin production (Cheng *et al.*, 1999).

Prostaglandins produced by cytokine-stimulated COX-2 within the spinal cord are important mediators of inflammatory pain and hyperalgesia (Vane *et al.*, 1998). COX-2 upregulation occurs in endothelial cells of the brain vasculature following inflammatory stimuli and is responsible for production of the fever-inducing PGE_2 (Cao *et al.*, 1996).

Due to the numerous activators of COX-2 and the many different conditions that lead to transcriptional activation of the COX-2 gene, it is difficult to identify specific signaling pathways. However, two pathways are heavily implicated in the regulation of COX-2 gene expression. The NF-κB signaling pathway is involved in the stimulation by inflammatory components and the MAPK signaling pathway in growth factor-mediated signaling (for review see Smith *et al.*, 2000).

The suggestion that the gastrotoxic effects of aspirin (discussed further in section 1.3.3) are due to the inhibition of housekeeping COX-1, whereas its anti-inflammatory effects are a consequence of inhibition of COX-2, led to the

development of selective COX-2 inhibitors in the hope that the beneficial effects could be retained without injury to the gastric mucosa (Crofford, 1997).

However, it is important to recognise that selective COX-2 inhibitors lose the cardiovascular benefit observed with aspirin as these are primarily mediated through COX-1. In addition, COX-2 has a role in sustaining vascular PGI_2 production (McAdam *et al.*, 1999). Recently, several of the new COX-2 inhibitors have been withdrawn due to mounting evidence of an increased risk of stroke, thought to be due to inhibition of COX-2 in endothelial cells leading to reduced generation of the antithrombotic and vasodilator agent, PGI_2, in relation to the unaffected COX-1 derived, TXA_2 (Fitzgerald, 2004).

1.5.3 Limitation of Use of Aspirin

The long term use of aspirin for pain and inflammation associated with conditions such as rheumatism and arthritis is limited because of its serious side-effects in the gastro-intestinal tract, reported to cause 16,000 deaths each year in the USA (Keeble & Moore, 2002). The first evidence for gastric damage caused by aspirin was presented in 1938 (Douthwaite & Lintott, 1938) and there is now clear evidence for an association between gastric and duodenal ulcers and NSAID use (AMIS, 1980; Levy, 1974; Silvoso *et al.*, 1979; Sun *et al.*, 1974; Wolfe *et al.*, 1999).

NSAIDs cause gastric mucosal injury through their topical irritant effect, but more importantly, through suppression of gastric prostaglandin synthesis (Rainsford & Willis, 1982; Wallace *et al.*, 1993a). Many components of mucosal defence rely on prostaglandins including mucus and bicarbonate secretion, blood flow (Ashley *et al.*, 1985; Gana *et al.*, 1987), epithelial cell turnover and repair, as well as mucosal

immunocyte function. It is believed, therefore, that inhibition of prostaglandin synthesis by aspirin depresses these defences, providing an opportunity for further damage by endogenous agents such as acid, pepsin and bile salts (Wolfe *et al.*, 1999).

1.5.3.1 Overcoming Gastrotoxicity

As mentioned above, COX-2-selective inhibitors were developed to avoid the gastric side-effects of aspirin under the assumption that the inhibition of housekeeping functions is COX-1-mediated and the anti-inflammatory effects are COX-2-mediated. The COX-2 selective inhibitor celecoxib has been demonstrated to have a more favourable gastric profile than other NSAIDs (Bensen *et al.*, 2000). The selective COX-2 inhibitor, NS-398 was shown to block pro-inflammatory prostaglandin synthesis in the air pouch model of inflammation without inhibiting gastric prostaglandin production, therefore avoiding gastric ulceration (Masferrer *et al.*, 1994). However, whilst celecoxib and rofecoxib were demonstrated to not produce gastric lesions in healthy rat mucosa when administered in animals without compromised gastrointestinal mucosa, they have been shown to aggravate and complicate existing ulcers (Laudanno *et al.*, 2001).

Interestingly, it has been more recently proposed that gastric damage is due to the dual inhibition of COX-1 *and* COX-2. A study demonstrated that whilst nonselective COX inhibitors produced severe gastric lesions, the selective COX-2 inhibitor, rofecoxib, did not induce damage and neither did the selective COX-1 inhibitor, SC-560. However, a combination of SC-560 and rofecoxib reintroduced the gastric lesions. The authors proposed the damage induced by conventional NSAIDs requires

both COX isoforms to be inhibited as inhibition of COX-1 alone led to an upregulation of COX-2 expression in the gastric mucosa (Tanaka *et al.*, 2001).

Other approaches in the quest to improve the cost : benefit balance of NSAIDs include enteric coating of drugs, aspirin pro-drugs (Ankersen & Senning, 1989; Carter *et al.*, 1980; Truelove *et al.*, 1980) and dual COX/5-LO inhibitors (Fiorucci *et al.*, 2001). Enteric-coated aspirin has a barrier that controls the location at which it is absorbed and so it passes through the stomach and is dissolved in the small intestine. Enteric-coated aspirin has been demonstrated to have no risk of peptic ulcer bleeding (Weil *et al.*, 1995), but whether or not it causes ulceration to the lower intestine is still under debate (Grossman, 1995; Weil *et al.*, 1995). The main disadvantage of the enteric coating is the time taken to obtain peak plasma levels of the drug. For normal formulations it is around 30 – 40 min, but for the enteric-coated drug, this increases to around 3-4 h (Patrono *et al.*, 2004). Reasons behind this are likely to include the fact the pH in the small intestine is much less acidic than the stomach and therefore the compound will be less ionised and absorbed more slowly, and also resulting in a reduction in the peak plasma concentration. The increased time taken to reach the point of absorption may also contribute to slower response.

Since it is becoming generally accepted that the 5-LO pathway is involved in the development and maintenance of inflammation, dual inhibitors of both the COX-2 and 5-LO pathways are being developed. A new dual COX-2/5-LO inhibitor has been shown to be effective in animal models of arthritis without being ulcerogenic (Inagaki, 2003). It remains to be seen, however, if these inhibitors will encounter the same problems as the COX-2 selective inhibitors.

A further novel target may be the newly discovered ALX receptor for LX and ATL (section 1.5.2.1.1) ALX receptor agonists may provide resolution-targeted anti-inflammatory therapies (Chiang *et al.*, 2005).

1.5.4 NO-Aspirin Hybrids

More recently, hybrid drugs, linking a cytoprotective molecule such as NO (Takeuchi *et al.*, 1998b) to the aspirin molecule have been developed (Cena *et al.*, 2003; del Soldato *et al.*, 1999) and appear to be promising in the search for a gastric-friendly NSAID.

NO has various effects on the gastric mucosa to increase mucosal defence. It increases blood flow in the gastric mucosa, promoting repair and removal of toxins (Wallace & Miller, 2000). It also increases secretion of protective gastric mucus (Brown *et al.*, 1993) and is thought to promote healing of gastric ulcers by promoting angiogenesis (Ma & Wallace, 2000). NO from NO-aspirins, like PGI_2, can inhibit neutrophil adhesion to the blood vessel wall (Lefer & Lefer, 1996). It is therefore possible that NO replaces the aspirin-inhibited PGI_2 as an inhibitor of neutrophil adherence and also of mucosal vasodilatation (Keeble & Moore, 2002). Wallace and colleagues documented the role of neutrophils in gastropathy (Asako *et al.*, 1992; Wallace, 1997). They reported neutrophil adherence to the vascular endothelium of the gastric microcirculation as one of the earliest events following NSAID administration to laboratory animals (Asako *et al.*, 1992). Furthermore, they showed, with the use of antibodies, that mucosal injury was avoided by preventing neutrophil adherence (Wallace *et al.*, 1993b). Since NO is known to prevent neutrophil

adhesion, the idea of combining an NO group to an NSAID was suggested to prevent gastropathy (Wallace, 1997).

The toxic effects of aspirin have previously been linked to actions of the carboxylic acid moiety (Rainsford & Whitehouse, 1976). NO-aspirin hybrids have been shown to have reduced acute gastric toxicity, thought to be primarily due to prevention of the carboxylic acid effect, rather than a direct protective effect of NO, although the latter may contribute (Cena *et al.*, 2003).

NCX4016 (section 1.4.1) and the related compound, NCX4215, (Wallace *et al.*, 1995) were the first NO-aspirins to be released, but now another family of NO-aspirin hybrid drugs named the furoxans (section 1.4.2) has been developed.

Various other NO-NSAID hybrids have been produced such as NO-flurbiprofen, NO-naproxen, NO-diclofenac, NO-ibuprofen and NO-paracetamol (Burgaud *et al.*, 2002; Keeble & Moore, 2002). Unlike aspirin, however, these NSAIDS inhibit COX reversibly and are therefore likely to be less selective antithrombotic agents.

1.5.4.1 NCX4016

Nicox Inc. produce the drug, NCX4016 (2 acetoxy-benzoate 2-(2-nitroxymethyl)-phenyl ester; commonly called nitroaspirin. Fig. 1.5). It comprises an aspirin molecule linked by an ester bond to a molecular 'spacer' molecule, which in turn is linked to an organic nitrate group (Fiorucci & Del Soldato, 2003).

NCX4016 has been demonstrated not to be ulcerogenic at equivalent concentrations as ulcerogenic aspirin. Furthermore, it causes a dose-dependent protection against gastric lesions induced by HCl / ethanol mix in rats (Takeuchi *et al.*, 1998a).

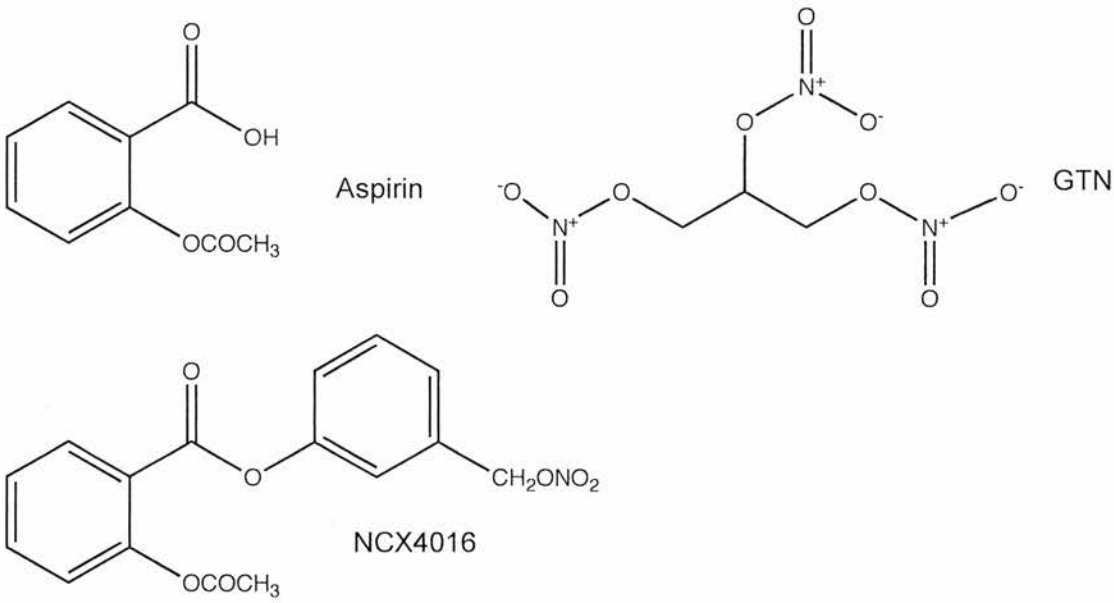


Fig. 1.5. Structural formula of aspirin, the organic nitrate compound GTN and NO-aspirin, NCX4016.

Mechanisms for the reduced gastric side-effects of NCX4016 may include reduced leukocyte infiltration to the gastric mucosal vasculature (Fiorucci *et al.*, 1999). In addition, it has been reported that NCX4016 spares the gastric mucosa in rats via inhibition of caspase activity through cGMP-dependent and -independent pathways (Fiorucci *et al.*, 1999). Caspases are a family of proteases that are involved in cytokine release and apoptosis (Creagh & Martin, 2001). Aspirin, but not NCX4016, caused increased activity of caspases 1 and 3. It is suggested that NO-aspirins cause a post-translational inactivation of caspases, possibly by S-nitrosation (Dimmeler *et al.*, 1997).

Inhibition of caspase 1 has also been proposed as a model of the anti-inflammatory effects of NCX4016 (Fiorucci *et al.*, 2000) that can be demonstrated using the carrageenan-induced hind paw oedema model (an acute model of inflammation), in which NCX4016 was demonstrated to cause similar effects to the parent compound (al-Swayeh *et al.*, 2000).

NCX4016 is an organic nitrate that relies upon enzymatic breakdown to yield NO (Wallace *et al.*, 2002) and this fact may limit its antiplatelet effects. Platelets have a poor capability of releasing NO from organic nitrates (Weber *et al.*, 1996) and the metabolism of both NCX4016, and the organic nitrate compound, GTN, has been reported to occur through identical mechanisms (Grosser & Schroder, 2000). It is therefore possible that compounds such as NCX4016 will fail to show additional, NO-mediated effects in platelets, at least *in vitro*. Despite these limitations, however, antiplatelet effects may still be retained *in vivo*, presumably through remote organic nitrate activation in vascular cells other than platelets (e.g. smooth muscle cells); a rather inefficient method of NO delivery specifically to platelets. Anti-platelet effects of NCX4016 have been demonstrated *ex vivo*, in animals and humans (Fiorucci *et al.*, 2004; Fiorucci *et al.*, 2003; Momi *et al.*, 2005; Wainwright *et al.*, 2002). In addition, it has been demonstrated to have beneficial effects *in vivo* in vascular cell types other than platelets, including protection of vascular endothelium in diabetic rats (Pieper *et al.*, 2002), reduction of blood pressure in hypertensive rats (Muscara *et al.*, 2001), prevention of restenosis in hypercholesterolemic mice (Napoli *et al.*, 2001) and reduction of infarct size in a model of cardiac ischaemia in pigs (Wainwright *et al.*, 2002).

1.5.4.2 Furoxans

A new range of drugs, in which a NO-donating moiety (furoxan group, fig. 1.6 and 1.7.) is linked by ester linkage to the aspirin molecule, appear to overcome the problem of gastric lesions (Cena *et al.*, 2003). However, the NO release mechanism is unlikely to require the same cellular machinery as that for organic nitrates,

suggesting that they might offer a degree of NO-mediated anti-platelet effects to complement those of the aspirin moiety.

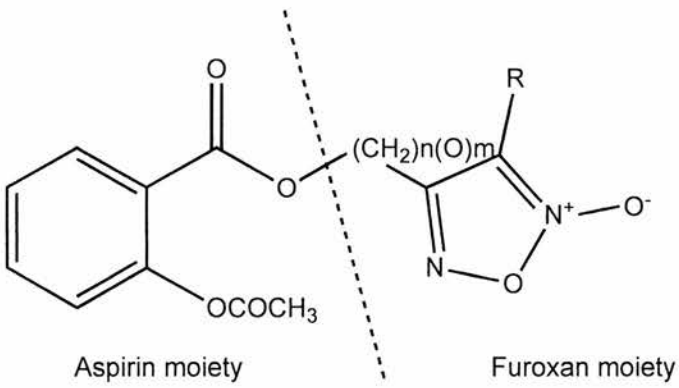


Fig. 1.6. General structural formula of a furoxan-aspirin hybrid drug.

Furoxan compounds B13 and B12, their respective aspirin hybrids (3-cyanofuroxan-4-yl)methyl 2-acetoxybenzoate (B8) and (3-carbamoylfuroxan-4-yl)methyl 2-acetoxybenzoate (B7) and their respective NO-free counterparts (furazans), 4-cyanofurazan-3-yl)methyl 2-acetoxybenzoate (B16) and (4-carbamoylfurazan-3-yl)methyl 2-acetoxybenzoate (B15) are shown in figure 1.7.

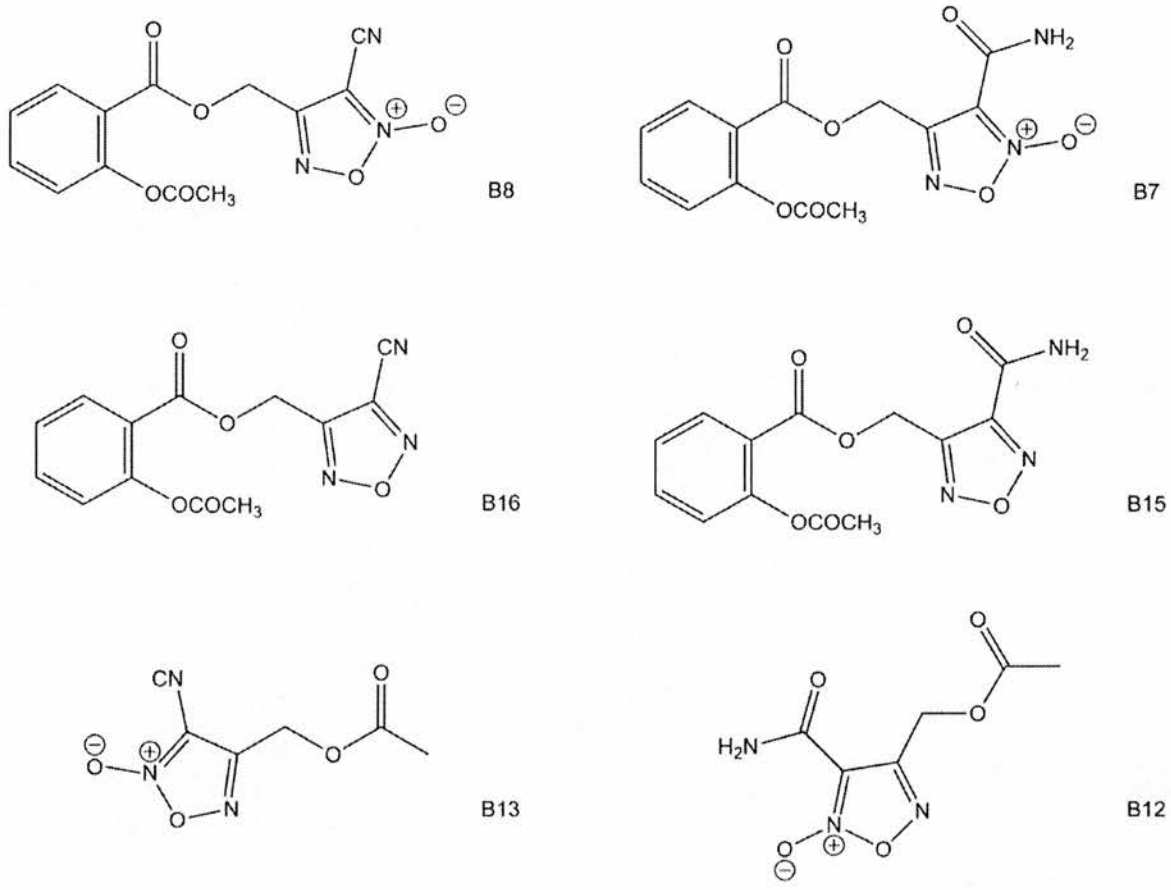


Figure 1.7. Structural formulae of furoxans (B8 and B7) and their NO-free equivalents, the furazans (B16 and B15) and their aspirin-free counterparts (B13 and B12).

The furoxan-aspirin hybrids have previously been demonstrated to be anti-inflammatory in the carrageenan-induced paw oedema model in rats (at a dose equimolar with 120 mg/kg aspirin); B8 was demonstrated to inhibit the paw volume increase by ~ 60 %, B7 gave a 50 % inhibition and aspirin gave a 55 % (Cena *et al.*, 2003).

The mechanisms of NO-release, antiplatelet and anti-inflammatory actions of these promising compounds have yet to be fully elucidated.

1.6 Project Aims

The aims of this project are to characterise the nitric oxide (NO)-releasing and COX-inhibiting effects of novel furoxan derivatives of aspirin in order to investigate the possible antithrombotic and anti-inflammatory effects with a view to possible protection against atherothrombosis.

The primary hypotheses that will be addressed are:

- That an aspirin-like action is retained by the hybrids and brings about COX inhibition *in vitro*.
- That the hybrid compounds bring about vasorelaxation through an NO-dependent mechanism.
- That NO-hybrids retain antiplatelet effects *in vitro*, relying on both the NO and aspirin moieties.
- That NO-release by the hybrids occurs intracellularly and is dependent on the presence of intracellular antioxidants.
- That anti-inflammatory effects are provided by NO- *and* COX-dependent mechanisms.

CHAPTER TWO

General Methods

2. General Methods

2.1. Electron Paramagnetic Resonance (EPR)

EPR is a spectrometric technique that can be used to detect oxidising free radicals. The technique utilises a chemical spin-trap, which reacts with free radical species that would otherwise be too short-lived for detection (e.g. NO, $O_2^{\cdot-}$, $^{\cdot}OH$), to form a stable radical adduct. The adduct is placed within a strong magnetic field and EPR spectrometry measures its absorption of electromagnetic (microwave) radiation.

In these studies, the spin trap agent used was 1-Hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine .HCl (CPH). The effectiveness of CPH as a spin trap in biological systems has been investigated elsewhere (Dikalov *et al.*, 1997).

The CPH spin adduct yield a typical 3-peak trace, centred around 3360 G (as shown in Fig. 2.1) when recorded by EPR due to the spin of the 3 nitrogen isotopes impacting upon the magnetic field.

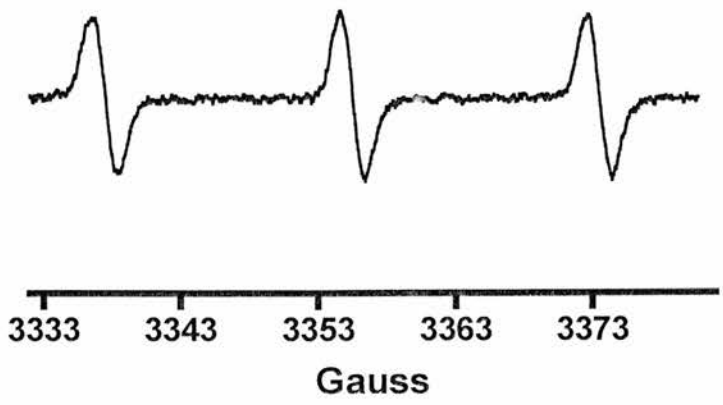


Fig. 2.1. A typical trace from the CPH-centred adduct in EPR

2.1.1 COX-1 Activity Assay

It has been previously demonstrated that the reduction of PGG_2 by the peroxidase element of COX to its corresponding alcohol concurrently causes an oxidation (Eling *et al.*, 1985). An assay was developed in which the spin-trapping agent CPH was oxidised to the stable adduct 3-carboxy-proxy (CP) under the action of the peroxidase. The adduct produced a characteristic signal, the intensity of which, could be recorded by EPR.

The specific experimental protocol is detailed in chapter three (section 3.2.1), but briefly, the COX assay was performed in 1 ml of sample mixture in Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 1.05 mM $MgSO_4$, 0.4 mM NaH_2PO_4, 12.5 mM $NaHCO_3$, 5.6 mM Glucose, 10 mM HEPES and 0.8 mM CaCl in dH_2O at pH 7.4). Ovine seminal COX-1 (100 units/ml; purchased from Sigma, Poole, UK) was incubated with an equimolar concentration of haematin for 5 min in an eppendorf tube at 37 °C prior to the assay start point to replace the haem lost in the purification process (Malkowski *et al.*, 2000).

Test drug (e.g. aspirin) was then added and incubated at 37 °C for a further 10 min before addition of the spin trapping agent, CPH. Immediately, a baseline reading was performed by EPR by taking a 50 µl sample from the incubation vessels containing the reaction mix into a glass micropipette tube (BLAUBRAND IntraMARK) which was then suspended within the core of the magnet (at 37 °C) in the EPR instrument. The intensity of the signal corresponding to formation of the radical adduct, 3-carboxy-proxyl was recorded by EPR spectrometry. The substrate AA (as sodium

salt; purchased from Sigma) was added after 2 min and three further EPR readings were taken 1.5, 4 and 6 min after the addition of AA. The suicidal nature of COX activation means that the period of activation is complete within approximately 1 min of AA addition.

The results were corrected for any auto-oxidation of spin-trap by subtraction of values recorded from a duplicate sample run in the absence of AA. Auto-oxidation was minimized by preparation of the spin trap, CPH, in ethylenediaminetetraacetic acid (EDTA) solution, which protected the spin trap against metal ion-induced oxidation.

The EPR signals were measured using a Miniscope (MS200) X-band spectrometer (Magnettech, Germany) with the following instrument settings: B0-field, 3356 Gauss; sweep width; 50 Gauss, sweep time, 30 sec; modulation amplitude, 1500 mGauss; microwave power, 20 mW.

2.2 Vasodilator Effects

2.2.1 Animals

Male Wistar rats (250-300 g) were purchased from Charles River (UK). They were allowed to settle for one week before being used for the experimental procedure. Animals were housed in groups in a controlled environment and fed a standard laboratory feed with water ad libitum.

2.2.2 Myography

Experiments were performed on thoracic aortas from male Wistar rats (250-300g). Rats were sacrificed by cervical dislocation and the aorta was then dissected free, excised and placed in ice-cold Krebs buffer of the following composition (mM): NaCl, 119; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.17; KH_2SO_4, 1.18; $NaHCO_3$, 25; EDTA, 0.027 and glucose, 5.5.

Adherent connective and adipose tissue was removed and the aorta was dissected into rings (approx 4 mm in length). Aortic rings were mounted onto fixed segment support pins of a Multi myograph with basal tension set to 80 mN (Mulvaney-Halpern, model 610M, Danish MyoTech, Aarhus, Denmark) connected to a MacLab (v 4e. AD Instruments, Hastings, UK). Once mounted, the rings were incubated at 37 °C in Krebs buffer bubbled with 5 % CO_2 in O_2 gas.

After rings were allowed to equilibrate for 20 min, 3 doses of KCl (125 mM in Krebs buffer) were introduced in order to test the contractile integrity of the rings. Vessels were washed with Krebs until fully relaxed between KCl additions and after the final addition. The endothelial integrity of the vessels was tested by testing endothelium-dependent relaxation in response to ACh (10 μM) before and after treatment with L-NAME (200 μM) to inhibit the activity of primarily eNOS. A relaxation after ACh of <10% was the criterion for L-NAME-induced inhibition.

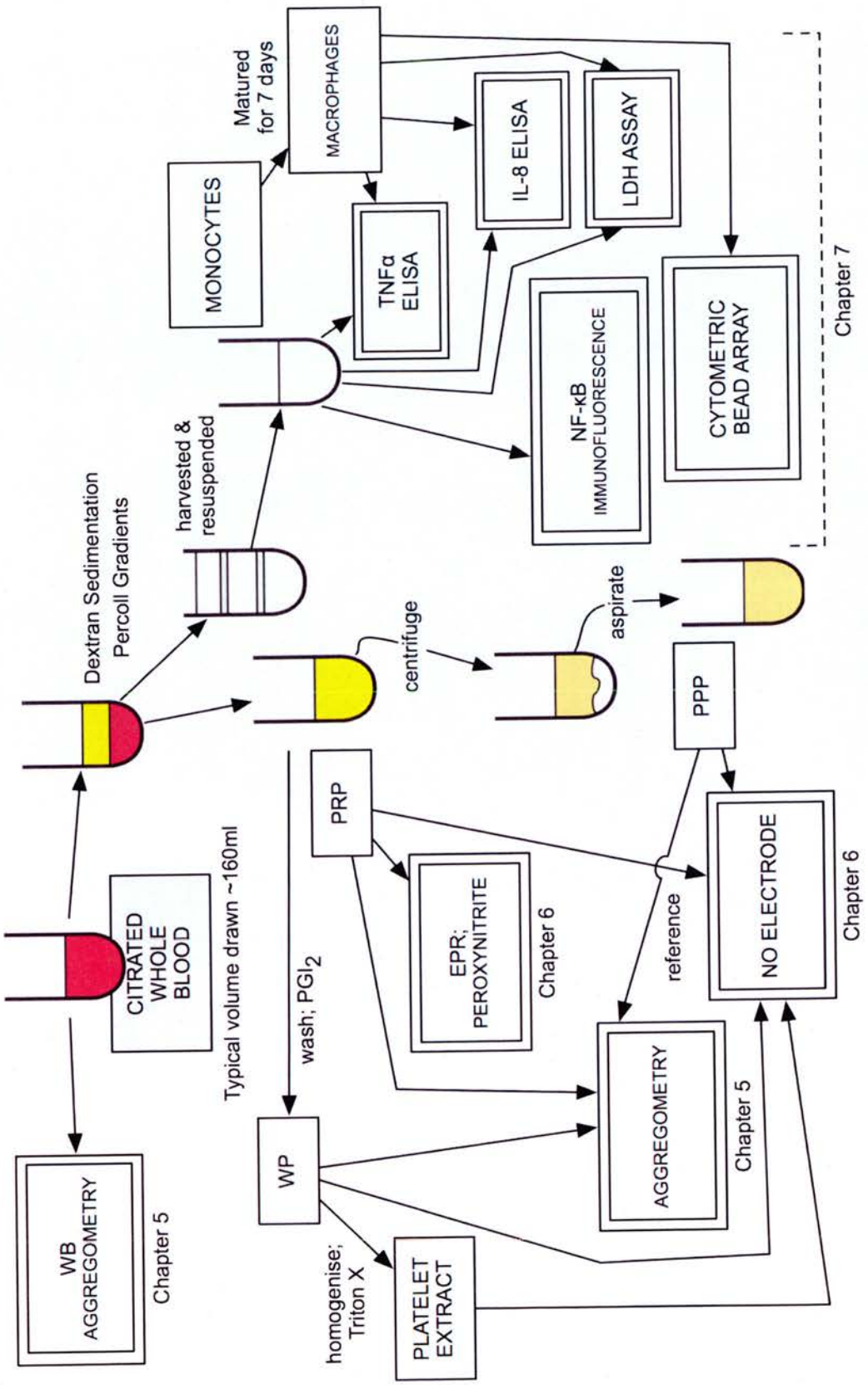
Following L-NAME-treatment, a concentration-response curve to α-1 adrenoceptor agonist phenylephrine (0.001 – 1 μM) was constructed for each ring and a suitable concentration selected to produce ~80% contraction (EC_{80}). This concentration was subsequently used for preconstricting the rings. The relaxation to increasing

concentrations of test compounds was investigated on rings preconstricted with phenylephrine as described in the individual experimental chapters.

2.3 Preparation of Platelets

Peripheral venous blood was drawn through a 19 gauge needle from the antecubital fossa of human volunteers who were non-smokers and had not taken any platelet-active agents during the previous 10 days. Blood was collected into 50 ml Falcon tubes containing 3.8 % sodium citrate and centrifuged at 350 *g* for 20 min at room temperature (RT) to obtain platelet rich plasma (PRP). This sample could be further centrifuged at 1200 *g* for 10 min to obtain platelet poor plasma (PPP). Washed platelet (WP) samples were obtained by adding 300 $ng.ml^{-1}$ prostacyclin to a 2 ml PRP sample before centrifuging at 1200 *g* for 10 min. The supernatant was then discarded and the pellet resuspended in Tyrode's buffer. Prostacyclin (300 $ng.ml^{-1}$) was again added before a second 10 min centrifugation (1200 *g*). Finally the pellet was resuspended in 2 ml Tyrode's buffer. The preparation and use of platelet products in this thesis (along with other blood products) is indicated in Fig. 2.2.

Fig. 2.2. Schematic diagram to show the preparation and use of human blood throughout this thesis.


WB
AGGREGOMETRY
Chapter 5
CITRATED
WHOLE
BLOOD
Typical volume drawn ~160ml
Dextran Sedimentation
Percoll Gradients
harvested &
resuspended
MONOCYTES
Matured
for 7 days
MACROPHAGES
TNFα
ELISA
IL-8 ELISA
LDH ASSAY
NF-κB
IMMUNOFLUORESCENCE
CYTOMETRIC
BEAD ARRAY
Chapter 7
centrifuge
aspirate
PRP
PPP
wash; PGI2
EPR;
PEROXYNITRITE
Chapter 6
reference
NO ELECTRODE
Chapter 6
WP
AGGREGOMETRY
Chapter 5
homogenise;
Triton X
PLATELET
EXTRACT

2.4 Platelet Aggregation

2.4.1 Turbidometric Platelet Aggregometry

Inhibition of platelet aggregation was measured using optical platelet aggregometry in a 4-channel aggregometer (CHRONO-LOG 470 VS, Labmedics) for the majority of the experiments described. Data were captured via an analogue digital converter (MacLab 4e, AD Instruments) and recorded using MacLab chart v3.3.7. Aggregation was recorded as a change in turbidity (light transmission) in PRP or WP measured against a PPP or Tyrode's buffer reference respectively.

Specific protocols are as described in the individual experimental chapters, but briefly, inhibition of aggregation by the various drugs was tested by incubating 0.5 ml aliquots of PRP or WP at 37 °C, stirring continuously at 1000 rpm. The samples were then treated with the test drug for 10 min before induction of aggregation with supra-maximal concentrations of collagen (2.5 $\mu g.ml^{-1}$). Aggregation was expressed as a percentage inhibition of control aggregation obtained with 2.5 $\mu g.ml^{-1}$ collagen (type I). Experiments were also performed with the addition of 50 μM of the cGMP inhibitor H-(1,2,4) oxadiazolo (4,3-a) quinoxallin-1-one (ODQ; Tocris Cookson, Bristol, UK), 15 min before addition of the test drug in order to determine the contribution of cGMP to any antiaggregatory effects.

2.4.2 Impedance Platelet Aggregometry

To measure the effects of the non-hybrid furoxan compound, B13, in whole blood, impedance platelet aggregometry was used. Blood samples were drawn by

venesection from the antecubital fossa of human volunteers. Collected blood was diluted 50:50 in PBS and placed in tubes incubated at 37 °C and stirred at 10000 rpm in a dual channel aggregometer (Chronolog Ca560, Labmedics), fitted with impedance electrodes. Aggregation was induced using 2.5 $\mu g.ml^{-1}$ collagen and responses were recorded for electrical impedance. Platelet aggregates between the two electrode probes impede the current between them and give a trace that is captured via an analogue digital converter (MacLab 4e, AD Instruments). Results were expressed as percentage inhibition of the response to a collagen only control. The precise protocol is detailed in section 5.2.1.1.

2.5 NO Release

2 ml samples of PRP, PPP, WP or Tyrode's buffer were incubated in a 2-channel aggregometer (Chronolog Ca560, Labmedics, Stockport, UK) at 37°C and stirred continuously at 6000 rpm. An isolated NO electrode (ISO-NO MARKII, World Precision Instruments, Stevenage, UK) was introduced into the cuvette and allowed to stabilise for at least 10 min until a stable baseline was achieved. Data were captured via an analogue digital converter (MacLab 4e, AD Instruments, Sussex, UK) and recorded using MacLab chart v3.3.7.

The instrument was calibrated using 2-(N,N-diethylamino)-diazenolate-2-oxide (DEA/NO; 0.1-1.6 μM; Fig. 2.3) in pH 4 buffer. DEA/NO is known to decompose rapidly at pH $\leq$ 5 to release two molecules of NO per molecule DEA//NO (Davies & Ren, 2001).

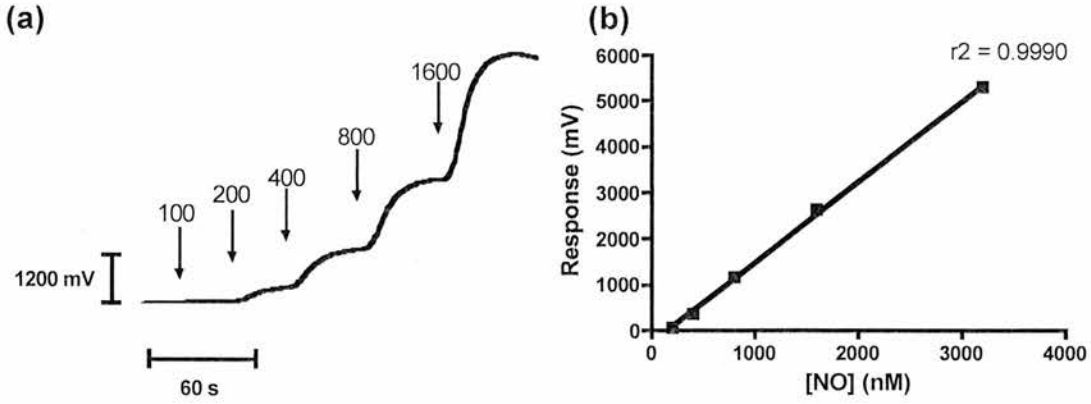


Fig. 2.3. (a) Example calibration trace and (b) graph for the NO electrode. Additions of DEA/NO (in nM) are indicated. Calibration assumes two molecules of NO are released per molecule of DEA/NO.

The precise experimental protocols are given in the individual experimental chapters, but briefly, the test drug was added to the cuvette and the release of NO was recorded for ten min before addition of the NO scavenger haemoglobin (10 μM) to confirm the contribution of NO to the signal.

In order to elucidate any involvement of endogenous reducing agents in the release of NO from the NO-aspirins, experiments were also carried out in Tyrode's buffer, reconstituted with approximate plasma and intracellular concentrations (as indicated in Table 2.1) of albumin and glutathione (GSH) and ascorbate, both alone, and in combination.

PLASMA	INTRACELLULAR
4 % albumin	Tyrode's buffer
3 μM GSH	3 mM GSH
50 μM ascorbate	1 mM ascorbate*

Table 2.1. Table to show the concentrations of endogenous reducing agents (GSH and ascorbate), both intracellularly and in plasma. To investigate the release of NO under plasma or intracellular conditions, 4% albumin or Tyrode's buffer, respectively, was reconstituted with the reducing agents at the concentrations indicated, both alone and in combination. *Only in the presence of 20 mM HEPES to buffer its acidic pH.

A platelet extract was also prepared from samples of PRP. A platelet count was determined using a Coulter A^c.T 8 Hematology Analyzer (Coulter Electronics, Luton, UK). The platelets were washed as above in the presence of prostacyclin (300 ng.ml^{-1}) to obtain a platelet pellet. The total platelet volume of the pellet was calculated by multiplying the number of platelets by average platelet volume (5.14×10^{-15} L; obtained from the haematology analyzer). The pellet was resuspended in 1 ml of 0.5 % Triton X in Tyrode's buffer for 1 h and the dilution of cell contents estimated using the above calculation. This was then homogenized by hand for 15 min and centrifuged at 12000 *g* for 10 min to give a cell extract. NO-aspirin hybrids were added to 1 ml of this supernatant in the assay as described above. Experiments were also carried out in the presence of the thiol alkylator, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), to determine the necessity of thiols in the release of NO.

Owing to the different profile of NO release by the various drugs, it was established that area under the curve (AUC) was more representative of NO release than peak concentration in these experiments.

2.6 Peroxynitrite Release

Due to its free radical nature, NO can rapidly react with reactive oxygen species (ROS). One particular example is the reaction with the ROS, superoxide (O_2^-) to form peroxynitrite ($ONOO^-$; equation 1; Saran *et al.*, 1990):

$$NO + O_2^- \longrightarrow ONOO^- \quad \textbf{(1)}$$

The enzyme superoxide dismutase (SOD) will prevent formation of peroxynitrite by removing superoxide through catalyzing its conversion to hydrogen peroxide (H_2O_2; equation 2):

$$O_2^- + 2H+ \xrightarrow{SOD} O_2 + H_2O_2 \quad \textbf{(2)}$$

Two sets of experiments were undertaken to determine whether the compounds B8 and B7 generate any peroxynitrite in addition to NO.

It has been demonstrated that EPR, using the spin trap agent, CPH, is an effective method of trapping peroxynitrite (Dikalov *et al.*, 1997). Samples of PRP were incubated with the test compound (e.g. B8, B7) and CPH ± SOD (500 $U.ml^{-1}$) at 37 °C. Control samples were performed without the test compound, and also, with the known peroxynitrite generator GEA 3162 (Taylor *et al.*, 2004). EPR readings (as described in section 2.1.1) were taken at 1, 10, 20, 30, 60 and 120 min and formation of the adduct 3-carboxy-proxyl recorded.

Experiments were also carried out using the NO electrode with PRP and PPP samples treated with B8 or B7. SOD (500 $U.ml^{-1}$) was added in order that it would unmask any further NO that might otherwise have formed peroxynitrite.

2.7 Preparation of Monocytes and Macrophages

Mononuclear cells were isolated from human blood using dextran sedimentation and Percoll gradients as follows: peripheral venous blood was drawn from the antecubital fossa of human volunteers (non-smokers; age 20-45. Fig. 2.2). Blood was collected into 50 ml Falcon tubes containing 3.8 % sodium citrate and centrifuged at 350 *g* for

20 min at RT. Following aspiration of the PRP, leukocytes were separated from the erythrocyte pellet by dextran sedimentation. 2.5 ml dextran (6 % in 0.9 % NaCl; Dextran 500 000, Pharmacia, Kent, UK) was added per 10 ml of pellet and the volume made up to 50 ml with saline, pre-warmed to 37 °C. This was left to sediment for not longer than 30 min at RT. The leukocyte rich upper layer was removed and transferred to a 50 ml Falcon tube and made up to 50 ml with saline, pre-warmed to 37 °C before centrifugation at 350 *g* for 6 min. The pelleted cells were resuspended in 2.5 ml of 55 % Percoll (Pharmacia; in PBS without Ca^{2+}).

Percoll gradients were prepared by overlaying 2.5 ml differing densities of Percoll at RT. The bottom layer was 89 % Percoll made in PBS (without Ca^{2+}). This was overlaid with 2.5 ml 68 % Percoll, and the 55 % layer containing the leukocytes was layered on top. The gradients were centrifuged at 700 *g* for 20 min. Mononuclear cells were harvested from the 55/68 % interface, washed twice in PBS without Ca^{2+} and then resuspended at a concentration of 4×10^6 cells/ml in ISCOVES DMEM (Sigma).

Autologous recalcified plasma (serum) was prepared by addition of 220 µl 1 M $CaCl_2$ to 10 ml of PRP in a sterile glass tube. This was incubated for 1 h at 37 °C. The serum was then separated from the platelet plug and refrigerated at 4 °C in a plastic tube.

2.8 Inflammatory Marker Studies

Mononuclear cells were plated out in 48-well plates at a concentration of 2×10^6 per well. After 1 h, non-adherent cells were removed by washing wells with Hank's

buffered salt solution (HBSS). Macrophages were derived from monocytes by culturing the monocytes in ISCOVES DMEM (supplemented with 10 % autologous serum) at 37 °C for a week, with the medium being changed after 3-4 days.

On day 7, the medium in each well was changed and fresh medium containing a test drug or DMSO vehicle added to wells with or without the inflammatory stimulant, LPS (1 – 100 $ng.ml^{-1}$ for pilot CBA studies. 10 $ng.ml^{-1}$ for all further studies). Cells were then incubated at 37 °C for 4 h before removal of the cell supernatants, which were subsequently frozen at -70 °C for future studies.

The same procedure was also carried out on mononuclear cells which had not been matured into macrophages with drug treatments and 4 h incubations taking place immediately after the final cell washing step of the blood preparation.

2.8.1 Cytometric Bead Array (CBA)

A human inflammation cytometric bead array kit that detects levels of IL-8, IL-1β, IL-6, IL-10, TNFα and IL-12p70 was purchased from BD Biosciences (Oxford, UK; cat no. 551811). The protocol was carried out as per kit instructions on supernatants from macrophages treated as described in section 6.2.2. The technique is based on using a series of spectrally discrete particles to capture and quantitate various soluble inflammatory markers. A mixed population of six beads with distinct fluorescence intensities, each coated with a specific antibody for either IL-8, IL-1β, IL-6, IL-10, TNFα and IL-12p70 were incubated with the test samples. The antibodies were conjugated with the fluorochrome phycoerythrin (PE), the fluorescence of which is detectable by flow cytometric analysis (FL3 channel of BD FACScan).

2.8.2 Enzyme-Linked Immunosorbent Assays (ELISAs)

Human TNFα and IL-8 ELISA kits were purchased from BD Biosciences (cat no; 550610 and 550999 respectively) and performed as per kit instructions on the supernatants removed from macrophage or monocyte plates. These TNFα and IL-8 ELISAs utilise a monoclonal antibody specific for TNFα (or IL-8) coated onto a 96-well plate. Any TNFα (or IL-8) present in the test samples binds to the immobilized antibody. The wells were then washed and a streptavidin-horseradish peroxidase conjugate mixed with a biotinylated anti-human TNFα (or IL-8) antibody added, giving an antibody-antigen-antibody "sandwich". A colour agent was incubated which produces a blue colour in direct proportion to the amount of TNFα (or IL-8) present in the initial sample. Finally, after a set time, a stop solution was used to stop this reaction, changing the colour from blue to yellow. The absorbances of each well were then read at 450 nm using a Thermo Labsystems Multiskan Ascent plate reader running Ascent software Version 2.6.

2.8.3 Lactate Dehydrogenase (LDH) Assay

The cytotoxic impact of the compounds was assessed by measuring the quantity of the enzyme LDH in the supernatant. This is a stable cytoplasmic enzyme present in all cells, which is rapidly released following damage to the plasma membrane. A kit was purchased from Roche (cat no 1 644 793) to quantify cell death by LDH quantification in the supernatants from treated cells (section 2.8). The kit works by a two-step reaction in which LDH catalyses conversion of a tetrazolium salt to a coloured formazan salt that absorbs light at 490 nm. The absorption recorded at 490 nm using a Thermo Labsystems Multiskan Ascent plate reader is proportional to the

number of damaged cells. The exact experimental protocol was as described in section 7.2.4.

2.8.4 Immunofluorescence for NF-κB p65 Subunit

NF-κB is a transcription factor responsible for the control of the gene expression of proinflammatory cytokine, TNFα. Normally, NF-κB is kept in an inactive state in the cytoplasm, bound to an inhibitory subunit named IκB. NF-κB exists as a dimer (can be either a homodimer or heterodimer). The most common form is a heterodimer composed of the p65/p50 subunits (Panwalkar *et al.*, 2004). The phosphorylation of IκB and its subsequent degradation leads to the activation of NF-κB. The NF-κB dimer containing the p65 subunit; the dominant factor in the induction of the TNFα gene, then translocates from the cytoplasm to the nucleus where it activates transcription of target genes such as TNFα (Baldwin, 1996; Kaltschmidt *et al.*, 1999; Liu *et al.*, 2000; Totzke *et al.*, 2006).

Immunofluorescence for the p65 subunit was used to visualise the translocation of NF-κB. Mononuclear cells were isolated and resuspended as described in section 2.7. 4×10^6 (1ml) cells were placed on a glass coverslip within a 6-well plate and left in an incubator (37 °C; 1 h) to adhere. Non-adherent cells were then removed by washing wells with HBSS. Cells were treated with the test compound and stimulated with LPS as described in chapter seven (section 7.2.5). The cell-covered coverslips were then washed 3 times with PBS and 1 ml of 3 % paraformaldehyde (in deionised water; dH_2O) was added and the cells left to fix (RT) for 20 min. Coverslips were then washed again 3 times with PBS and then incubated for 10 min at RT with 1 ml of 50 mM glycine to quench aldehyde groups. Following another 3 PBS washes, 1

ml of blocking solution (10% sheep serum in 0.2 % fish skin gelatin (Sigma)) was added to the coverslips and they were left overnight at 4 °C.

The following morning, after washing the cells, 100 µl of primary antibody (NF-κB p65 mouse anti-human (BD Biosciences, cat no. 610868); 1:50 dilution in blocking solution) was added to the cells. An hour later, following three PBS washes, 100 µl of a 1:250 dilution (in blocking solution) of secondary antibody (Alexa Fluor® 488 goat anti-mouse IgG; Invitrogen, cat no. A-11001) was added and left to incubate for 1 hr at RT in the dark. The green fluorescent secondary antibody is FITC-labelled and is excited at 488 nm and excited at 530 nm.

Finally, three PBS washes, followed by three dH_2O washes were carried out to prevent crystal formation. Coverslips were then mounted onto slides using Moviol (Calbiochem, Merck, Nottingham, UK) and the slides were then stored in the dark at 4 °C.

2.8.4.1 Microscope Image Capturing

Images from the immunofluorescence slides were captured with a camera connected to a Zeiss Axiovert S100 microscope using Improvision Openlab 3.1.5 software. Images were captured at a magnification of x100 using oil immersion.

2.9 Materials

General laboratory reagents (such as buffer salts) were supplied by Sigma and Fisher. Other reagents were diluted in either Tyrode's buffer, PBS, DMSO, dH_2O, 0.01 M NaOH or ISCOVES DNEM. Vehicles and suppliers are detailed in Table 2.2.

Company locations are listed in Table 2.3. Furoxan-aspirins, furoxan compounds, furazan-aspirins and NCX4016 were synthesized (Cena *et al.*, 2003) and generously supplied by Università degli studi di Torino, Torino, Italy.

REAGENT	VEHICLE	SUPPLIER
AA	DMSO	Sigma
Albumin	Tyrode's buffer	Sigma
Ascorbate	Tyrode's buffer	Sigma
Aspirin	PBS	Sigma
Collagen (type I; fibrils; equine)	Supplied as water soluble solution (suspension)	Labmedics
COX-1; ovine	Supplied as water soluble solution	Sigma
CPH	10 mM EDTA in dH_2O**	Axxora
cPTIO	PBS	Sigma
DEA/NO	0.01 M NaOH	Axxora
DTNB	DMSO	Sigma
Furoxans/Furoxan aspirin	1% DMSO in PBS	University of Turin
Glutathione	Tyrode's buffer	Sigma
Gliotoxin	ISCOVES DNEM	Sigma
Haemoglobin	H_2O*	Sigma
Hematin (bovine)	DMSO	Sigma
Human serum albumin	Tyrode's buffer	Sigma
Indomethacin	1% DMSO in PBS	Sigma
L-NAME	Krebs	Sigma
LPS	ISCOVES DNEM	Sigma
NS-398	1% DMSO in PBS	Sigma
ODQ	DMSO	Tocris Cookson
PGI_2	PBS	Sigma
Phenylephrine	Krebs	Sigma
Salicylic Acid	PBS	Sigma
SOD	PBS	Sigma
Triton-X-100	Tyrode's buffer	Sigma

Table 2.2. Suppliers and vehicles for reagents.
* Met-haemoglobin was reduced to the ferro form by sodium dithionate (57.4 μM), and excess dithionate was then removed by dialysis (Martin *et al.*, 1985).
** Minimises metal ion-induced auto-oxidation of the spin trap.

COMPANY	LOCATION
Axxora	Nottingham, UK
BD Biosciences	Oxford, UK
Fisher	Loughborough, UK
Labmedics	Stockport, UK
Pharmacia	Kent, UK
Sigma Aldrich	Poole, UK
Tocris Cookson	Bristol, UK
World Precision Instruments	Stevenage, UK

Table 2.3. Companies and locations.

2.10 Statistics

Statistical analyses are as stated in the individual experimental chapters. All statistical tests were performed using GraphPad Prism version 4 (GraphPad Software, San Diego, USA). * is used to represent a p value between 0.01 and 0.05, ** represents a p value of 0.001 to 0.01 and *** represents p values less than 0.001. P values greater than 0.05 were deemed not significant. Where expressed, data are in the form mean ± standard error of the mean (S.E.M.).

2.11 Figures

Graphs for this thesis were produced using GraphPad Prism version 4. Other diagrams and figures were produced using OmniGraffle version 4 (The Omni Group, Seattle, USA) and Illustrator CS2 (Adobe, Uxbridge, UK). Structural formulae were drawn using ChemDraw version 10.0 (CambridgeSoft, Cambridge, MA, USA).

CHAPTER THREE

Assessment of the COX-1 Activity of Furoxan-Aspirin Hybrids by a Novel EPR-Based Assay

3. Assessment of the COX-1 Activity of Furoxan-Aspirin Hybrids by a Novel EPR-Based Assay

3.1 Introduction

Prostaglandins are derived from arachidonic acid (AA) in a pathway dependent on the Prostaglandin H_2 synthase (PGHS; EC 1.14.99.1) family of enzymes. PGHS is more commonly known as COX, referring to the first enzymatic activity of the enzyme. COX converts AA to prostaglandin H_2, the precursor of all prostanoids. The enzyme contains two active sites: a COX site, where AA is converted into the hydroperoxy endoperoxide, prostaglandin G_2 (PGG_2), and a haem with peroxidase activity that reduces PGG_2 to PGH_2 (Chandrasekharan & Simmons, 2004).

Two structurally similar COX isoforms exist (COX-1 and COX-2) which are encoded by different genes and the expression of which varies between tissues (Simmons *et al.*, 2004). COX-1 is often referred to as the 'house-keeping' isoform due to its regulatory functions in many tissues. COX-2 is virtually undetectable under normal conditions in most tissues and is often referred to as the 'inducible' isoform due to its tendency to be expressed in response to inflammatory stimuli. The exception to this is in the brain and spinal cord, where COX-2 is constitutively expressed and plays a role in nociception signalling (Hoffmann, 2000).

The importance of COX as a therapeutic target has long been highlighted by the actions of aspirin (Vane, 1971; Vane & Botting, 2003), the first drug of the family of NSAIDs for use as analgesics, anti-inflammatory agents and antithrombotic agents. In contrast to other NSAIDs, such as indomethacin, which reversibly bind at the COX active site (Dannhardt & Kiefer, 2001), aspirin causes an irreversible inhibition of COX by rapidly and selectively acetylating the hydroxyl group of a serine residue (Ser 530) near the C-terminus of the enzyme, forming an impediment to the binding of AA (DeWitt & Smith, 1988; Roth & Majerus, 1975; Roth *et al.*, 1975). The ensuing irreversible COX inhibition requires *de novo* synthesis of the enzyme for subsequent production of prostaglandins.

The alteration of the chemical structure of aspirin, as has occurred in the generation of the NO-aspirins, may impede its activity. It is therefore vital to ensure the activity of the parent compound is not prevented, and thus, an assay to check that the NO-aspirins retain a COX-inhibitory action was developed.

It is surprising that relatively few assays for the activity of the COX enzyme have been developed. Amongst the most popular techniques is the measurement of thromboxane B_2 (TXB_2), the stable metabolite of one of PGH_2–derived TXA_2, as a marker of COX activity (Gierse *et al.*, 2005). Alternatively, measurement of oxygen consumption using an oxygen electrode (Gierse *et al.*, 1999; Kulmacz & Wang, 1995), assays using radio-labelled substrate (Schneider *et al.*, 2005) and immunoassays for the prostaglandin products (Gierse *et al.*, 1999) can also be applied. More recently, a commercial chemiluminescent assay (Axxora, Nottingham, UK) has been developed for use on purified enzyme. The assay involves the use of

labelled substrate that generates a luminescent product under the action of the hydroperoxidase element of COX, most likely via the generation of oxidising free radicals.

EPR (methods chapter, section 2.1) has been previously utilised by others to measure free radicals generated by COX as a measure of its activity (Schreiber *et al.*, 1989; Tsai *et al.*, 1992). However these techniques can involve complex mechanisms to trap the short-lived radical species. For instance, in an *in vitro* assay using purified COX, liquid nitrogen was utilised to stop the reaction and was required to stabilise the tyrosyl radical species generated, in order that it could be recorded by EPR (Tsai *et al.*, 1992). Another technique which recorded COX activity in mouse keratinocytes relied on the use of the antioxidant glutathione to stabilize the generated radical before trapping it with DMPO (Schreiber *et al.*, 1989).

A convenient novel assay was therefore developed in order to assay the activity of COX-1. The reduction of PGG_2 by the peroxidase element generates the corresponding alcohol. This reaction has previously been demonstrated to concurrently oxidise aminopyrine molecules to aminopyrine free radicals (Eling *et al.*, 1985). Here, a spin-trapping agent, CPH, is oxidised to $CP^{\bullet}$ under the action of the peroxidase (Fig. 3.1), in a similar fashion to that previously seen with aminopyrine. CP is a stable adduct which is detectable by EPR.

The studies in this chapter were carried out in order to validate this technique for assaying COX-1 activity *in vitro*, in order that it could be further utilised to test the hypothesis that the newly developed NO-aspirin compounds retain an anti-COX activity.

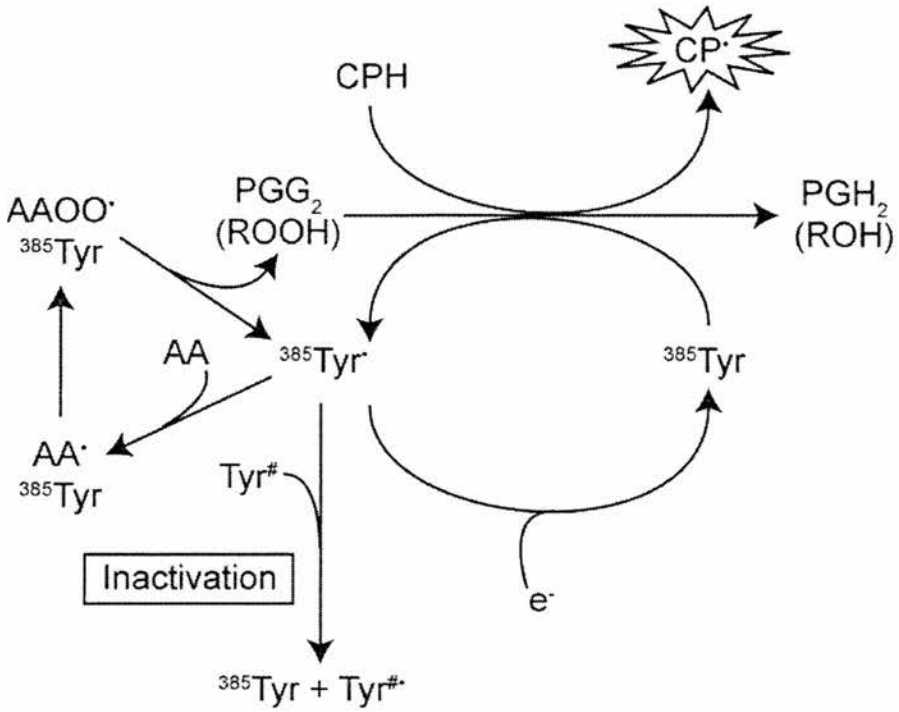


Fig. 3.1. Schematic diagram showing the peroxidase activity of COX. The process requires prior formation of a tyrosine radical from a tyrosine residue in close proximity to the haem group (Tyr 385). The tyrosyl radical is either recycled or participates in the suicide inactivation of the enzyme (Smith *et al.*, 2000). Following incorporation of oxygen and formation of PGG_2, the peroxidase reduces the peroxyl moiety to the equivalent alcohol. The process allows for the concomitant oxidation of spin-trap CPH to CP, which is detected by EPR.

3.2 Methods

3.2.1 Measurement of COX-1 Activity

The activity of COX-1 was assessed using a novel EPR-based assay. The assay was performed at 37°C in 1 ml of Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 1.05 mM $MgSO_4$, 0.4 mM NaH_2PO_4, 12.5 mM $NaHCO_3$, 5.6 mM Glucose, 10 mM HEPES and 0.8 mM $CaCl_2$ in deionised water at pH 7.4). 100 units/ml ovine seminal COX-1 was incubated with 1.5 μM haematin (5 min, 37 °C) prior to the assay. Data from the manufacturers of the COX-1 reveal that 1 unit of enzyme consumes 1 nanomole of oxygen at 37 °C in the presence of 1 μM haematin and 100 μM AA. 10 μl of the stock furoxan-aspirin, their furazan equivalent, NCX4016, indomethacin, NS398 (Merck Biosciences, Nottingham, UK), aspirin or salicylic acid (SA) or a vehicle control (DMSO, 1 %) was added and left to incubate for a further 10 min prior to addition of the spin-trap, CPH (1 mM; as described in section 2.1). Final drug concentration investigated in this study was 100 μM for all drugs. Further concentrations (10 μM and 1 mM) of aspirin and salicylic acid were also investigated. At this point (time = -2 min), a baseline EPR measurement was taken (MS200, Magnettech, Germany. Instrument settings: B0-field, 3356 Gauss; sweep width; 50 Gauss, sweep time, 30 sec; modulation amplitude, 1500 mGauss; microwave power, 20 mW). After 2 min (time = 0), 100 μM AA (as sodium salt) was added. Further EPR readings were taken at 1.5, 4 and 6 min. The suicidal nature of COX-1 activation means that the period of activation is complete within ~1 min of AA addition (Simmons *et al.*, 2004).

The results are corrected for any auto-oxidation of spin-trap by subtraction of values recorded from a duplicate sample run in the absence of AA. The intensity scale on the y-axis of all graphs is an arbitrary scale based upon the area under the curve of the first derivative traces generated.

3.2.2 Statistics

Statistical analysis was by 2-way ANOVA or by 1-way ANOVA and Dunnett's post-test carried out using GraphPad Prism version 4. * is used to represent a p value between 0.01 and 0.05, ** represents a p value of 0.001 to 0.01 and *** represents p values less than 0.001. P values greater than 0.05 were deemed not significant. Where expressed, data are in the form mean ± s.e.m.

3.3 Results

3.3.1 Time-dependent Adduct Generation by COX-1

Addition of AA caused a time-dependent increase in the characteristic 3-line EPR spectrum for a spin-adduct with the unpaired electron in the vicinity of a nitrogen atom. Traces of the 3-line spectrum of the CP adduct obtained with a control sample and samples containing COX-inhibitor aspirin can be viewed in Fig 3.2.

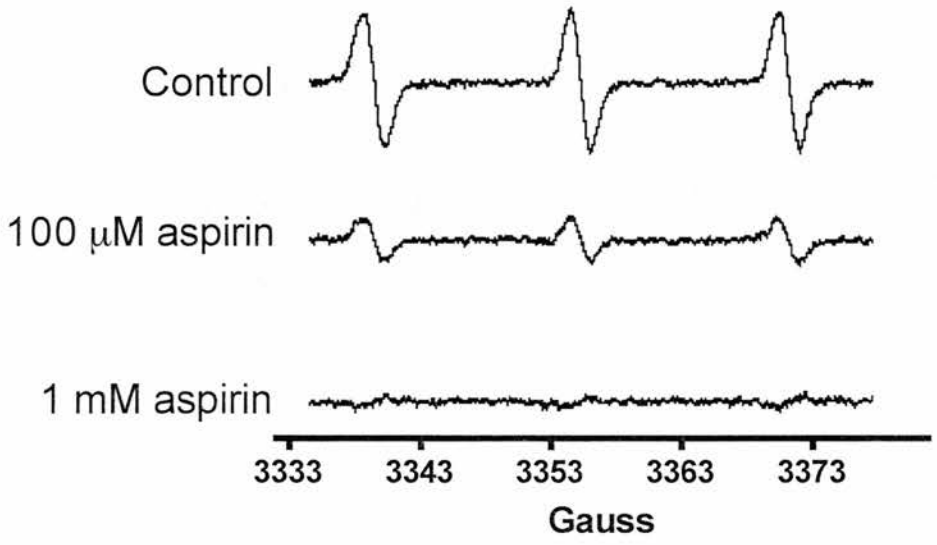


Fig. 3.2. Sample EPR spectra obtained in the absence (control; COX + AA) and presence of aspirin (100 mM or 1mM) after correction for background autoxidation. EPR settings: B0-field, 3356 Gauss; sweep width; 50 Gauss, sweep time, 30 sec; modulation amplitude, 1500 mGauss; microwave power, 20 mW.

The majority of the reaction was complete by the time the first reading was taken. The signal developed rapidly before the first reading (244 intensity units.min^{-1}), but subsequently slowed to a relatively constant rate (65 intensity units.min^{-1}) over the following 4.5 min of the assay (Fig 3.3. n = 9-12); the equivalent experiment without AA failed to show the initial rapid rise and was significantly lower than the AA-treated sample throughout ($p=0.02$, 2-way ANOVA, repeated measures).

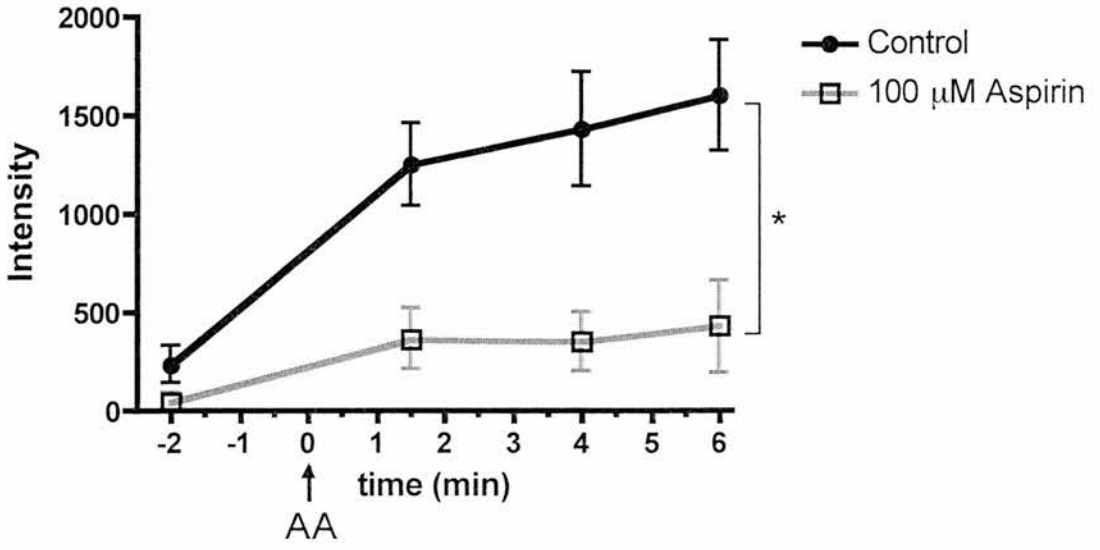


Fig. 3.3. Mean data for development of EPR signal intensity (AU) with time in the absence (control; COX + AA) and presence of aspirin (100 mM). In both cases, substrate (AA) was added at t=0 min. P=0.02, 2-way ANOVA, repeated measures: n=9-12. Values are mean ± SEM.

From these data, it was determined that the point t=1.5 min was an appropriate point at which to compare spin-adduct generation between control and NSAID-treated COX-1, given that spin-adduct generation in response to AA had peaked – subsequent adduct formation was at an equivalent rate in control and AA-treated samples and was likely to be due to non-specific auto-oxidation of CPH.

3.3.2 Inhibitory Effect of Aspirin and Salicylic Acid

The impact of pre-incubation of COX-1 with different concentrations of aspirin (10 μM – 1 mM) is shown in Fig 3.4. The lower end of this range was representative of the known therapeutic range for human antiplatelet effects and the higher end for aspirin-mediated anti-inflammatory effects (Mitchell *et al.*, 1993; Osnes *et al.*, 1996; Sils *et al.*, 1988). Results indicate that aspirin concentrations of 100 μM and 1 mM caused significant inhibition of spin-adduct formation at the 1.5 min time-point (to 72 ± 11 and 100 ± 16 % of control respectively; $P<0.05$ for both compared to control, 1-way ANOVA followed by Dunnett's post-hoc analysis).

Parallel experiments with SA (10 μM – 1 mM; n = 6) showed that a 10 min pre-incubation with SA failed to significantly inhibit generation of the spin-adduct, even at the highest concentration ($P>0.05$).

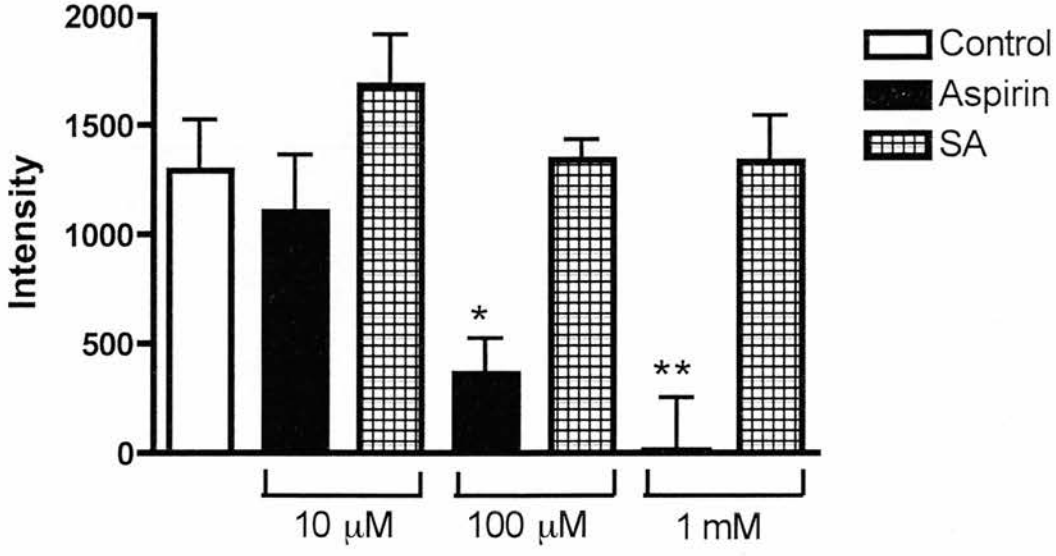


Fig. 3.4. Effect of aspirin and salicylic acid (10 μM – 1 mM) on EPR signals generated from COX-1 after treatment with substrate (AA). In each case, incubations with aspirin or SA were for 10 min prior to the baseline EPR reading (t=-2 min, not shown). AA was added at t=0 min and readings shown were taken at t=1.5 min.*P<0.05, **P<0.01; 1-way ANOVA with Dunnett's post-hoc test vs. control: n=8-10. Values are mean ± SEM.

3.3.3 Comparative Pharmacology of COX-1 and COX-2 Inhibitors

The assay was further verified by the screening of a COX-2 selective inhibitor, NS398 (Fig. 3.5). Equivalent concentrations (100 μM) of the recognized non-selective COX inhibitors, aspirin and indomethacin both significantly ($P<0.05$ and $P<0.01$ respectively; 1-way ANOVA with Dunnett's post-hoc test) inhibited generation of the EPR-detectable spin adduct at 1.5 min (72 ± 11 and 114 ± 10 % inhibition of control response respectively), but the COX-2-selective inhibitor, NS398 had no effect ($P>0.05$) on this assay of COX-1, despite its use at a concentration which is in excess of that required to significantly inhibit COX-2 (Futaki *et al.*, 1994).

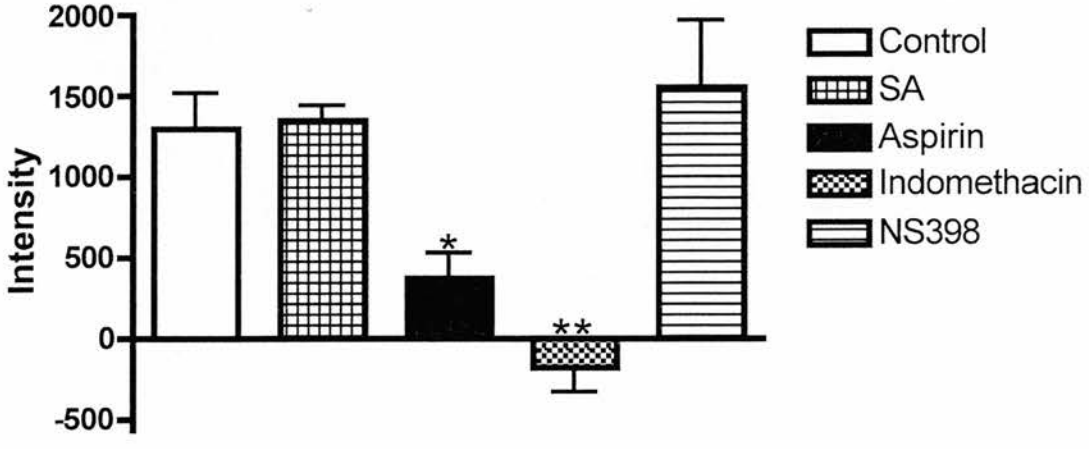


Fig. 3.5. Comparative effects of SA and recognized NSAIDs (all 100 μM) on EPR signal intensity measured at t=1.5 min. *P<0.05, **P<0.01; 1-way ANOVA with Dunnett's post-hoc test vs. control: n=6-10. Values are mean ± SEM.

3.3.4 Inhibitory Effect of NO-Aspirin Compounds

A significant inhibition of COX activity was observed with the furoxan-aspirins, B8 and B7 (100 μM; $p<0.05$ and $p<0.01$ respectively; 1-way ANOVA with Dunnett's post-hoc test vs. control, n=6). Even greater inhibition was demonstrated with the NO-free furazan equivalents, B16 and B15 (100 μM, $p<0.01$; 1-way ANOVA with Dunnett's post-hoc test vs. control for both, n=6). The furazan compounds demonstrated a significantly greater inhibition of COX than aspirin ($p<0.01$ for both as assessed by Student's t-test). No significant difference was observed between inhibition with the furoxan hybrids and their furazan equivalents ($p>0.05$ for both, as assessed by Student's t-test). The nitrooxy-ester, NCX4016 (100 μM) also abolished COX activity ($p<0.01$; 1-way ANOVA with Dunnett's post-hoc test vs. control, n=6). The NO donor DEA/NO (100 μM) failed to significantly inhibit generation of the spin-adduct (100 μM, n=6; Fig 3.6).

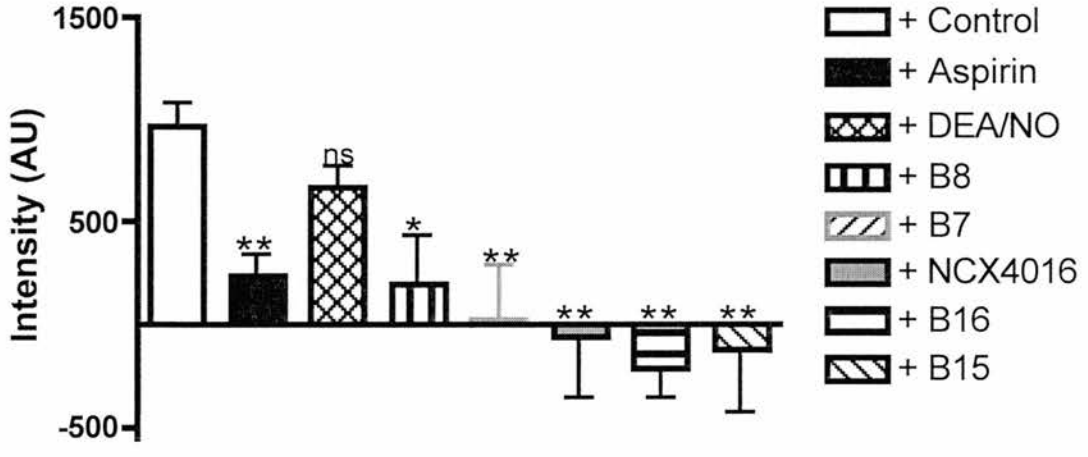


Fig. 3.6. Effect of aspirin, furoxans, furazans and NCX4016 (all 100 μM) on EPR signals generated from COX-1 after treatment with substrate (AA). In each case, drug incubations were for 10 min prior to the baseline EPR reading. Readings shown were taken 1.5 min after the addition of AA. *P<0.05, **P<0.01; 1-way ANOVA with Dunnett's post-hoc test vs. control: n=6-10. Values are mean ± SEM.

3.4 Discussion

In order to investigate the whether the hybrid drugs retained a COX-inhibitory action *in vitro*, a simple, reliable assay was first developed and verified.

3.4.1 Assay Development and Verification

The principle of the assay was based on the concomitant oxidation (Fig. 3.1) which occurs with the reduction of PGG_2 to its corresponding alcohol by the peroxidase element of COX (Eling *et al.*, 1985). This oxidation can be exploited in the absence of antioxidant GSH to oxidise spin-trapping agent CPH to the stable adduct, $CP^{\bullet}$, which generates a characteristic 3-line EPR spectrum. The amplitude of the EPR signal is proportional to the amount of adduct generated.

Results indicated that isolated COX-1 enzyme generated sufficient adduct generation upon addition of the enzyme substrate, AA, to be easily detectable by EPR. The initial peak in the detected signal recorded at the first reading, had by the subsequent time-points slowed to a rate that was equivalent to the signal generation from substrate-free enzyme, most likely due to autoxidation of the spin trap. The loss of specific enzyme-mediated adduct generation is unsurprising, given the well-recognised suicidal nature of activated COX-1 (Simmons *et al.*, 2004; Smith *et al.*, 2000). From these time-course experiments, a 1.5 min time-point was selected for subsequent comparative studies, because by this time the AA-dependent adduct generation was complete but the signal was not significantly enhanced by autoxidation of the spin trap.

Aspirin pretreatment (10 min) was shown to have a concentration-dependent

inhibitory effect in the published range (Mitchell *et al.*, 1993), but the acetyl-free counterpart, SA, failed to significantly inhibit enzyme activity. The lack of effect of SA indicates that aspirin-mediated inhibition of the enzyme is dependent on the acetyl group, the moiety involved in COX inhibition, and not due to a non-specific antioxidant effect. SA was not expected to have any effect in this assay because it is known not to affect COX-1 or 2 activity (Wu, 2000) except in intact cells (Mitchell *et al.*, 1993), possibly by suppressing COX-2 transcription in response to exogenous stimuli (Xu *et al.*, 1999). Furthermore, the reversible, non-specific COX inhibitor, indomethacin (Kulmacz & Lands, 1985), was demonstrated to have a powerful inhibitory effect. Addition of the COX-2 specific inhibitor, NS398, failed to cause inhibition. NS398 specifically inhibits COX-2 by inducing a slow structural transition of the enzyme upon binding that results in significant and irreversible inhibition of COX-2 (Copeland *et al.*, 1994). Unsurprisingly, it had no effect in this assay of COX-1, despite being used at a concentration known to inhibit COX-2 (Futaki *et al.*, 1994). Signal intensities shown in the figures are after subtraction of an AA-free control from a parallel experiment to account for autooxidation of the spin trap. A negative signal (as observed with indomethacin for example) therefore indicates that the control value was greater than the AA-treated sample, which might suggest that AA has a slight antioxidant effect on its own.

The assay was sensitive to the inhibitory effects of conventional NSAIDs (aspirin and indomethacin) and not to SA or a COX-2 specific inhibitor. These results confirmed the assay to be a relevant screen for NSAIDs with different modes of inhibitory action and therefore it was used to screen the NO-aspirin compounds.

3.4.2 COX-inhibitory Effect of NO-Aspirin Compounds

Both the nitrooxy-ester NCX4016, and the furoxan-aspirin hybrids were demonstrated to significantly inhibit COX-1. The alterations to the chemical structure by the hybrids perhaps play a role in bringing about the inhibition that was observed to be greater than that by the unaltered aspirin compound. The powerful inhibitory effect of the furoxan-aspirin hybrids in the purified COX-1 assay, together with the equally powerful effects of the furazan equivalents and the lack of significant inhibition observed with the NO donor DEA/NO, suggests that the COX-1-inhibitory activity of B8 and B7 is more likely to be aspirin-mediated than NO-mediated.

This assay demonstrates that the furoxan-aspirin hybrids retain an aspirin-like action *in vitro*. At face value, these data would indicate that the compounds have potential to be antithrombotic through inhibition of COX-1. However, it is important to recognize that the COX-inhibitory effect might be lost in biological media or *in vivo*, depending on how these compounds are hydrolysed under physiologically relevant conditions. Previous studies (Cena *et al.*, 2003) have demonstrated complete hydrolysis of the furoxan-aspirin acetyl group in serum. This feature would suggest that in plasma, there would be at least a partial loss of the acetyl group, resulting in formation of the SA equivalent. As SA was demonstrated to have no effect in this assay of COX activity, any loss of acetyl group may prove to limit the effectiveness of the drug. Other groups have also demonstrated that COX inhibition by the hybrid NCX4016 has a similar requirement for the acetyl-group but not the NO moiety (Corazzi *et al.*, 2005).

3.4.2.1 NO Impact on COX-inhibitory Effect

Interestingly, it might be expected that the NO moiety of the hybrids could influence the COX-inhibitory action of the aspirin moiety because NO has been previously reported to both inhibit and stimulate activity of the COX enzyme. NO-mediated inhibition of thromboxane generation has been reported to occur via a cGMP-independent mechanism in platelets (Tsikas *et al.*, 1999). NO-mediated inhibition of COX-1 may be a possible mechanism for this effect (Kanner *et al.*, 1992; Tsai *et al.*, 1994). In contrast, however, NO has also been reported to increase the production of eicosanoids such as PGI_2 through activation of COX in endothelial cells, again in a cGMP-independent manner (Davidge *et al.*, 1995). There is a greater amount of evidence for an NO-mediated activation of COX (for review see Salvemini, 1997). Activation of COX by NO has been observed in various *in vitro* (Hajjar *et al.*, 1995; Landino *et al.*, 1996; Salvemini & Masferrer, 1996; Salvemini *et al.*, 1993) as well as *in vivo* systems (Salvemini & Masferrer, 1996). The contrast in the reported impact of NO on COX may be due to the COX isotype present. It has further been shown that the interaction between NO and COX is not a direct one (Tsai *et al.*, 1994) and thus the opposing effects could be a result of differing intermediary pathways. A further possible explanation is that the NO-related species, peroxynitrite, is responsible for the effect (Landino *et al.*, 1996). In such case, the formation of peroxynitrite would not occur in purified enzyme studies and so would not be observed (Davidge *et al.*, 1995). The results in this chapter reveal the inhibition of COX-1 obtained with the furoxan hybrids not to be significantly different from that obtained with their furazan equivalents. These data indicate that the NO moiety has no impact in any manner on the COX-1-inhibition by the aspirin moiety *in vitro*.

However, as discussed above, in an *in vivo* situation, this may alter due to indirect effects of the NO on COX or as a consequence of the formation of NO-related species such as peroxynitrite, which may act to increase COX activity (Landino *et al.*, 1996).

3.4.3 Summary, Future Directions and Therapeutic Implications

The assay presented in this chapter was used as an initial screen to ensure that modification of the aspirin molecule to include an NO-donating moiety had not impaired its ability to inhibit COX. The data show that, at least *in vitro,* an aspirin-like inhibition of COX-1 does occur.

This gives the furoxan NO-aspirins potential to retain the beneficial cardiovascular effects of aspirin. Aspirin irreversibly inhibits COX via an acetylation reaction (DeWitt & Smith, 1988; Roth & Majerus, 1975; Roth *et al.*, 1975). The aspirin-mediated reduction in cardiovascular risk comes about through inhibition of platelet aggregation by action on platelet COX-1. This results in reduced production of TXA_2, a vital element in the induction of irreversible platelet aggregation (FitzGerald, 1991; Hamberg *et al.*, 1975). Due to the anucleate nature of platelets, recovery of full platelet COX activity only takes place after ~ 10 days following platelet turnover (Burch *et al.*, 1978).

This chapter demonstrates that the furoxan NO-aspirin compounds, along with the organic nitrate compound, NCX4016, retain the ability to inhibit COX *in vitro* and thus have the potential to be antithrombotic through aspirin-mediated inhibition of platelet COX-1. Further studies to ensure the effect is retained *in vivo* and to discover the contribution of NO will help determine their effectiveness as antithrombotic drugs.

CHAPTER FOUR

Vasodilator Effects of Furoxans and Furoxan-Aspirin Hybrid Drugs

4. Vasodilator Effects of Furoxans and Furoxan-Aspirin Hybrid Drugs

4.1 Introduction

In healthy blood vessels, eNOS-derived NO is generated by the endothelium in response to shear stress, hypoxia and endogenous mediators (Arnet *et al.*, 1996; Hori *et al.*, 1998; Rapoport *et al.*, 1983) where it mediates vasodilatation in order to increase local blood flow. NO released by the endothelium acts directly upon the adjacent smooth muscle cells to bring about relaxation. The mechanism is virtually exclusively cGMP-dependent; NO acts on cytosolic guanylate cyclase to increase cGMP, which results in a reduction of intracellular calcium in smooth muscle cells and thereby relaxation (Collins *et al.*, 1986; Gruetter *et al.*, 1981; Ignarro *et al.*, 1987; Liu *et al.*, 1992; Miller *et al.*, 2000; Rapoport *et al.*, 1983).

Endothelial dysfunction, as typically occurs in atherosclerosis, is often defined as decreased endothelial production of NO, causing the endothelium to become proinflammatory and prothrombotic, with reduced capacity for vasodilatation. In addition to its role in atherosclerosis (discussed in chapter one), endothelial dysfunction in epicardial vessels and those of the coronary microcirculation is also thought to play a role in effort-related angina (Egashira *et al.*, 1993; Gordon *et al.*, 1989; Quyyumi *et al.*, 1992; Zeiher *et al.*, 1995). The vasodilatory role of NO is exploited by the use of NO-donating organic nitrate compounds such as GTN and ISDN, which are traditionally used to treat exercised-induced angina. The organic

nitrates rapidly dilate veins and coronary arteries, thus reducing cardiac work and improving the blood supply to the heart (Abrams, 1885).

The action of aspirin in the blood vessel is an interesting one due to the role of COX in the production of the vasoconstricting substance TXA_2 (Hamberg *et al.*, 1975). However, the vasodilatory substance PGI_2, which plays a role in overcoming the vasoconstrictive effects of TXA_2 is also COX-derived (Bunting *et al.*, 1976; Dusting *et al.*, 1977).

The ability of NO to cause vasodilatation was utilised in this chapter as a screening tool to determine whether the furoxan compounds and their respective aspirin hybrids were NO-active. The hypothesis that the furoxan compounds (B13 and B12) and their NO-aspirin counterparts (B8 and B7), but not their NO-free furazan hybrids (B16 and B15), cause vasorelaxation was investigated in isolated rat aortas using myography. Temporal experiments were also conducted with the hybrid compounds in order to establish the durability of the vasodilator effect and the ease with which the drug can be washed out.

4.2 Methods

4.2.1 Vasorelaxant Effects of Furoxan-Aspirins in Isolated Aortae

Myography experiments were performed on isolated rings of thoracic aorta from male Wistar rats, dissected and calibrated as detailed in section 2.2. Mounted rings were bathed in Krebs buffer at 37 °C and bubbled with 5 % CO_2 in O_2 gas. The contractile integrity of the rings was first investigated using KCl as described in the methods chapter (section 2.2.2). The rings were treated with L-NAME (200 μM) to prevent generation of any eNOS-derived NO. The α_1 adrenoceptor agonist, phenylephrine, was used to achieve smooth muscle contraction. A concentration-response curve to phenylephrine (0.001 – 1 μM) was constructed in order to determine the concentration for each ring that gave ~ 80 % of the maximum achievable contraction (EC_{80}).

Relaxation to the furoxan compounds, B13 (1 pM – 30 nM), and B12 (0.1 – 300 μM), furoxan-aspirin hybrids, B8 (0.3 nM - 3 μM) and B7 (30 nM - 100 μM), and their NO-free counterparts, B16 (0.3 nM - 3 μM) and B15 (30 nM - 100 μM), were investigated by the cumulative addition of increasing concentrations (chosen following pilot experiments) of the test drug on rings preconstricted with phenylephrine (EC_{80} concentration). Peak relaxations were recorded and results expressed as a percentage relaxation of the contraction to the EC_{80} concentration of phenylephrine.

4.2.2 Duration of Action of Furoxan-Aspirin, B7, in Isolated Aortae

Experiments were also undertaken to determine the rate of NO release from the compounds. This was as above but, following the addition of a single concentration of B8 (30 nM) or B7 (3 μM), the vessels were left to incubate.

In order to determine whether compounds were trapped in tissue or formed durable NO stores, the ease with which the drug was washed out was investigated with B7. After administration of a single concentration (3 μM), the myography chamber was emptied either every 10 or every 30 min and new Krebs, containing the EC_{80} dose of phenylephrine added until the full contraction to phenylephrine was restored.

4.2.3 Statistics

Statistical analysis was by 2-way ANOVA carried out using GraphPad Prism version 4. * is used to represent a p value between 0.01 and 0.05, ** represents a p value of 0.001 to 0.01 and *** represents p values less than 0.001. P values greater than 0.05 were deemed not significant. Where expressed, data is in the form mean ± s.e.m.

4.3 Results

4.3.1 Vasorelaxant Effects of Furoxan Compounds in Isolated Aortae

The myography experiments revealed that B13 induced concentration-dependent relaxation of phenylephrine-contracted rat aortic rings (log EC_{50} = -9.96 ± 0.09. Fig. 4.1). Preincubation with ODQ (50 μM) abolished the effect completely ($p < 0.001$; 2-way ANOVA, B13 + ODQ vs. B13 alone). In the presence of 500 μM cPTIO (log EC_{50} = -8.88 ± 0.08) there was a partial, but significant ($p < 0.001$; 2-way ANOVA, B13 + cPTIO vs. B13 alone) inhibition.

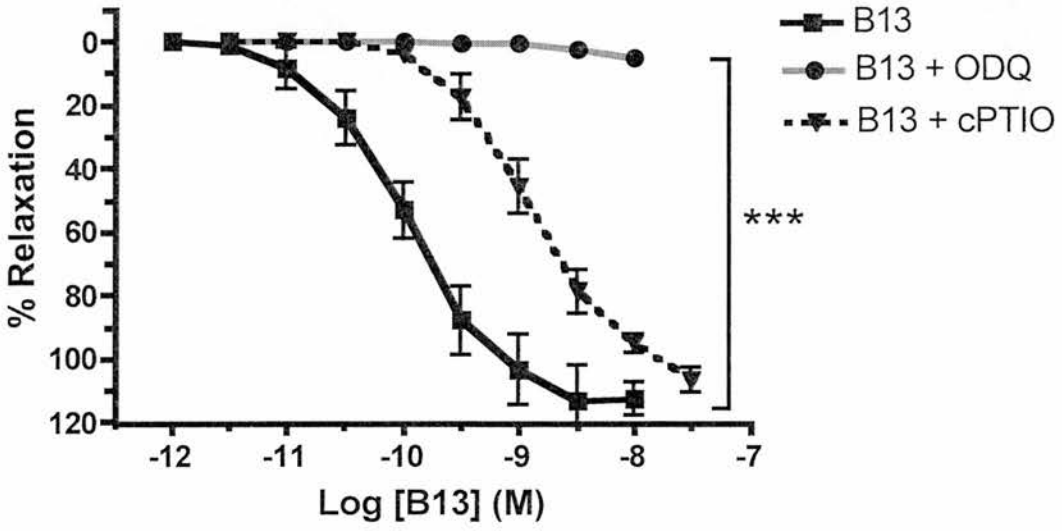


Fig. 4.1. Vasodilator effect of furoxan compound B13. Isolated rat aortic rings were preconstricted with an EC_{80} dose of phenylephrine and the peak relaxation to B13 (1 *p*M - 30 nM) ± ODQ (50 μM; 15 min pre-treatment) were recorded. n=6. Values are mean ± s.e.m. *** = $p < 0.001$ 2-way ANOVA with Bonferroni post-test, B13 + cPTIO and B13 + ODQ vs. B13 alone.

B12 caused concentration-dependent relaxation of phenylephrine-contracted rat aortic rings (log EC_{50} = -5.31 ± 0.04. Fig. 4.2). Preincubation with ODQ (50 μM) inhibited the effect fully ($p < 0.001$; 2-way ANOVA, B12 + ODQ vs. B12 alone), whereas preincubation with 500 μM cPTIO (log EC_{50} = -4.4 ± 0.09) gave a partial, but significant ($p < 0.001$; 2-way ANOVA, B12 + cPTIO vs. B12 alone) inhibition.

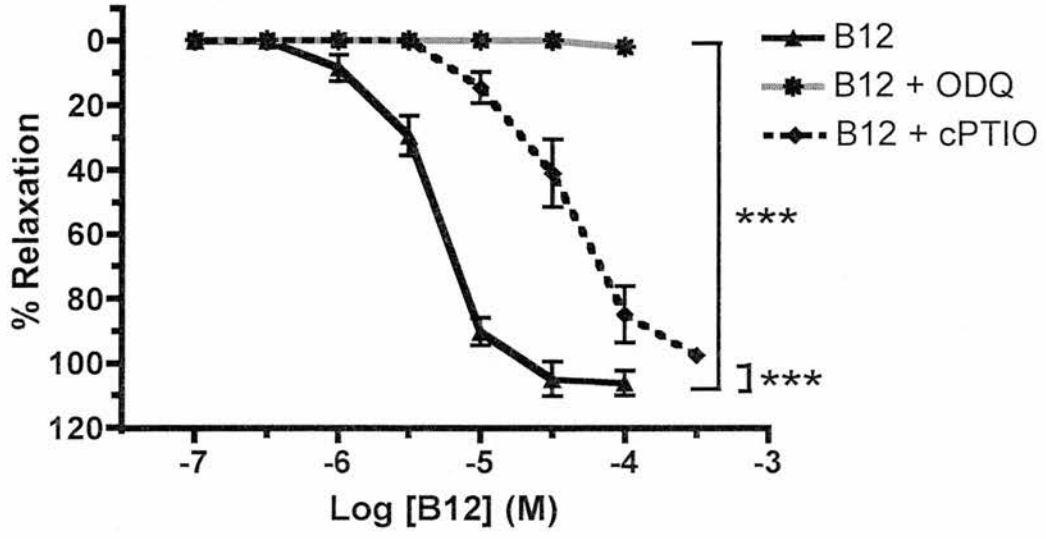


Fig. 4.2. Vasodilator effect of furoxan compound B12. Isolated rat aortic rings were preconstricted with an EC_{80} dose of phenylephrine and the peak relaxation to B132 (0.1 - 300 μM) ± ODQ (50 μM; 15 min pre-treatment) were recorded. n=6. Values are mean ± s.e.m. . *** = $p < 0.001$ 2-way ANOVA with Bonferroni post-test, B12 + cPTIO and B12 + ODQ vs. B12 alone.

4.3.2 Vasorelaxant Effect of B8 in Isolated Aortae

B8 (0.3 nM - 3 μM) evoked concentration-dependent relaxation in isolated rat aortic rings preconstricted with phenylephrine. Data revealed the log EC_{50} of B8 to be -8.11 ± 0.15 (Fig. 4.3). Relaxation to B8 was completely abolished following preincubation with 50 μM ODQ ($p<0.0001$. 2-way ANOVA B8 vs. B8 + ODQ). At

the same concentrations, B16, the NO-free equivalent of B8, failed to cause relaxation. ($p<0.0001$. 2-way ANOVA B8 vs. B16. Fig 4.3).

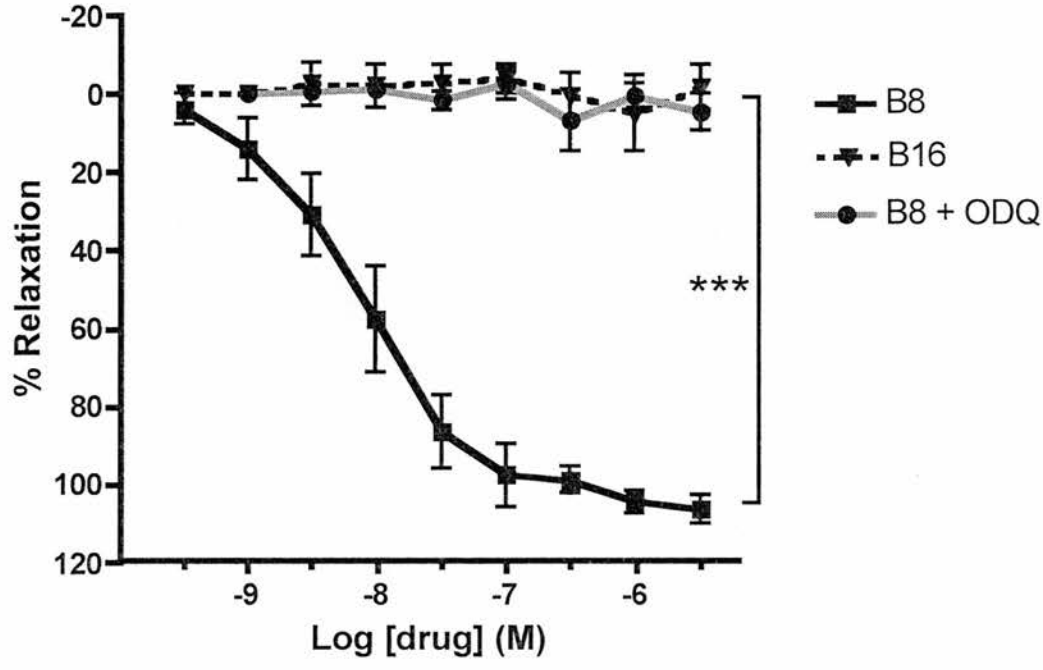


Fig. 4.3. Vasodilator effect of furoxan-aspirin compound B8 and its NO-free equivalent B16. Isolated rat aortic rings were preconstricted with an EC_{80} dose of phenylephrine and the peak relaxation to B8 (0.3 nM - 3 μM) ± ODQ (50 μM; 15 min pretreatment) or B16 (0.3 nM - 3 μM) were recorded. *** = $p<0.0001$. 2-way ANOVA B8 vs. B16/B8+ODQ. n=6. Values are mean ± s.e.m.

4.3.3 Vasorelaxant Effect of B7 in Isolated Aortae

The furoxan-aspirin B7 (30 nM - 100 μM) also evoked concentration-dependent relaxation of phenylephrine-contracted isolated rat aortic rings, with a log EC_{50} of -6.09 ± 0.05 (Fig. 4.4). Preincubation with ODQ (50 μM) prevented this B7-induced relaxation ($p<0.0001$. 2-way ANOVA B7 vs. B7 + ODQ). At the same concentrations, B15, the NO-free equivalent of B7, failed to cause significant relaxation ($p<0.0001$. 2-way ANOVA B7 vs. B15. Fig 4.4).

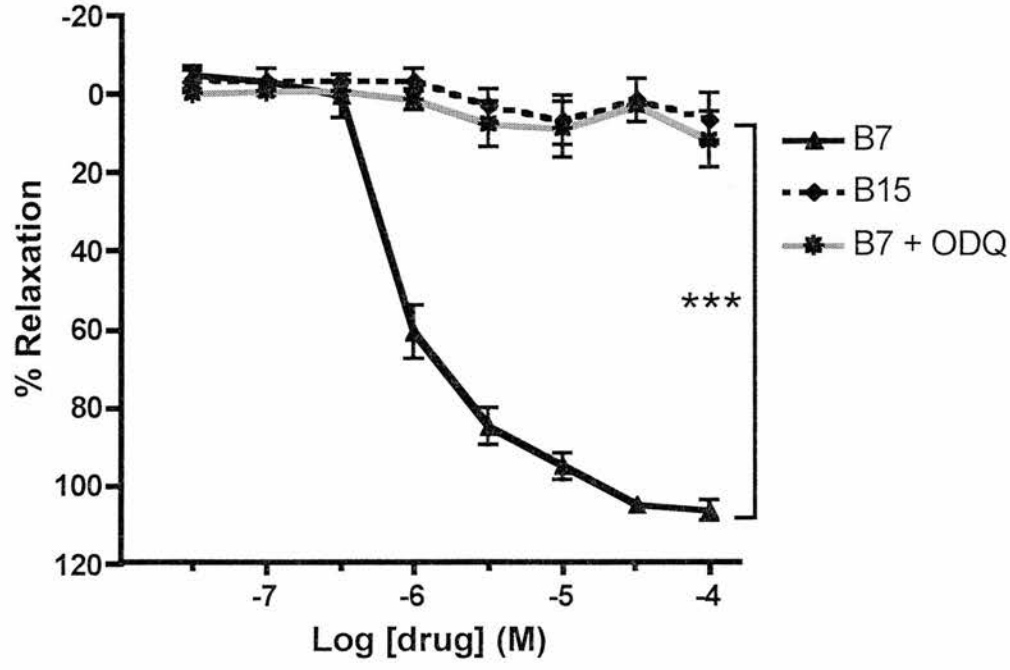


Fig. 4.4. Vasodilator effect of furoxan-aspirin compound B7 and its NO-free equivalent B15. Isolated rat aortic rings were preconstricted with an EC_{80} dose of phenylephrine and the peak relaxation to B7 (30 nM - 100 μM) ± ODQ (50 μM; 15 min pretreatment) or B15 (30 nM - 100 μM) were recorded. *** = $p<0.0001$. 2-way ANOVA B7 vs. B15/B7+ODQ. n=6. Values are mean ± s.e.m.

4.3.4 Duration of Vasorelaxation Effect of B7 in Isolated Aortae

Preliminary data revealed that the effect of B8 (30 nM) was transient, and an approximate 90 % recovery was observed within 10 min. By contrast, the effect of B7 at (3, 1 and 0.3 μM) persisted beyond 3 h. A typical trace (from n = 6) of the relaxation achieved with both drugs is shown in Fig. 4.5.

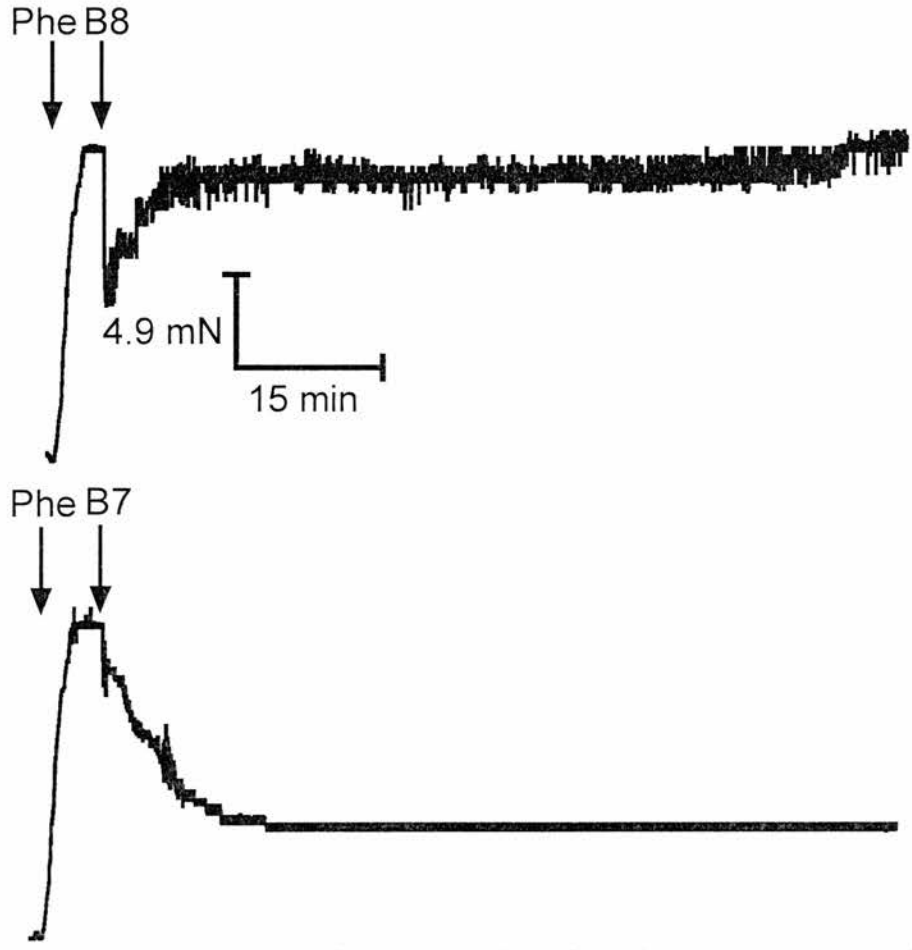


Fig. 4.5. Typical traces (of n = 6) obtained from the rat aorta myography experiments following a single concentration (30 nM) of B8 (top) or a single concentration (3 μM) of B7 (bottom). The response to B7 was typically sustained for longer than 3 h.

The reversibility of the effect on washout was then tested using the longer acting of the two hybrids, B7. Experiments were conducted in which every 10 or 30 min, the B7 (3 μM)-containing Krebs was replaced by fresh phenylephrine (EC_{80})-containing Krebs. Data revealed that the contraction was generally restored within 4 washes, irrespective of the timeframe (Fig. 4.6).

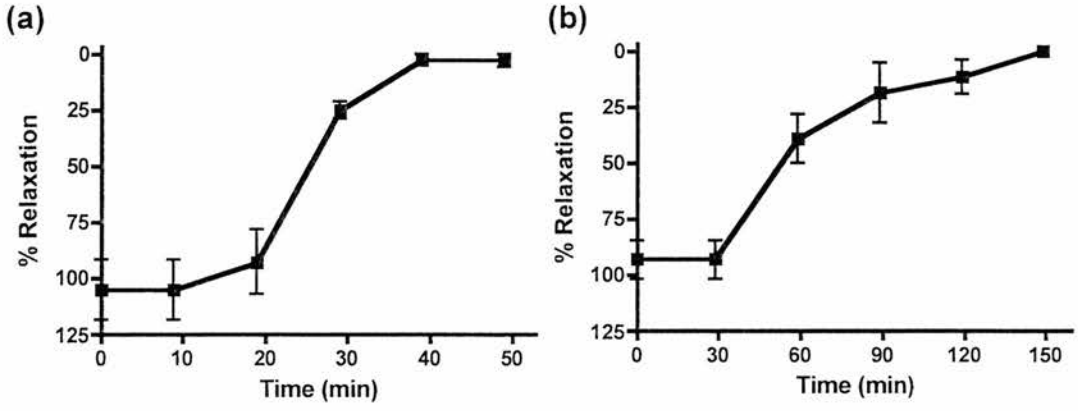


Fig. 4.6. Duration of action of a single (3 μM) dose of furoxan-aspirin compound B7. (a) shows the effect of a single wash after every 10 min and (b) shows the effect of a single wash after every 30 min. n=6 for each. Values are mean ± s.e.m.

4.4 Discussion

4.4.1 Furoxan-Induced Vasodilatation of Rat Aortic Rings

Both furoxan compounds (B13 and B12) induced concentration-dependent relaxation of phenylephrine-contracted rat aortic rings with log EC_{50} of -9.96 ± 0.09 and -5.31 ± 0.04 respectively. In the case of both furoxans, the effect was demonstrated to be entirely cGMP-dependent as indicated by the ability of the sGC inhibitor, ODQ, to abolish the responses. These data imply a role for NO in the furoxan-mediated vasodilatation. The use of L-NAME in the organ bath demonstrates that the activity of B13 and B12 does not depend on NO release from vascular endothelial cells and instead suggests a direct donation of NO from the compounds to overcome the lack of endogenous NO.

Incubation with the NO scavenger, cPTIO, gave a significant partial inhibition of the effect of both B13 and B12. The failure of cPTIO to fully abolish the effect of the furoxan is likely due to the locality of action. The literature is unclear as to the locality of action of cPTIO; it has been reported to act both intracellularly (Yi *et al.*, 2002) and extracellularly (Amano & Noda, 1995; Doran *et al.*, 2003). The data obtained here would imply that cPTIO acts in a compartment that is largely inaccessible to the NO released by the furoxans and thus does not fully prevent the action of the NO released. However, it may also be the case that the furoxans B13 and B12 release their NO in closer proximity to sGC, and thus that reaction occurs too quickly for cPTIO to have an effect.

The remarkable ability of B13 to cause vasorelaxation at concentrations in the picomolar range is an exciting revelation. The existing NO donor drug, DEA/NO, has been reported to have a EC_{50} of 35 nM in rat aortic rings preconstricted with norepinephrine (Sausbier *et al.*, 2000). The EC_{50} of 110 pM obtained in this study reveals B13 to be ~300-fold more potent than DEA/NO. DEA/NO is considered a primarily extracellular NO donor (Crane *et al.*, 2005; Hirasaki *et al.*, 1996). It has previously been hypothesised that cGMP-dependent NO effects correspond with intracellular release of NO (Crane *et al.*, 2005; Miller *et al.*, 2004). Following this hypothesis, the cGMP nature of the effect by the furoxans would imply their NO is released intracellularly. The release in closer proximity to the sGC could account for the extremely high potency of B13 in particular.

4.4.2 NO-Aspirin-Induced Vasodilatation

The furoxan-aspirin compounds, B8 and B7 evoked concentration-dependent relaxation in isolated rat aortic rings preconstricted with phenylephrine. As revealed by the log EC_{50}s, B8 was approximately 100 times more potent than B7. In the case of both furoxan-aspirin compounds, preincubation with the guanylate cyclase inhibitor, ODQ, completely abolished the relaxation, indicating a role for the NO:sGC pathway in this response.

Further evidence for the involvement of NO in the furoxan-aspirin mediated relaxation comes from the response to the furazan-aspirin equivalents. At the same concentrations as their respective furoxan counterparts, NO-free B16 and B15 failed to reverse the phenylephrine-induced contraction. These data suggest that the

relaxation achieved by B8 and B7 is completely NO-mediated and unlikely to involve aspirin-mediated COX inhibition under these conditions.

The use of L-NAME in the organ bath demonstrates that the activity of the compounds B8 and B7 does not depend on NO release from vascular endothelial cells and instead indicates a direct donation of NO from the compounds.

The vasorelaxant effects of the organic nitroaspirin, NCX4016, have been investigated previously by others. NCX4016 caused vasodilatation in norepinephrine -preconstricted rat tail arteries in a process that relies on cGMP and not the aspirin moiety (Rossoni *et al.*, 2002). These data fit with the findings for the furoxan NO-aspirins in that the effect is all NO and specifically cGMP-mediated.

4.4.3 NO-Mediated Vasodilatation

NO causes vasodilatation by a cGMP-dependent mechanism, whereby the increase in cGMP reduces intracellular calcium in smooth muscle cells causing them to relax (Collins *et al.*, 1986; Gruetter *et al.*, 1981; Ignarro *et al.*, 1987; Liu *et al.*, 1992; Rapoport *et al.*, 1983). The lack of response observed in this study following preincubation with ODQ, further confirms the role of cGMP in the NO-mediated vasorelaxation to furoxans.

A further mechanism of NO-induced vasodilatation is via NO-mediated stimulation of the potent vasorelaxant compound, PGI_2. NO does this by a cGMP-independent stimulation of the activity of the enzyme COX (Davidge *et al.*, 1995). However, due to the possible activity of the aspirin moiety (chapter three) of these compounds, this mechanism is unlikely to play a role in this situation. Indeed, the cGMP-dependent

nature of the response indicated by the ODQ data indicates that it does not play a role.

4.4.4 Aspirin-Mediated Vasodilatation?

The dual inhibition by aspirin in the generation of vasoconstricting substance TXA_2 (Hamberg *et al.*, 1975) and the vasodilatory substance PGI_2 (Bunting *et al.*, 1976; Dusting *et al.*, 1977) gives uncertainty as to whether aspirin will give a vasodilatory or constrictive response.

TXA_2 acts through a specific G-protein coupled receptor known as the TP receptor. Stimulation of the TP receptor leads to phospholipase C activation, release of inositol triphosphate and an increase in the intracellular Ca^{2+} level, thus triggering smooth muscle contraction (Habib *et al.*, 1997; Narumiya *et al.*, 1999). The source of the vasoconstricting TXA_2 is platelet COX-1 (Patrono *et al.*, 2005) and due to their anucleate nature, following aspirin inhibition, full TXA_2 recovery will only take place as a function of platelet turnover (Burch *et al.*, 1978). However, it is important to note that as this study uses isolated aortic rings, there is no source of platelet-derived TXA_2 in this model, and so no contractile response for the aspirin to inhibit.

In contrast, in the vasculature, PGI_2 is predominantly COX-2-derived and the main source is endothelial cells (Fitzgerald, 2004). PGI_2 brings about endothelium-dependent vasorelaxation by action on IP receptors (Coleman *et al.*, 1994). Stimulation of the IP receptors brings about vascular smooth muscle cell relaxation in a process that relies upon cAMP (Coleman *et al.*, 1994; Halushka *et al.*, 1989). Interestingly, studies with cultured vascular smooth muscle cells demonstrate that the

vasorelaxant substance PGI_2 is produced only on stimulation by thrombin (Weksler *et al.*, 1978; Whiting *et al.*, 1980) or when in co-culture with platelets (Weksler *et al.*, 1977) and thus suggest that PGI_2 is produced endogenously to overcome the vasoconstrictive effects of TXA_2. It was further shown that, following treatment with aspirin, PGI_2 is restored within only a couple of hours (Whiting *et al.*, 1980).

It is generally accepted that in *in vivo* situations, aspirin treatment will result in a vasodilatory effect due to the predominant inhibition of platelet-derived TXA_2 over that of endothelium-derived PGI_2. Due to platelets only possessing COX-1 (Patrono, 1994) and aspirin being nearly 170-fold more selective for COX-1 than COX-2 (Vane *et al.*, 1998), low dose aspirin that is effective at inhibiting platelet COX-1–derived TXA_2 without affecting endothelial COX-2-derived PGI_2 (FitzGerald, 2002; Patrono *et al.*, 2001) can be administered. These facts, combined with the ability of the endothelial cells to restore PGI_2 production quickly (Fuster *et al.*, 1993; Weksler *et al.*, 1978), suggest that aspirin will bring about a vasodilatory action. Indeed, the reason behind the recent withdrawal of COX-2-specific inhibitors is due to an imbalance between vascular PGI_2 and platelet TXA_2, resulting in an increased risk of stroke. The increased risk of thrombus is thought to be due to inhibition of COX-2 in endothelial cells leading to reduced generation of the anti-thrombotic and vasodilator agent, PGI_2, in relation to the unaffected COX-1 derived, TXA_2 (Fitzgerald, 2004).

It is proposed that the lack of platelets in the isolated preparation used here could be the reason behind the lack of vasodilatory response to NO-free furazans B16 and B15 and that the situation may alter in *in vivo* situations.

The data here show that whilst the addition of the aspirin group decreases the potency of B13 (Table 4.1) by approximately 70-fold, the addition of aspirin to B12 to form B7 caused a slight increase in potency. It is likely that the release of NO and hence the effect of the drug is related to its structure and thus NO release requires investigating.

DRUG	Log EC_{50}
B13	-9.96 ± 0.09
B12	-5.31 ± 0.04
B8	-8.11 ± 0.15
B7	-6.09 ± 0.05

Table. 4.1. Log EC_{50} s obtained in the rat aorta myography experiments.

4.4.5 B7 – Duration and Reversibility of Action

The data here show that, in the case of B8, NO is released transiently, and that within approximately 10 min 90 % of the contraction to phenylephrine is recovered. For B7 the onset of the relaxation is slower but the effect was sustained for longer and exceeded the life of the tissue preparation.

The reversibility of the effect of the compounds was investigated with the wash-out experiment. Data obtained in this study demonstrated that whilst a single concentration of the furoxan-aspirin compound B7 would last in excess of three hours, it was fairly easily washed out of the system. The wash-out experiments were carried out at two different time frames (10 and 30 min) and revealed that rather than persisting for a set period of time, the response to the drug persisted as a function of

washes. If it is to be presumed from the previous data that the drug releases NO intracellularly, due to the speed at which a response was seen with the myography apparatus (within seconds), it appears that the drug enters the cell quickly. The susceptibility of B7 to rapid washout implies it is not being trapped in the intracellular compartment but, instead, is simply equilibrating across the membrane. It is important to note that this study is likely a poor predictor of the duration of action *in vivo*. The drug is likely to respond differently in different vessels and the wash-out with phenylephrine-containing Krebs does not likely reflect that of blood flowing through the vessel *in vivo*. Furthermore, factors such as metabolism by cytochrome P450s and clearance will undoubtedly play a role *in vivo*. It may be that alteration of the chemical structure could alter the equilibrium across the cell membrane leading to less diffusion of unaltered drug out of the cell.

4.4.6 Summary and Future Directions

The data obtained here reveal the furoxan compounds, particularly B13, to be promising and extremely potent vascular NO donor drugs. B13 caused relaxation of vascular smooth muscle *in vitro* at concentrations in the picomolar range, demonstrating it, to my knowledge, to be the most potent vasodilator NO-donor drug yet discovered.

The data here clearly show a concentration-dependent vasodilatation by the furoxan NO-aspirins in isolated rat aortae. The effect is NO-mediated, as demonstrated by lack of response with the furazan equivalents, and the susceptibility to ODQ pre-treatment is indicative of an exclusively cGMP-dependent effect. The inclusion of

the aspirin moiety has a more dramatic impact on the potency of B13 than B12, most likely due to the effect of the structural change on the NO release.

In addition to their aspirin-like action revealed in chapter three, the furoxan hybrids have an NO mediated action and, therefore, both elements of the hybrid have the potential to be active, at least *in vitro*. Their NO-mediated action appears to vary with structure and thus the NO-release mechanism requires further exploration.

CHAPTER FIVE

Comparative Pharmacology of Novel Furoxan and Furoxan-Aspirin Hybrid Compounds in Platelets

5. Comparative Pharmacology of Novel Furoxan and Furoxan-Aspirin Hybrid Compounds in Platelets

5.1 Introduction

The NSAID, aspirin, is the most commonly used prophylactic drug in the prevention of coronary thrombotic events (ISIS-2, 1988; Manson *et al.*, 1991; PHS-Committee, 1989). As previously discussed, aspirin reduces COX-1-generated TXA_2, therefore inhibiting platelet aggregation for the lifetime of the platelet (Burch *et al.*, 1978; DeWitt & Smith, 1988; FitzGerald, 1991; Hamberg *et al.*, 1975; Roth & Majerus, 1975; Roth *et al.*, 1975). The unfortunate limitation of the long-term use of aspirin is that it can cause gastrointestinal disorders, including ulceration (Cameron, 1975; Seager & Hawkey, 2001; Tramer *et al.*, 2000; Wallace, 1997), due mainly to the inhibition of prostaglandins that normally protect the gastric mucosa (Robert *et al.*, 1979; Schoen & Vender, 1989; Wallace, 1997; Whittle, 1977).

NO-aspirins, or aspirin esters containing an NO-donor moiety, were developed in the hope of overcoming the gastric side-effects of aspirin through various gastroprotective effects of drug-derived NO.

Whilst NO hybrids of aspirin were primarily designed to protect against damage to the gastric mucosa, there may be additional antiplatelet benefits of drug-derived NO. NO displays antithrombotic actions through its ability to inhibit platelet adhesion (Radomski *et al.*, 1987b; Radomski *et al.*, 1987d) and aggregation (Pasqui *et al.*, 1991; Radomski *et al.*, 1990; Radomski *et al.*, 1987c). Inhibition of platelet aggregation occurs primarily via stimulation of cGMP; the platelet aggregation response to sodium

nitroprusside has been shown to be entirely cGMP dependent (Sogo *et al.*, 2000). However, cGMP-independent signalling mechanisms have also been identified (Crane *et al.*, 2005; Gordge *et al.*, 1998; Homer & Wanstall, 2002; Sogo *et al.*, 2000; Trepakova *et al.*, 1999).

The release of NO from an existing nitroaspirin, NCX4016, and glyceryl trinitrate has been reported to occur through identical mechanisms (Grosser & Schroder, 2000). Platelets have a poor capability of releasing NO from organic nitrates like these (Weber *et al.*, 1996) and thus it is possible that compounds such as NCX4016 will fail to show additional, NO-mediated effects in platelets, at least *in vitro*. A further limitation of NCX4016 is the probability of tolerance with long-term or high dose use; there is no evidence to suggest that NCX4016 will not be susceptible to the tolerance problems associated with long-term or high dosage use of traditional organic nitrates (Munzel *et al.*, 2005).

However, the furoxan series of NO-aspirin hybrids do not possess an organic nitrate group and might not be susceptible to the same failings. The NO moiety has already been demonstrated to be active in vascular smooth muscle cells (chapter four), but in this chapter the effects of the hybrid-derived NO will be examined in platelets, where the aspirin moiety also has potential for action (chapter three). Here, the mechanism of action of two furoxan-aspirin hybrid drugs, B8 and B7 (Fig. 1.7), with different NO-releasing properties (Cena et al., 2003) are investigated in human platelets i*n vitro* along with their parent counterparts B13 and B12 (Fig. 1.7), which do not possess an aspirin moiety in order to test the hypothesis that the hybrids have antiplatelet action contributed to by both elements. The antiplatelet effects are compared with that of existing nitroaspirin compound, NCX4016 (Fig. 1.5), which has an alternative NO-releasing moiety.

5.2 Methods

5.2.1 Platelet Aggregometry

The effect of the furoxans on platelet aggregation was examined by two techniques. Turbidometric platelet aggregometry allowed the evaluation of the impact of plasma components on the efficacy of the drugs. Impedance aggregometry was utilised to examine the effects in whole blood where the optical density of the samples prevented the use of the turbidometric technique. Impedance aggregometry allowed determination of whether the drug could withstand the NO scavenging effect of haemoglobin in erythrocytes.

5.2.1.1 Impedance Platelet Aggregometry

Impedance aggregometry was used to investigate the ability of the furoxan compound, B13, to withstand the NO-scavenging properties of endogenous scavenger haemoglobin. Human blood was collected as described in chapter two (section 2.2) and diluted 50:50 in PBS. 1 ml samples were placed in tubes incubated at 37 °C and stirred at 1000 rpm in a dual channel aggregometer, fitted with impedance electrodes. Following a 10 min preincubation with either DEA/NO or B13 (100 and 300 μM), aggregation was induced using 2.5 $\mu g.ml^{-1}$ collagen and electrical impedance recorded for the subsequent 5 min. Results are expressed as percentage inhibition of the response to a collagen-only control.

5.2.1.2 Turbidometric Platelet Aggregometry

The effect of the furoxans and furoxan-aspirin hybrids on platelet aggregation was examined by optical (turbidometric) platelet aggregometry to allow the evaluation of the impact of plasma components on the efficacy of the drugs.

Inhibition of platelet aggregation was measured in a 4-channel aggregometer as described in section 2.4.1. Aliquots (0.5 ml) of either PRP or WP (prepared as in section 2.3) were incubated at 37 °C and constantly stirred at 1000 rpm. Platelet samples were treated with either B13 (1 nM – 10 μM), B12 (3 – 300 μM), B8 (10 nM – 3 μM), B7 (10 μM – 1 mM), the respective NO-free furazan equivalents of B8 and B7; B16 (10 nM – 3 μM) or B15 (10 μM – 1 mM), the organic nitrate, NCX4016 (3 μM - 0.3 mM), aspirin or SA (3 μM – 1 mM) for 10 minutes before induction of aggregation with supra-maximal concentrations of collagen (2.5 $\mu g.ml^{-1}$). Aggregation was then recorded for 5 min following addition of the agonist. Experiments were also performed with the addition of the cGMP inhibitor ODQ (50 μM) 15 min before addition of the test drug in order that the contribution of cGMP to the antiaggregatory effects could be determined. Aggregation was expressed as a percentage inhibition of control aggregation obtained in response to 2.5 $\mu g.ml^{-1}$ collagen.

5.2.2 Statistics

Statistical analysis was by either students' t-test or 2-way ANOVA carried out using GraphPad Prism version 4. * is used to represent a p value between 0.01 and 0.05, ** represents a p value of 0.001 to 0.01 and *** represents p values less than 0.001. P

values greater than 0.05 were deemed not significant. Where expressed, data are in the form mean ± s.e.m.

5.3 Results

5.3.1 Impedance Platelet Aggregometry

Whole blood aggregometry was limited by failure of B13 to remain in solution at concentrations above 300 μM. B12 also failed to stay in solution at concentrations high enough (> 300 μM) to obtain an effect in this system.

The data obtained demonstrate that the furoxan B13 causes significantly greater inhibition of collagen (2.5 $\mu g.ml^{-1}$)-induced platelet aggregation than that obtained with the NO donor compound, DEA/NO, in whole blood at both 100 μM and 300 μM (for both concentrations, $p < 0.05$ as analysed by Student's t-test; Fig. 5.1). 300 μM B13 gave a 37.7 ± 6.8 % inhibition, whereas an equimolar concentration of DEA/NO gave 11.0 ± 7.0 %. At 100 μM, B13 gave 21.3 ± 6.7 % and DEA/NO 0.7 ± 6.3 %.

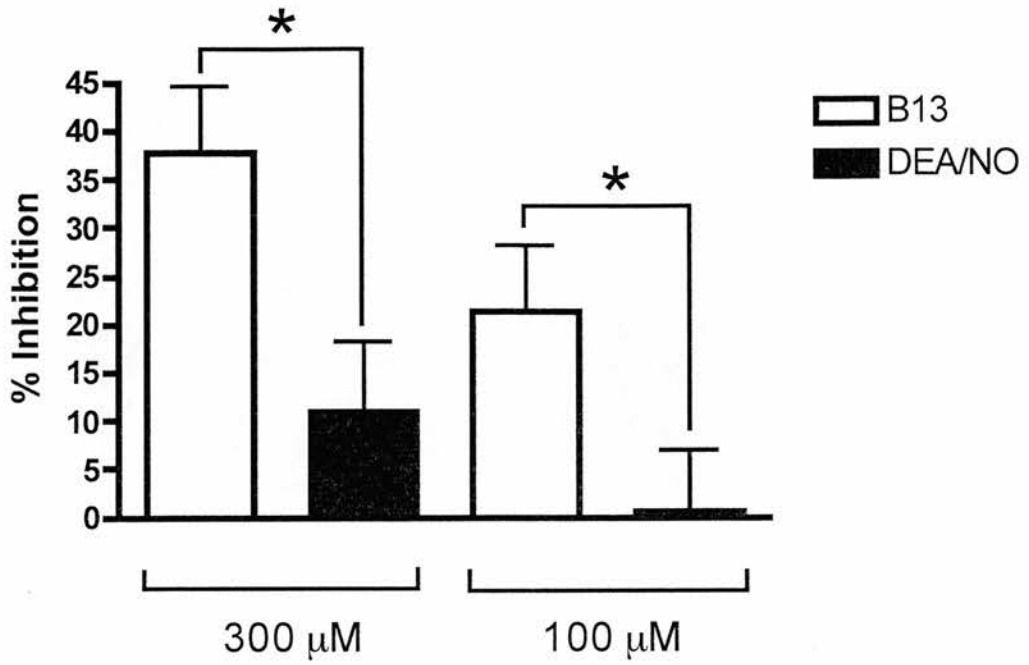


Fig. 5.1. Inhibition of collagen (2.5 μg ml^{-1})-induced platelet aggregation in whole blood by the furoxan compound, B13 (100 and 300 μM). n = 6 - 7. Values are mean ± s.e.m. * = significant difference ($p < 0.05$) as analysed by students' t-test.

5.3.2 Turbidometric Platelet Aggregometry

5.3.2.1 Effect of Furoxan B13

The furoxan B13 caused concentration-dependent inhibition of collagen-induced platelet aggregation in both PRP and WP (log IC_{50} = -6.4 ± 0.2 and -6.9 ± 0.2 respectively). No significant difference between effect in PRP and WP ($p = 0.71$) as assessed by 2-way ANOVA; Fig. 5.2). The effect was significantly inhibited by preincubation with the sGC inhibitor, ODQ, in both PRP and WP ($p = 0.020$ and 0.0002, respectively; 2-way ANOVA vs. B13 alone, n = 6).

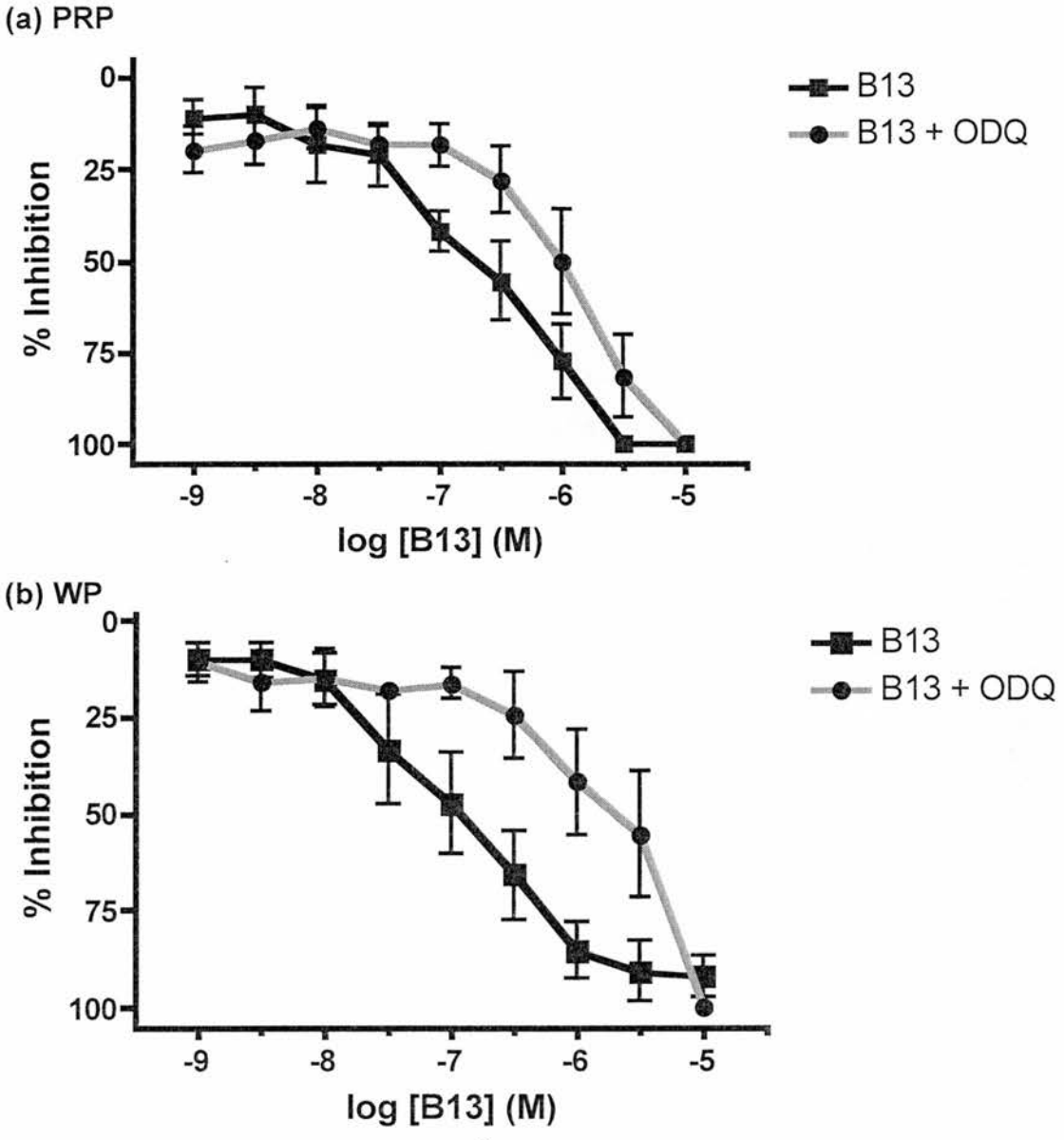


Fig. 5.2. Inhibition of collagen (2.5 μg ml^{-1})-induced platelet aggregation in (a) PRP and (b) WP by the furoxan compound, B13 (1 nM – 10 μM) and the impact of the guanylate cyclase inhibitor, ODQ (50 μM; 15 min preincubation) on this effect; n = 6. Values are mean ± s.e.m. Statistical analysis by two-way ANOVA demonstrated that preincubation with ODQ significantly inhibited the effect of B13 (p = 0.020 in PRP and 0.0002 in WP).

5.3.2.2 Effect of Furoxan B12

B12 also caused concentration-dependent inhibition of collagen-induced platelet aggregation in both PRP and WP but at approximately 300x higher dose than B13 (log IC_{50}s in PRP and WP are -3.8 ± 0.2 and -4.1 ± 0.2 respectively. p = 0.0172 as

assessed by 2-way ANOVA; Fig. 5.3). Preincubation with the sGC inhibitor, ODQ caused significant inhibition of the effect of B12, in WP ($p < 0.0001$; 2-way ANOVA; $n = 6$) but not in PRP.

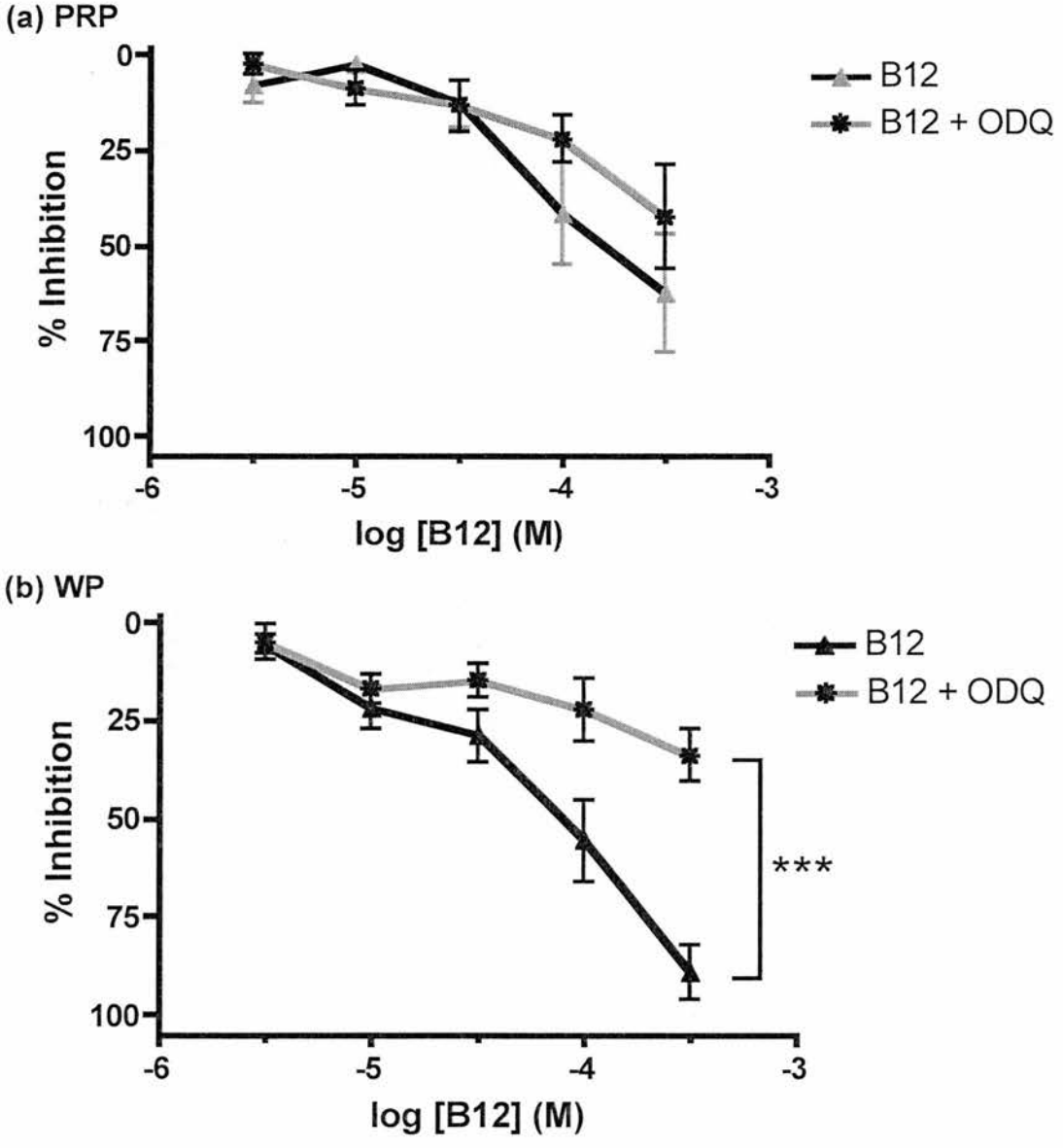


Fig. 5.3. Inhibition of collagen (2.5 μg ml^{-1})-induced platelet aggregation in (a) PRP and (b) WP by the furoxan compound, B12 (3 – 300 μM) and the impact of the guanylate cyclase inhibitor, ODQ (50 μM; 15 min preincubation) on this effect. $n = 6$. Values are mean ± s.e.m. Statistical analysis by two-way ANOVA revealed preincubation with ODQ significantly inhibited the effect of B12 in WP (***; $p < 0.0001$).

5.3.2.3 Effect of Aspirin, Salicylic Acid and NCX4016

Aspirin (3–300 μM) caused concentration-dependent inhibition of collagen-induced platelet aggregation in PRP and the effect was enhanced in WP, but SA failed to show an inhibitory effect, even at concentrations of 300 μM (Fig 5.4; n=6-8). NCX4016 (3-300 μM) had no effect on collagen-induced platelet aggregation in PRP (Fig. 5.4a; n=6), but did cause concentration-dependent inhibition of platelet aggregation in WP that was significantly enhanced compared to aspirin (Fig 5.4b, P=0.002; n=6). Responses to NCX4016 were insensitive to the soluble guanylate cyclase inhibitor, ODQ (15 min preincubation; P=0.88, NCX4016 + ODQ vs. NCX4016 alone in WP; Fig. 5.4b).

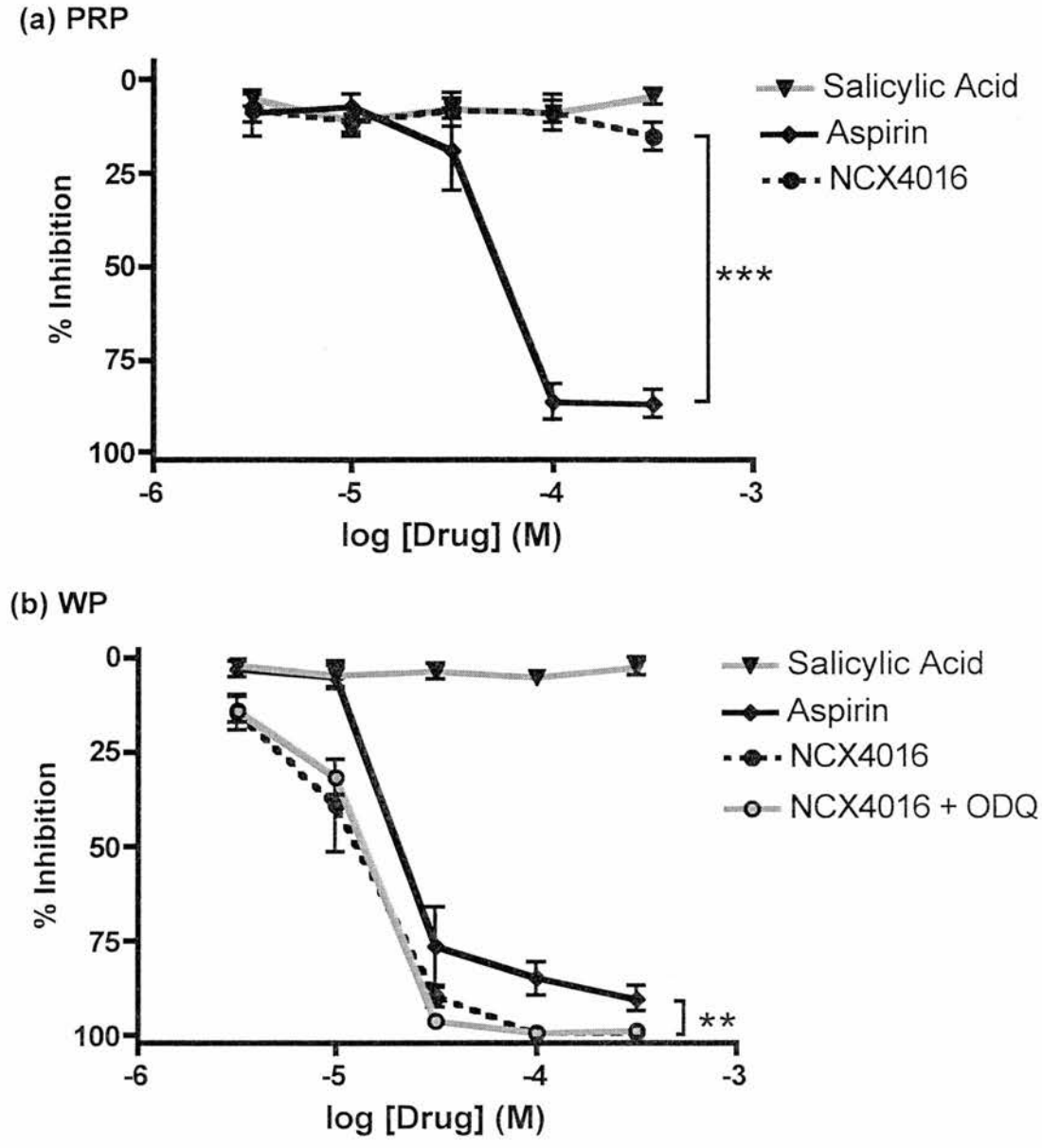


Fig. 5.4. Collagen (2.5 μg.ml^{-1})-induced platelet aggregation in PRP and WP. **a**. Effect of NCX4016, salicylic acid and aspirin on collagen-induced platelet aggregation in PRP. *** P<0.0001, n = 6-7. **b**. The effect of the guanylate cyclase inhibitor, ODQ (50 μM; 15 min preincubation), on responses to NCX4016 in WP. ** P = 0.002, n = 6. Values are mean ± SEM. Statistical analysis by 2-way ANOVA.

5.3.2.4 Effect of B8

B8 (10 nM–3 μM) caused concentration-dependent inhibition of collagen-induced platelet aggregation in PRP at concentrations ~100-fold lower than for aspirin (B8 log IC_{50} in PRP = -6.21 ± 0.07; Fig. 5.5a. See Fig 5.4a for aspirin effect.). ODQ significantly inhibited the responses to B8 in PRP (Fig. 5.5a; n = 6-8; $P<0.0001$), whilst the NO-free, structurally related furazan derivative of B8 (B16; 10 nM-1 mM) was considerably less effective at inhibiting platelet aggregation than B8 in PRP (Fig. 5.5a; $P<0.0001$; n = 8).

The effects of B8 in PRP were largely mirrored in WP: B8 was found to be a powerful inhibitor of collagen-induced aggregation (log IC_{50} = -6.23 ± 0.06) and its actions were significantly inhibited by ODQ (Fig 5.5b; $P<0.0001$; n = 6-8). B16 had a significantly greater inhibitory effect on platelet aggregation in WP compared to PRP (log IC_{50} = -4.27 ± 0.05; Fig 5.5b; $P<0.0001$. PRP B16 vs. WP B16; n = 6-8).

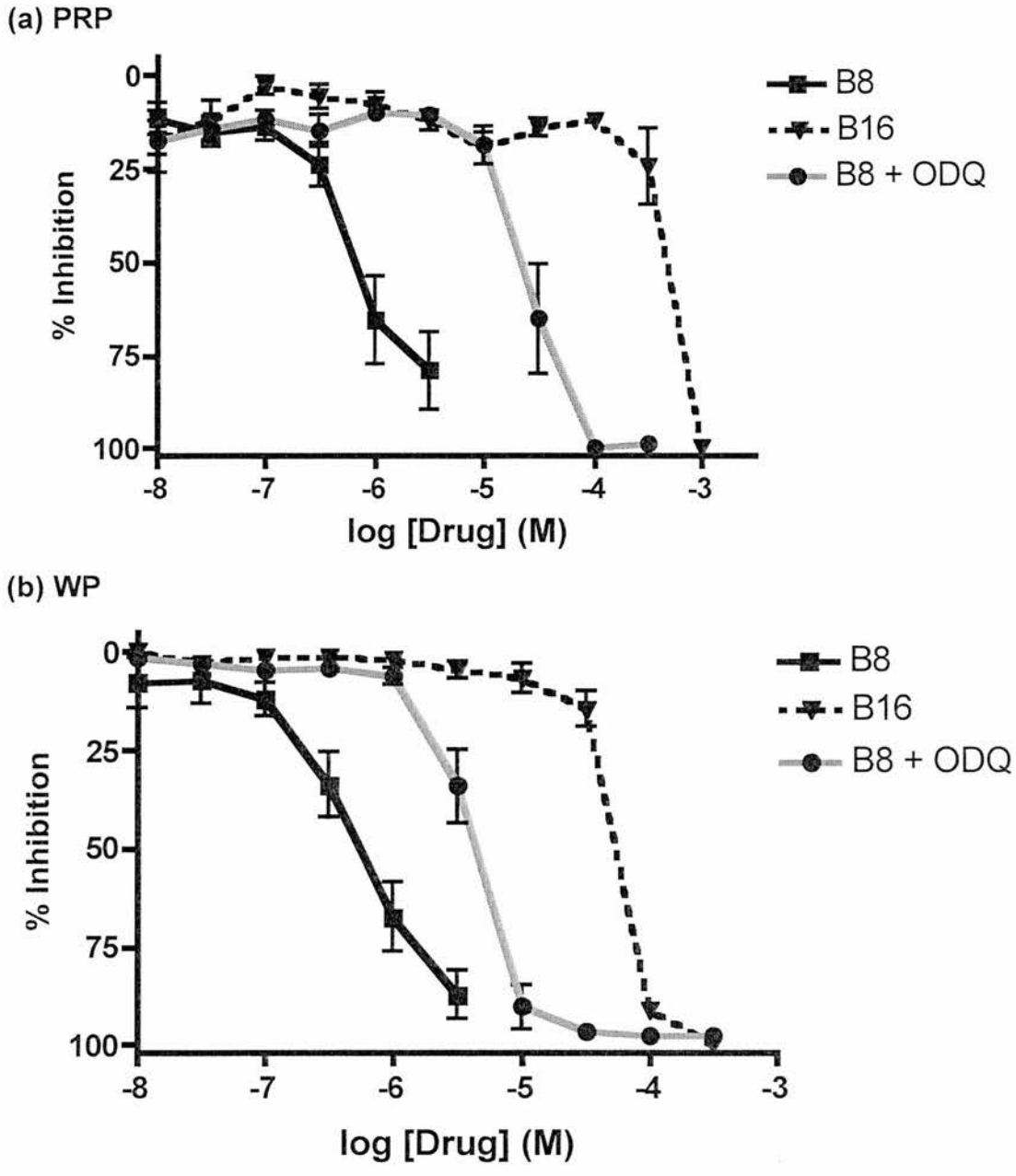


Fig. 5.5. Collagen (2.5 $\mu g.ml^{-1}$)-induced platelet aggregation in PRP and WP. **a**. Effect of B8 (± ODQ; 50 μM, 15 min preincubation) and its NO-free equivalent, B16, on collagen-induced platelet aggregation in PRP. $P< 0.0001$ (B8 + ODQ vs. B8 alone, n = 6 – 9). **b**. The effect of ODQ (50 μM) on responses to B8 in WP. $P< 0.0001$ (+ ODQ vs. B8 alone), n = 6 – 9.

5.3.2.5 Effect of B7

B7 (10 μM–1 mM) was less effective than B8 and aspirin at inhibiting collagen-induced platelet aggregation in PRP (B7 log IC_{50} = -3.43 ± 0.7). ODQ significantly inhibited the response of B7 in PRP (Fig. 5.6a; n = 6-7. P<0.0001). B15 was ineffective in PRP.

In order to make a direct comparison, concentrations studied with WP were dictated by the PRP response curve. B7 (10 μM-1 mM) was considerably more effective in WP (log IC_{50} = -4.6 ± 0.9; Fig 5.6 b) than PRP. The inhibitory effects of B7 in WP were significantly attenuated by ODQ (P=0.002, 2 way ANOVA; n=6-7). In stark contrast to the findings in PRP, B15 was found to be a powerful inhibitor of platelet aggregation in WP (B15 log IC_{50} in WP = -4.22 ± 0.1); indeed, at concentrations of 100 μM or more, it was as effective as B7 under these conditions.

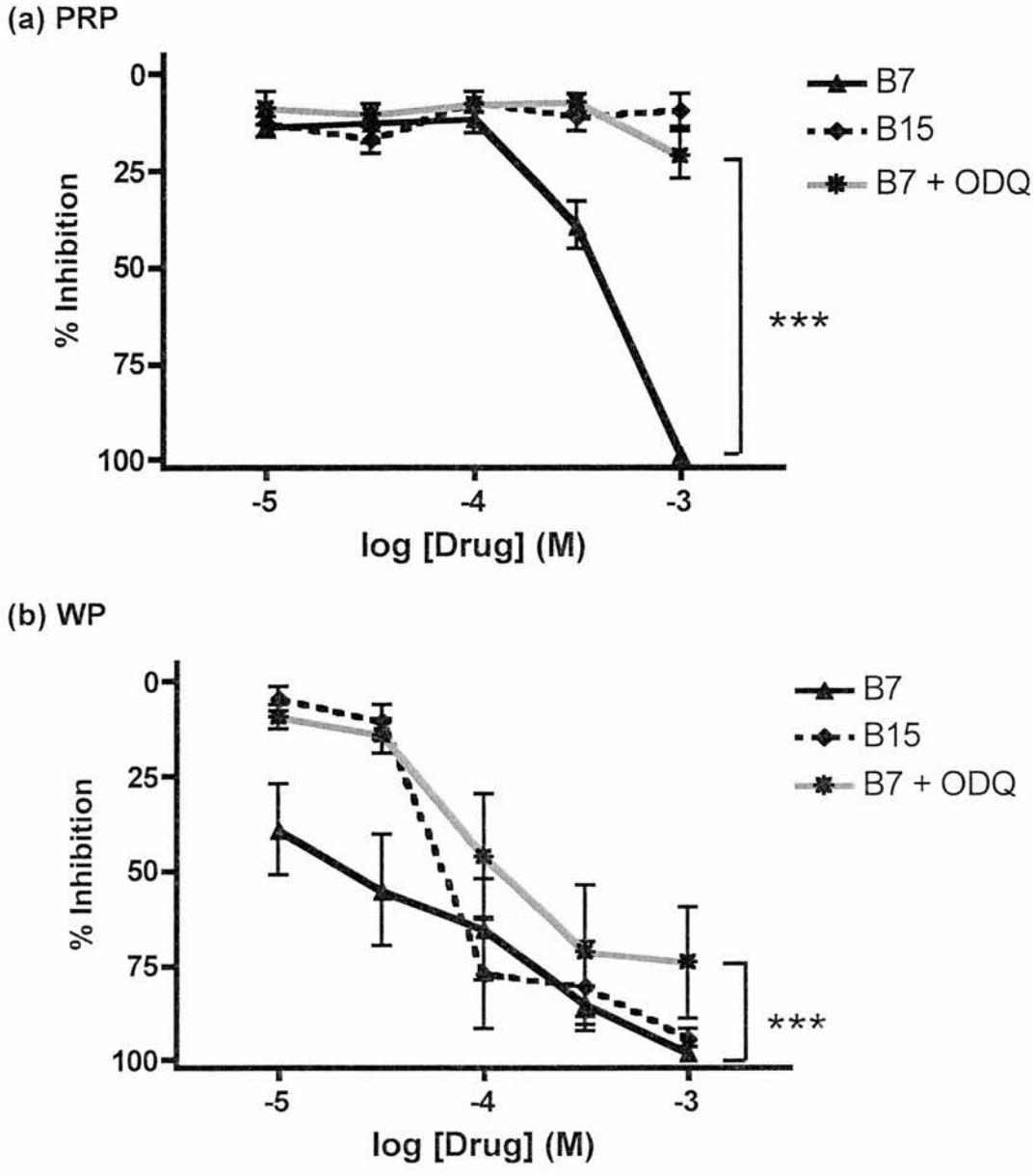


Fig. 5.6. Collagen (2.5 μg.ml^{-1})-induced platelet aggregation in PRP and WP. **a**. Effect of B7 (± ODQ; 50 μM) and its NO free equivalent B15 on collagen-induced platelet aggregation in PRP. *** P<0.0001, n = 6 – 7. **b**. The effect of ODQ (50 μM) on the B7 response in WP. *** P< 0.0001, n = 6 – 7. Values are mean ± SEM. Statistical analysis by 2-way ANOVA.

5.4 Discussion

These studies provide evidence that the both furoxans and furoxan-aspirin hybrid drugs provide concentration-dependent inhibition of collagen-induced platelet aggregation *in vitro*. The effect was determined to be sGC-dependent by use of cGMP inhibitor, ODQ.

5.4.1 Furoxan-Induced Inhibition of Platelet Aggregation

The endogenous metalloprotein, haemoglobin, is found in erythrocytes where it functions to transport oxygen in the blood by use of its iron-containing core (For review see Habler & Messmer, 1997). However, NO can also react with haemoglobin and so, haemoglobin can act as an endogenous mechanism to limit NO bioactivity (Gladwin *et al.*, 2004; Kosaka *et al.*, 1989). This feature can be exploited experimentally and haemoglobin is often used as an NO scavenger. NO reacts with haemoglobin in its oxygenated state to form methaemoglobin and nitrate but also reacts with deoxy-haemoglobin to form iron-nitrosyl-haemoglobin. This reaction, were it not for the containment of haemoglobin in the erythrocyte and the effect of plasma streaming, would consume all available NO (Butler *et al.*, 1998; Gladwin *et al.*, 2004; Lancaster, 1996; Vaughn *et al.*, 2000).

Whole blood impedance aggregometry was therefore utilised to determine the impact of cellular haemoglobin on the function of the furoxans. Collected data was limited by the failure of B12 to stay in solution at concentrations high enough to obtain an effect. For the same reason, B13 data above 300 μM were not collected.

The data did, however, reveal some interesting information. At concentrations which cause similar inhibition of platelet aggregation in PRP (data not shown), the effect of B13 and DEA/NO are significantly different in whole blood. At both concentrations studied, B13 caused significantly greater inhibition of collagen-induced platelet aggregation than that of the NO donor compound DEA/NO in whole blood. These data show that, despite requiring higher concentrations than necessary to have an effect in PRP, B13 can overcome the NO-scavenging effects of haemoglobin. The fact that in PRP these concentrations yield similar results with B13 and DEA/NO, but in the presence of haemoglobin, the antiplatelet effect of B13 is greater, shows B13 to be a more effective inhibitor of platelet aggregation than DEA/NO under physiological conditions. Furthermore, the data shows potential for B13 to retain an antiplatelet effect *in vivo*.

The *in vitro* study of the scavenging effect of haemoglobin on the antiplatelet activity of NO donors is not commonplace. However, one study shows that the effect of NO-donors, S-nitrosoglutathione and RIG 200 are subject to inhibition by haemoglobin (Megson *et al.*, 2000). The results of this study may indicate that the NO released by B13 is released in a compartment less accessible to haemoglobin than to DEA/NO, S-nitrosoglutathione and RIG 200. The obvious inference from such data is that NO from B13 is released inside platelets, whilst that from other NO donors is released predominantly extracellularly. Further support for the theory of intracellular release of NO comes from the hypothesis that cGMP-dependent vasorelaxation (as demonstrated in chapter four) is correlated with intracellular release of NO (Crane *et al.*, 2005; Miller *et al.*, 2004).

Turbidometric assessment revealed the furoxans B13 and B12 to both cause concentration-dependent inhibition of collagen-induced platelet aggregation in both PRP and WP, although B13 did so at approximately 1000-fold lower concentrations.

The effect of B13 in PRP and WP was determined to be cGMP-dependent as demonstrated by significant attenuation of responses by the sGC inhibitor, ODQ. There was no significant difference between the inhibition of aggregation by B13 in PRP and in WP, suggesting that plasma has no impact on the process. The lack of impact of plasma on the response could again be an indication that the NO is released by these compounds in a compartment inaccessible to the plasma – such as inside the cell. As with the data obtained in chapter four, the cGMP-dependent nature of the response would indicate an intracellular release of NO (Crane *et al.*, 2005).

There was a significant difference in the B12-induced inhibition of aggregation in PRP compared to WP, with higher concentrations required in PRP to achieve the same effect. The differential contribution of cGMP on the response in PRP and WP may be dependent on their release location as hypothesised by Crane. (Crane *et al.*, 2005). The data reveal that B12-induced inhibition of aggregation in WP is cGMP-dependent but that in PRP, ODQ data indicate that B12 acts via a cGMP-independent mechanism - possibly implying a role for plasma in the extracellular release of NO from B12.

5.4.2 NO-Aspirin-Induced Inhibition of Platelet Aggregation

The results show that two novel furoxan-aspirin hybrids drugs effectively inhibit collagen-induced platelet aggregation and that the relative contribution of NO to the

inhibitory effect is dependent on the characteristics of the specific furoxan involved. The potent effects of B8 were considered to be largely NO-dependent on account of the fact that the closely related NO-free furazan, B16, had only a very weak antiplatelet effect. B7 was a less potent inhibitor of aggregation than B8; but the effects were also primarily NO-mediated in PRP, where the furazan counterpart was largely ineffectual. However, in WP, it was apparent that the antiplatelet activity of B7 comprised both NO-dependent and -independent components. The existing nitrooxy-ester, nitroaspirin (NCX4016), was also an effective anti-platelet agent in WP but the effects were entirely sGC-independent and were altogether lost in PRP.

The furoxan-aspirin hybrid drugs inhibited platelet aggregation in both WP and PRP, although the effects of both compounds, and B7 in particular, were attenuated in PRP. The comparatively weak inhibitory effects of the NO-free furazan counterparts is indicative of a major role for NO in platelet inhibition, whilst the inhibitory effect of ODQ confirmed that a major component of the effects were sGC-dependent, especially in PRP. The impact of ODQ on responses was less pronounced in WP, particularly in the case of B7, due in part to the greater influence of NO-independent effects, as illustrated by increased sensitivity of WP to the furazan counterparts of B7 and B8. The effects of the furazan derivatives were weak compared to aspirin, but were nevertheless more potent than SA under the conditions of these experiments. The lower activity of these agents in PRP compared to WP is in keeping with the complete hydrolysis of the furoxan-aspirin acetyl group in serum (Cena *et al.*, 2003), resulting in formation of relatively ineffectual SA. These hybrid molecules are likely to undergo hydrolysis at two positions: firstly, at the acetyl group, converting aspirin to SA and secondly at the other ester linkage to release the furoxan. The order and

rates at which hydrolysis occurs will greatly influence the ability of these compounds to retain aspirin activity.

The high levels of NO release by B8 and the loss of its aspirin effect in PRP imply that its antiplatelet actions are mainly NO-mediated. However, B7, which has an IC_{50} closer to that of aspirin, does display a cGMP-independent antiplatelet effect in WP implying a role for aspirin. This characteristic reveals it to be a more suitable hybrid candidate, allowing a role for both NO and aspirin. This is in contrast to B8 where NO dominates. However, the unfortunate loss of the aspirin effect in PRP is an issue that needs to be addressed by appropriate modification of the chemical structure.

The finding that NCX4016 is an effective inhibitor of platelet aggregation in WP is in keeping with previous *in vitro* studies using NCX4016 (Lechi *et al.*, 1996) and the related drug, NCX4125 (Minuz *et al.*, 1995; Wallace *et al.*, 1995). However, the results here go on to show that the effect is lost in PRP, possibly due to sequestration by a plasma constituent such as albumin or due to breakdown in the plasma to the inactive SA. Interestingly, the inhibitory effect of NCX4016 was significantly enhanced compared to aspirin but was not affected by the sGC inhibitor, ODQ.

Whilst a cGMP-independent effect of NCX4016-derived NO cannot be ruled out, the known inability of platelets to effect NO release from organic nitrates (Weber *et al.*, 1996), and the COX assay data (chapter three) demonstrating powerful anti-COX activity of NCX4016, suggests that the antiplatelet action of NCX4016 is most likely NO-independent. Although this study shows NCX4016 to be a poor inhibitor of platelet function, its effects in other cells types may still make it a useful cardiovascular drug. Anti-platelet effects may still be retained *in vivo* through remote

nitrooxy-ester activation in cells other than platelets (e.g. smooth muscle cells), although this would appear to be a rather inefficient method of NO delivery specifically to platelets. Nevertheless, antiplatelet effects of NCX4016 have been demonstrated *ex vivo*, in animals and humans (Fiorucci *et al.*, 1999; Fiorucci *et al.*, 2004; Momi *et al.*, 2005; Wainwright *et al.*, 2002). Furthermore, NCX4016 has beneficial effects *in vivo,* where it has been demonstrated to protect the vascular endothelium in diabetic rats (Pieper *et al.*, 2002), to reduce blood pressure in hypertensive rats (Muscara *et al.*, 2001), to prevent restenosis in hypercholesterolemic mice (Napoli *et al.*, 2001) and to reduce infarct size in a model of cardiac ischemia in pigs (Wainwright *et al.*, 2002).

An interesting observation with these data is that whilst the IC_{50} of B7 is approximately 200-fold less than for its aspirin-free equivalent B12 in PRP and around 500-fold less in WP, the addition of an aspirin group to B13 to form B8 does not impact on its IC_{50}. This implies an NO-independent role for B7 to complement the NO effect. These data further confirm the dominance of the NO moiety of B8 and once again show that B7 acts as a better hybrid by allowing action of both of its components.

5.4.3 Summary, Future Directions and Therapeutic Implications

The data show the furoxan compound B13 to be an effective inhibitor of platelet aggregation and to be more resistant than the NO donor DEA/NO, to the scavenging effects of haemoglobin probably on account of intraplatelet release of NO.

The main drawback of the furoxan compounds B12 and B13 is their solubility. Whilst it cannot be ruled out that the drugs would still retain an action *in vivo* at concentrations that are soluble in biological media, alteration to the chemical structure may be necessary to achieve beneficial effects.

Whilst addition of the aspirin molecule to B13 to form B8 did not impact on its effects in WP or PRP, addition of aspirin to B12 did yield a more potent drug. Both the hybrids B7 and B8 did, however, cause concentration-dependent, NO-induced antiplatelet effects, with B8 being approximately 1000-fold more potent.

Previous studies have shown that both furoxan-aspirins (Cena *et al.*, 2003) and NCX4016 (Fiorucci *et al.*, 1999; Fiorucci & Del Soldato, 2003; Fiorucci *et al.*, 2003) achieve their original goal of a preferential gastrotoxicity profile over aspirin, suggesting either a masking of the toxic aspirin effect or an active NO element in the gastrointestinal tract. However, the activity of both the NO and aspirin elements of the hybrids in platelets is questionable. NCX4016 has no antiplatelet action in PRP and in WP it fails to demonstrate aspirin-independent effects, possibly due to sequestration by a plasma constituent. Unlike NCX4016, the furoxans demonstrate aspirin-independent effects on platelet aggregation in both PRP and WP *in vitro,* but their ability to retain the action of aspirin is compromised, particularly in PRP. The furoxans appear to display a more effective antiplatelet activity over NCX4016, but further molecular modifications are necessary in an effort to retain the aspirin actions in addition to achieving the added benefits of NO.

In summary, whilst the furoxan hybrid examples tested in these experiments carry some limitations, it is probable that with chemical modification to retain an aspirin-

like action in PRP, drugs of this class may become promising anti-thrombotic therapies.

CHAPTER SIX

NO-Release Mechanism of Novel Furoxan and Furoxan-Aspirin Hybrids in Platelets

6. NO-Release Mechanism of Novel Furoxan and Furoxan-Aspirin Hybrids in Platelets

6.1 Introduction

There are a variety of mechanisms by which NO donors release NO. Organic nitrate compounds such as GTN, ISDN and the NO-aspirin hybrid, NCX4016, require bioactivation by enzyme catalysis (Chen *et al.*, 2002; Grosser & Schroder, 2000; Sydow *et al.*, 2004). The requirement for such bioactivation is thought to be at the root of the phenomenon of nitrate tolerance that limits their use; vascular mitochondrial aldehyde dehydrogenase, the enzyme that accomplishes bioactivation undergoes nitrate-mediated inhibition (Chen *et al.*, 2002; Hinz & Schroder, 1998; Sydow *et al.*, 2004) .

The commonly-used experimental NO-donor, DEA/NO, does not require bioactivation and will spontaneously release NO when in solution at a rate dependent on temperature, pH and the nature of the nucleophile (Homer & Wanstall, 1998; Horstmann *et al.*, 2002; Keefer *et al.*, 2001; Ramamurthi & Lewis, 1997). The rapid release of NO and its very short half life of around 1 min at physiological temperature and pH (Homer & Wanstall, 1998) limit its therapeutic potential.

It is important to characterise the NO release mechanism of the furoxan compounds in order to fully understand their actions. Data from chapters four and five have led to the hypothesis that the NO released by the furoxan hybrids is intracellular, but the mechanism behind NO release remains to be elucidated.

There are numerous reports of paradoxical actions of NO in biological systems; it can

be both cytotoxic and cytoprotective (Hattori *et al.*, 2004; Keira *et al.*, 2002; Polte *et al.*, 1997) and both pro- and antioxidant (Joshi *et al.*, 1999; Rubbo *et al.*, 1995; Struck *et al.*, 1995). It has been proposed that these paradoxical effects can be explained by the precise NO-related species generated; peroxynitrite in particular is thought to be responsible for many of the deleterious effects of 'NO' (Shaw, 2006). For this reason, experiments were conducted in order to test the hypothesis that the hybrid compounds release no peroxynitrite in platelet-containing samples.

In this chapter, the mechanisms of NO release from furoxans and furoxan-aspirin hybrid drugs are investigated in human platelets *in vitro*. Furthermore, the possibility of peroxynitrite release was studied in order to determine the specificity of the hybrids as true NO donors.

6.2 Methods

6.2.1 NO Electrode Measurements

The furoxan and furoxan-hybrid compounds were investigated in the same manner, with the only exception being the use of Krebs as the buffer for the furoxan B13 experiments. The choice of buffer was decided to investigate the remarkable effects observed with B13 in the myography experiments. However, it is important to note that pilot experiments revealed no difference in release between samples in Tyrode's compared to Krebs buffer.

NO release was detected using an NO electrode, as described in section 2.5. The apparatus was set up and calibrated with DEA/NO (0.1-1.6 μM) as described in section 2.5. 2 ml samples of PRP, PPP, WP (prepared as in section 2.3) or Tyrode's/Krebs buffer were incubated in a 2-channel aggregometer (Chronolog Ca560); 37 °C and stirred continuously (6000 rpm). An isolated NO electrode was introduced into the cuvette and allowed to stabilise for at least 10 min until a steady baseline was achieved. The test compound, either B13 (3 μM), B8 (100 μM) or B7 (500 μM) was added to the cuvette and the release of NO was recorded for 10 min before addition of the NO scavenger, haemoglobin (10 μM) to scavenge the NO.

The involvement of endogenous reducing agents in the release of NO from the drug was investigated in a further series of experiments. Experiments were carried out in buffer reconstituted with approximate plasma and intracellular concentrations of GSH and ascorbate (as indicated in table 2.1), both alone, and in combination. Recordings were made from buffer ± 4% albumin, with the addition of varying

concentrations of GSH (3 μM or 3 mM) ± 50 μM ascorbate before the drug. Ascorbate (1 mM) was also used, but only in the presence of 10 mM HEPES to buffer its acidic pH.

To further investigate the possibility of intracellular release of NO from the furoxan-aspirin hybrids suggested by the data from chapter five, a platelet cell extract was prepared as described in section 2.5. 1 ml aliquots of the extract were incubated with either B8 (100 μM) or B7 (500 μM) and the NO release was recorded as described above. A further set of experiments were carried out following a 10 min preincubation of the platelet extract with 500 μM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) before NO release from B8 (100 μM) or B7 (500 μM) being recorded. Owing to the different profile of NO release by the different drugs, it was established that AUC was more representative of NO release than peak concentration in these experiments; results are quoted as AUC over the 10 min incubation (mmol.min).

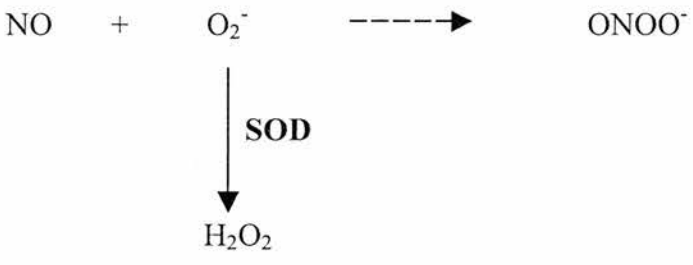
6.2.2 Peroxynitrite Release

The possible release of peroxynitrite by the compounds was investigated by two techniques making use of the enzyme SOD as outlined in section 2.6.

EPR was used to detect whether B8 and B7 released any peroxynitrite. Samples of PRP were incubated with either 100 μM B8 or B7 along with the spin-trap, CPH (10 mM), ± SOD (500 $U.ml^{-1}$) at 37 °C. Control samples were performed without the test compound and in addition, with the known peroxynitrite generator GEA 3162. EPR readings (as described in section 2.1.1) were taken periodically for 120 min and formation of the adduct 3-carboxy-proxyl ($CP^{\bullet}$) recorded.

The NO electrode was also used to determine if any peroxynitrite was released. The furoxans B8 (100 μM) and B7 (500 μM) were incubated in samples of PRP and PPP as before, but with the addition of 500 $U.ml^{-1}$ SOD before recording with the NO electrode for 10 min.

The enzyme superoxide dismutase (SOD) will prevent formation of peroxynitrite by removing superoxide through catalyzing its conversion to hydrogen peroxide (below)



6.2.3 Statistics

Statistical analysis was by 1- or 2-way ANOVA or by students' *t*-test. Bonferroni or Dunnett's post-test were carried out as appropriate. Tests were performed using GraphPad Prism version 4. * is used to represent a p value between 0.01 and 0.05, ** represents a p value of 0.001 to 0.01 and *** represents p values less than 0.001. P values greater than 0.05 were deemed not significant. Where expressed, data are in the form mean ± s.e.m.

6.3 Results

6.3.1 NO Electrode Measurements; Effect of Endogenous Antioxidants on NO Release from B13

Data for the compound B12 were not obtained due to its failure to stay in solution at a concentration high enough to allow detection by the extracellular electrode. The compound B13 (≥ 3μM) was found to release detectable NO in this system.

The electrode data revealed that the amount of NO released from B13 in Krebs buffer was barely detectable (Fig. 6.1a). NO generation from platelet-containing media (PRP and WP) was considerably enhanced (mean NO release = 10.45 ± 2.60 mmol.min and 10.67 ± 2.91 mmol.min respectively, Fig 6.1 b. $P < 0.01$; PRP vs. Krebs buffer alone, and $P < 0.05$; WP vs. Krebs buffer alone. Both 1-way ANOVA with Dunnett's post-test). In PPP the release was not significantly different from that in Krebs buffer (2.49 ± 0.31 mmol.min. $P > 0.05$; PPP vs. Krebs alone, 1-way ANOVA with Dunnett's post-test). Reconstitution of Krebs buffer with approximate plasma concentrations of either albumin (4%), GSH (3 μM) or ascorbate (50 μM) singly (0.29 ± 0.19 mmol.min, 0.25 ± 0.10 mmol.min, 0.73 ± 0.39 mmol.min respectively) or in combination (2.08 ± 0.72 mmol.min) failed to enhance the NO generation from B13 ($P > 0.05$ vs. Krebs alone, 1-way ANOVA).

Following the reconstitution of Krebs buffer with approximately intracellular concentration of GSH (3 mM), NO generation was enhanced (5.32 ± 1.29 mmol.min) as with the addition of the intracellular concentration of ascorbate (1 mM; 4.08 ±

1.97). A combination of GSH and ascorbate caused a considerable increase in NO generation from B13 (16.02 ± 1.70 mmol.min, n=6, Fig 6.1b, P<0.01 vs. individual components alone, 1-way ANOVA with Bonferroni post-test) to that beyond the release with platelet-containing samples. No significant difference was observed between WP and PRP samples ($p > 0.05$, 1-way ANOVA).

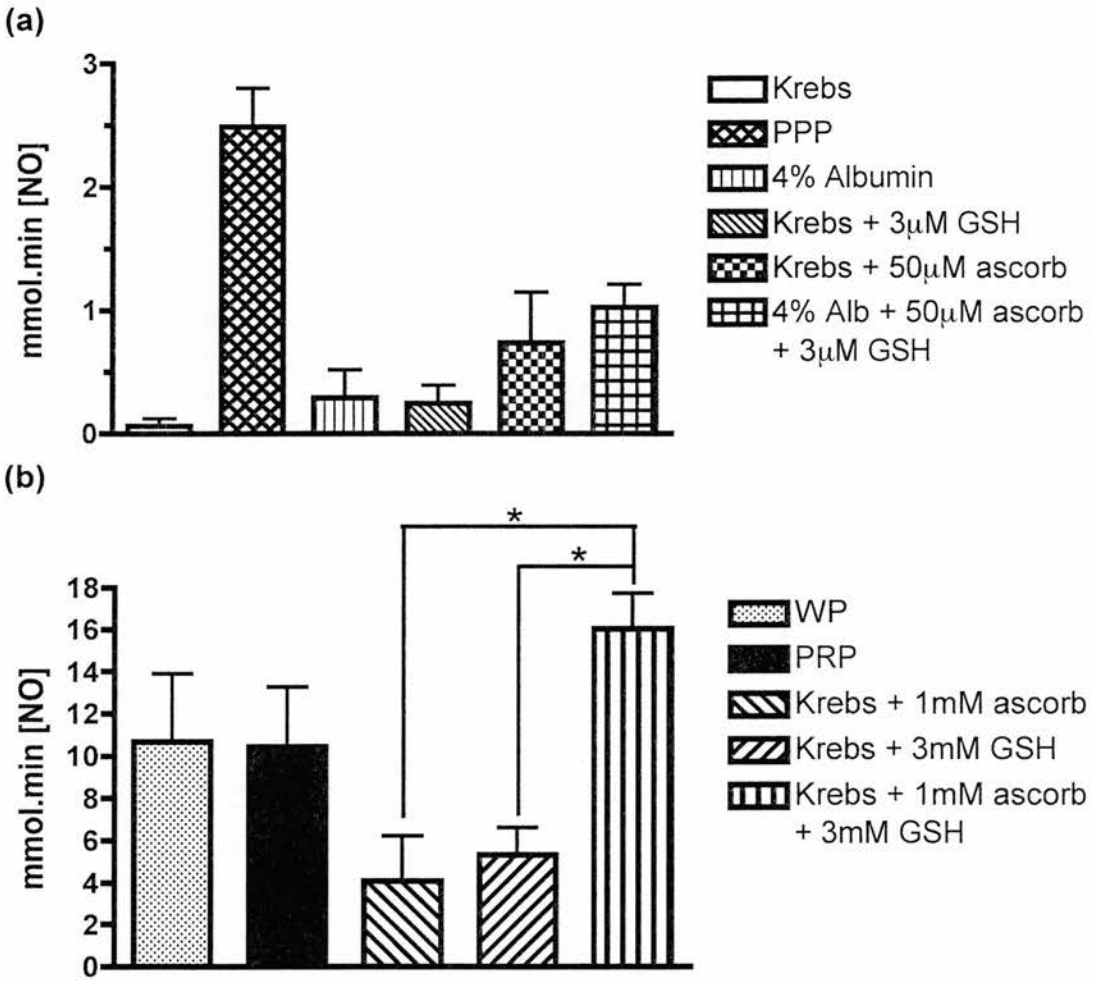


Fig. 6.1. NO release recorded over 10 min from B13 (3 μM) in various media. (a) NO release from B13 in media related to plasma conditions: Krebs buffer was reconstituted with approximate plasma concentrations of the plasma constituents, albumin (4%), GSH (3 mM) and ascorbate (50 mM). 1-way ANOVA revealed no significant difference in NO release between samples (b) Shows typical NO release from B8 in media related to platelet conditions: Krebs buffer contained approximate intracellular concentrations of glutathione and ascorbate (3 and 1 mM respectively). * = $p < 0.05$; combination of intracellular components vs. individual components alone, 1-way ANOVA with Bonferroni post-test. GSH = Glutathione. Ascorb = ascorbate. n = 6-7, values are mean ± s.e.m.

6.3.2 NO Electrode Measurements; NO Release from NO-Aspirin Hybrids

Experiments were performed in the presence of NCX4016 (100 μM) but NO release was undetectable in samples of Tyrode's buffer, PRP and WP (n = 6-8 for each. Data not shown). Sample recordings of NO generation from B8 and B7 in the presence and absence of GSH and ascorbate are shown in Fig. 6.2.

Similar to NCX4016, NO release from the furoxans B8 and B7 was undetectable in Tyrode's buffer alone (Fig. 6.2). However, it was detected in samples of PRP, WP, PPP or Tyrode's buffer in the presence of approximate plasma concentrations of albumin (4 %), GSH (3 μM) or ascorbate (50 μM; Fig. 6.3b, 6.4b).

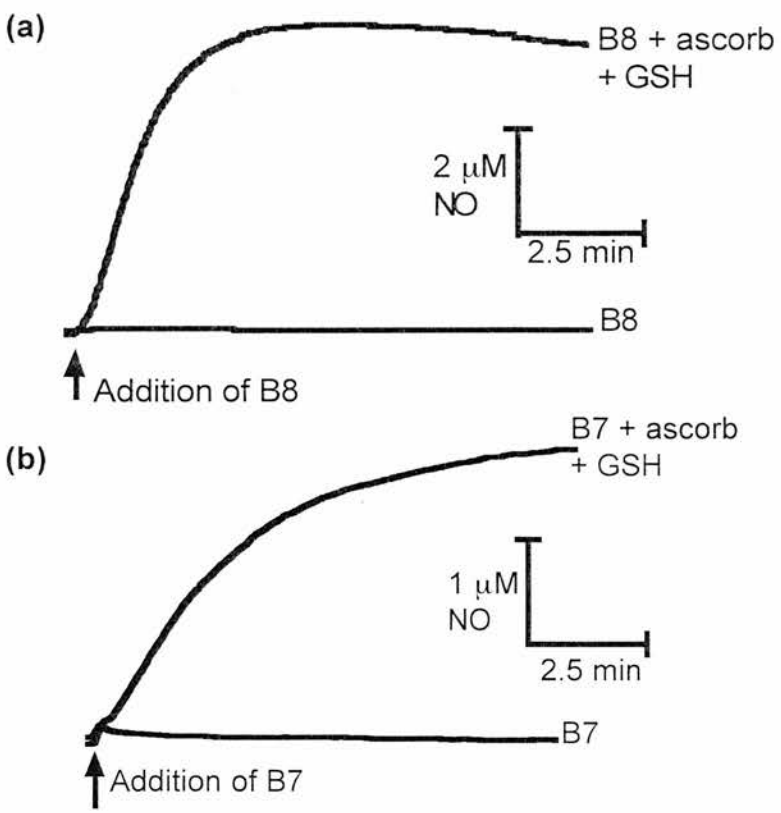


Fig. 6.2. Shows typical 10 min traces of NO release recorded using the NO electrode in Tyrode's buffer with or without ascorbate (1 mM) and GSH (3 mM). **(a)** is following addition of B8 (100 μM) and **(b)** is following addition of B7 (500 μM).

6.3.2.1 NO Release from B8; Effect of Endogenous Antioxidants

NO was released from B8 in platelet-containing samples (PRP and WP; mean NO release = 33.2 ± 2.9 mmol.min and 33.0 ± 2.5 mmol.min respectively; Fig 6.3b), and in PPP (mean NO release for B8 = 21.0 ± 2.8 mmol.min; Fig 6.3a).

Reconstitution of Tyrode's buffer with approximate plasma concentrations of either albumin (4%), GSH (3 μM) or ascorbate (50 μM) enhanced NO generation from B8. A combination of these three constituents failed to enhance NO release from B8 beyond the effects seen with the individual components alone (2.7 ± 0.7 mmol.min; Fig. 6.3a; $P > 0.05$ vs. GSH alone, $P > 0.05$ vs. ascorbate alone, both 1-way ANOVA with Bonferroni post-test).

Reconstitution of Tyrode's buffer with approximately intracellular concentrations of GSH (3 mM) generated marginally less NO from B8 (14.4 ± 2.5 mmol.min; Fig 6.3b) compared with samples containing platelets. Ascorbate (1 mM) in Tyrode's buffer caused minimal NO release from B8 (2.2 ± 0.9 mmol.min; Fig. 6.3b), but a combination of GSH and ascorbate caused a considerable increase in NO generation from B8 (247 ± 19 mmol.min, n = 6, fig 6.3b, $P<0.001$ vs. individual components alone, 1-way ANOVA with Bonferroni post-test). No significant difference was observed between WP and PRP samples ($p > 0.05$, 1-way ANOVA).

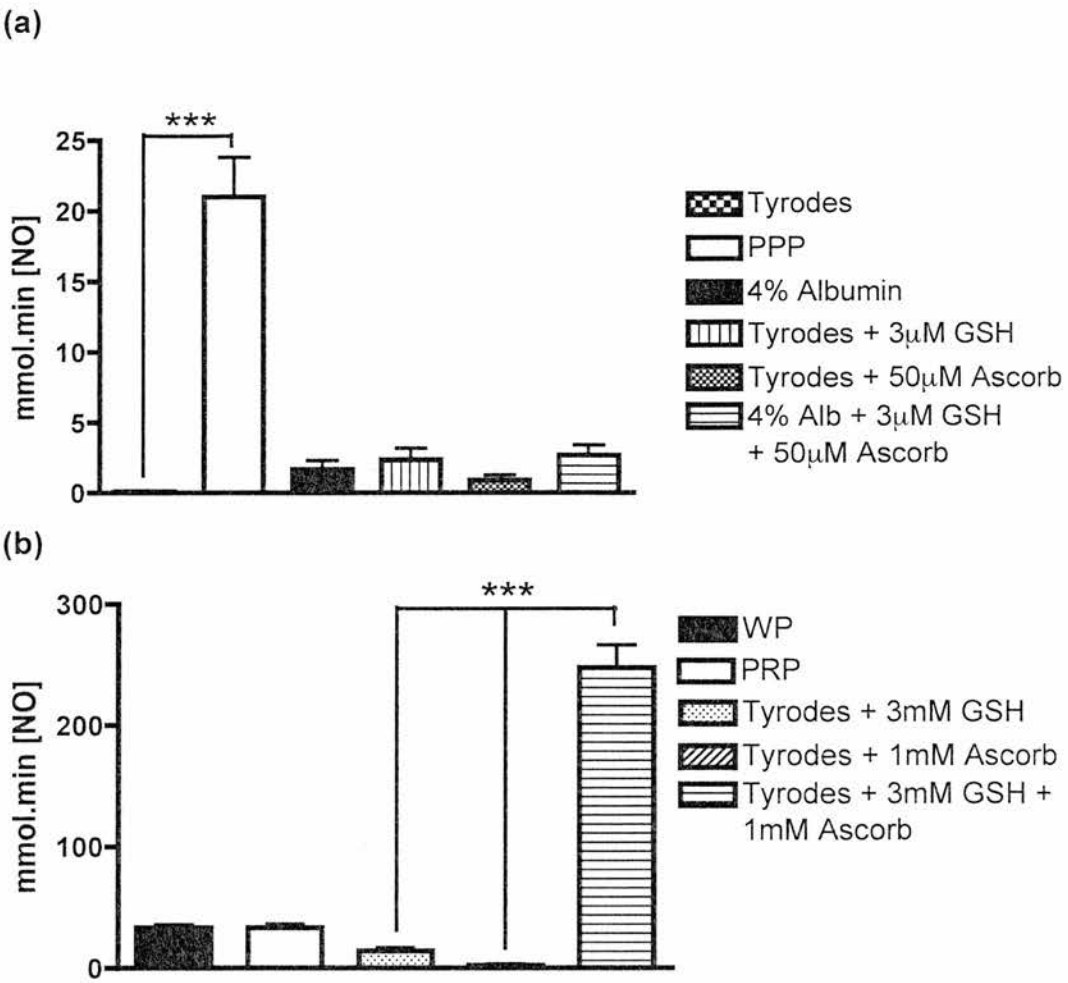


Fig. 6.3. NO release recorded over 10 min from B8 (100 μM) in various media. **a**. NO release from B8 in media related to plasma conditions: Tyrode's buffer was reconstituted with approximate plasma concentrations of the plasma constituents, albumin (4%), GSH (3 μM) and ascorbate (50 μM). **b**. shows typical NO release from B8 in media related to platelet conditions: Tyrode's buffer 3 mM and 1 mM are approximate intracellular concentrations of glutathione and ascorbate respectively. *** = $p < 0.001$ as revealed by 1-way ANOVA with Bonferroni post-test. n = 6 – 7. GSH = Glutathione. Ascorb = ascorbate. Values are mean ± SEM.

6.3.2.2 NO Release from B7; Effect of Endogenous Antioxidants

Relatively low levels of NO were released from B7 in PRP and WP (1.6 ± 0.4 mmol.min and 3.8 ± 0.7 mmol.min respectively; n = 6, Fig 6.4b) despite the higher drug concentration used. Reconstitution of the Tyrode's buffer with approximate plasma concentrations of either albumin (4%), GSH (3 μM) or ascorbate (50 μM) enhanced NO generation from B7 and a combination of these three constituents had an additive effect on the release of NO from B7 (3.8 ± 0.8 mmol.min), where release was equivalent to that seen in PPP (1-way ANOVA with Bonferroni post-test $P < 0.05$, Fig. 6.4a).

Reconstitution of Tyrode's buffer with approximately intracellular concentrations of GSH (3 mM) increased NO release from B7 (8.5 ± 1.1 mmol.min). Ascorbate (1 mM) in Tyrode's buffer caused minimal NO release from B7 (3.1 ± 1.6 mmol.min), but a combination of GSH and ascorbate caused a considerable increase in NO generation from B7 (47 ± 4 mmol.min n=6, Fig 6.4b).

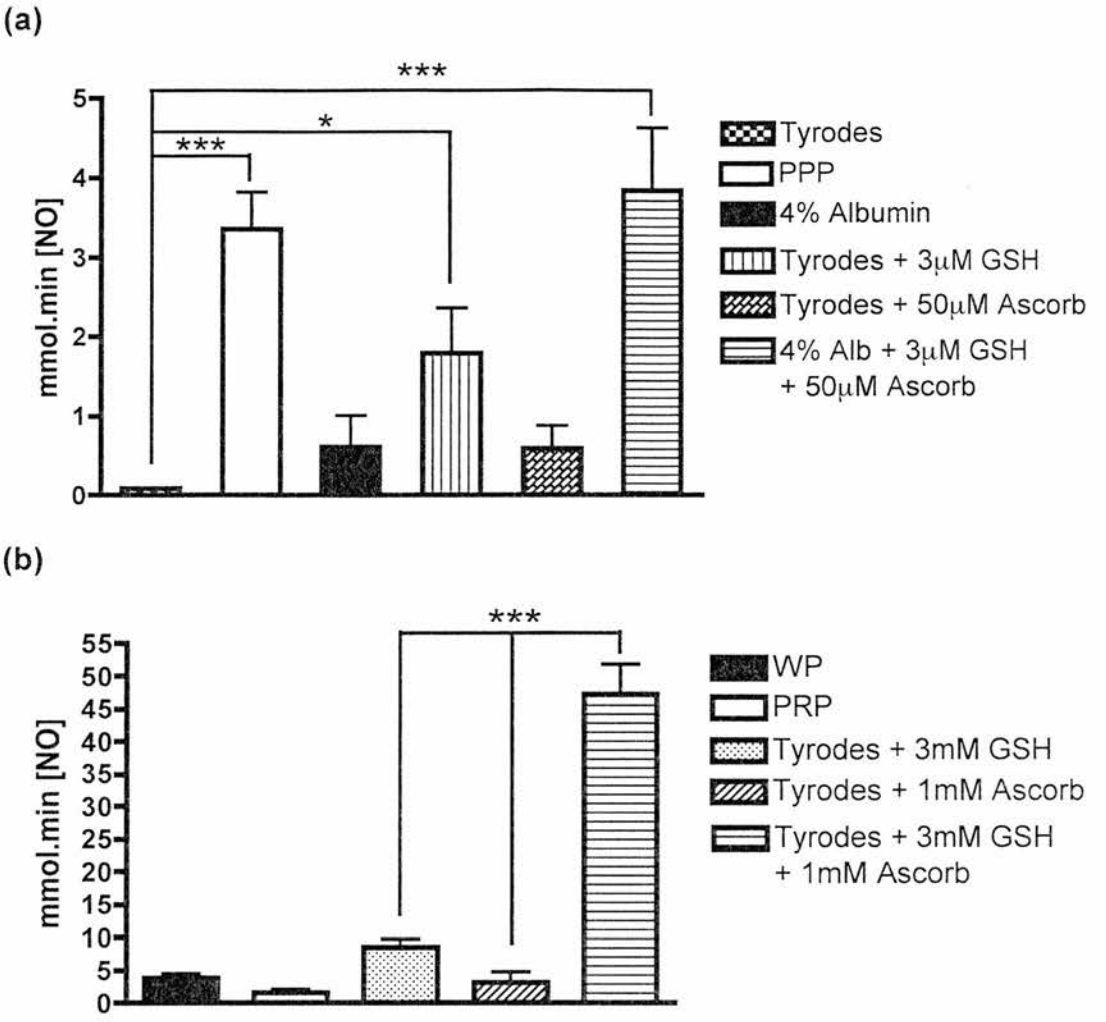


Fig. 6.4. NO release recorded over 10 min from B7 (500 μM) in various media. **a**. NO release from B8 in media related to plasma conditions: Tyrode's buffer was reconstituted with approximate plasma concentrations of the plasma constituents, albumin (4%), GSH (3 μM) and ascorbate (50 μM). **b**. shows typical NO release from B8 in media related to platelet conditions: Tyrode's buffer 3 mM and 1 mM are approximate intracellular concentrations of glutathione and ascorbate respectively. * = $p < 0.05$. *** = $p < 0.001$ as revealed by 1-way ANOVA with Bonferroni post-test. n = 6 – 7. GSH = Glutathione. Ascorb = ascorbate. Values are mean ± SEM.

6.3.2.3 NO Release in a Platelet Cell Extract

The possibility of intracellular release of NO from the furoxan-aspirin hybrids was further investigated using a platelet cell extract. The data showed a relationship between the dilution factor of platelet extract and the amount of NO released from both B8 and B7 (Fig. 6.5a and b respectively). NO generation by B8 (100 μM) failed to increase at platelet extract dilutions below ~1 in 30, with a maximum NO generation of ~90 mmol.min. Pre-treatment of platelet extracts with the thiol alkylator, DTNB (500 μM), all but abolished NO generation.

A similar pattern was observed with B7 (500 μM), but the amount of NO generated in the presence of platelet extracts was >10-fold lower than from B8, despite the higher drug concentration. In the case of B7, dilutions of platelet extract lower than ~1 in 75 failed to generate detectable NO, but there was a direct relationship between NO generation and dilution factor at dilutions of 1 in 75 – 1 in 25 (Fig. 6.5b). DTNB again showed a marked inhibitory effect on NO generation in the presence of platelet extract. Colorimetric analysis of DTNB-treated samples yielded intracellular total reduced thiol concentrations of 22 ± 4 mM (corrected for dilution; n = 5).

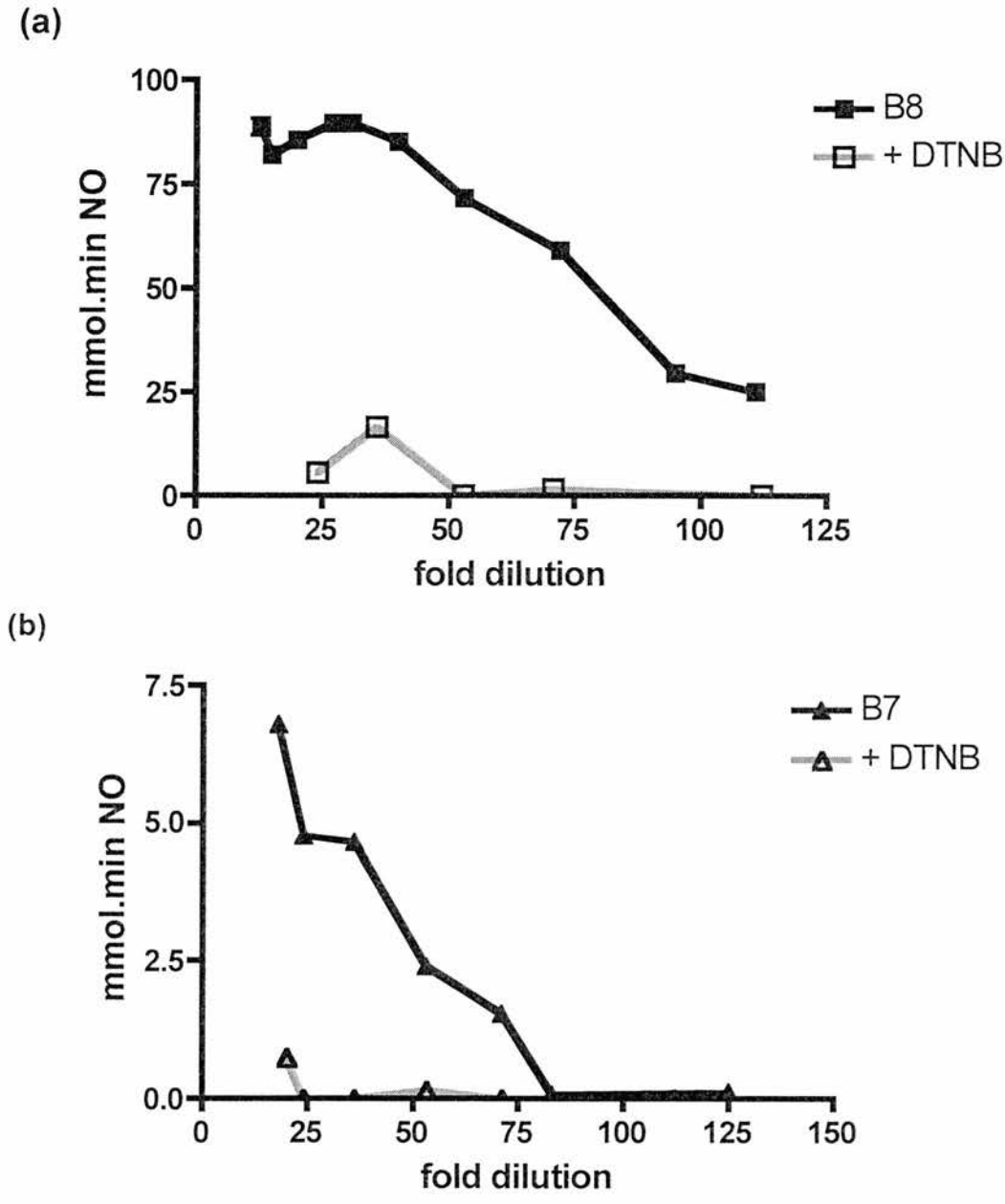


Fig. 6.5. A sample of WP was suspended in 1 ml triton-X and then homogenized to release platelet extract before centrifugation to remove membrane fraction. The dilution factor on x-axis was calculated by volume of triton–X (1 ml) / (number of platelets × average platelet volume). **a**. NO release from 16 platelet extracts treated with 100 μM B8. The grey line shows samples treated in the same way but with a 10 min preincubation with 500 μM DTNB before addition of B8. **b**. NO release from 13 platelet extracts treated with 500 μM B7. The grey line shows samples treated in the same way but with a 10 min preincubation with 500 μM DTNB before addition of B7.

6.3.3 Peroxynitrite Release

In the case of both B8 (100 μM) and B7 (500 μM), incubation with the enzyme SOD (500 $U.ml^{-1}$), had no effect on the amount of NO released as detected by the NO electrode (Fig. 6.6. $p = 0.83$ for B8 ± SOD and $p = 0.54$ for B7 ± SOD).

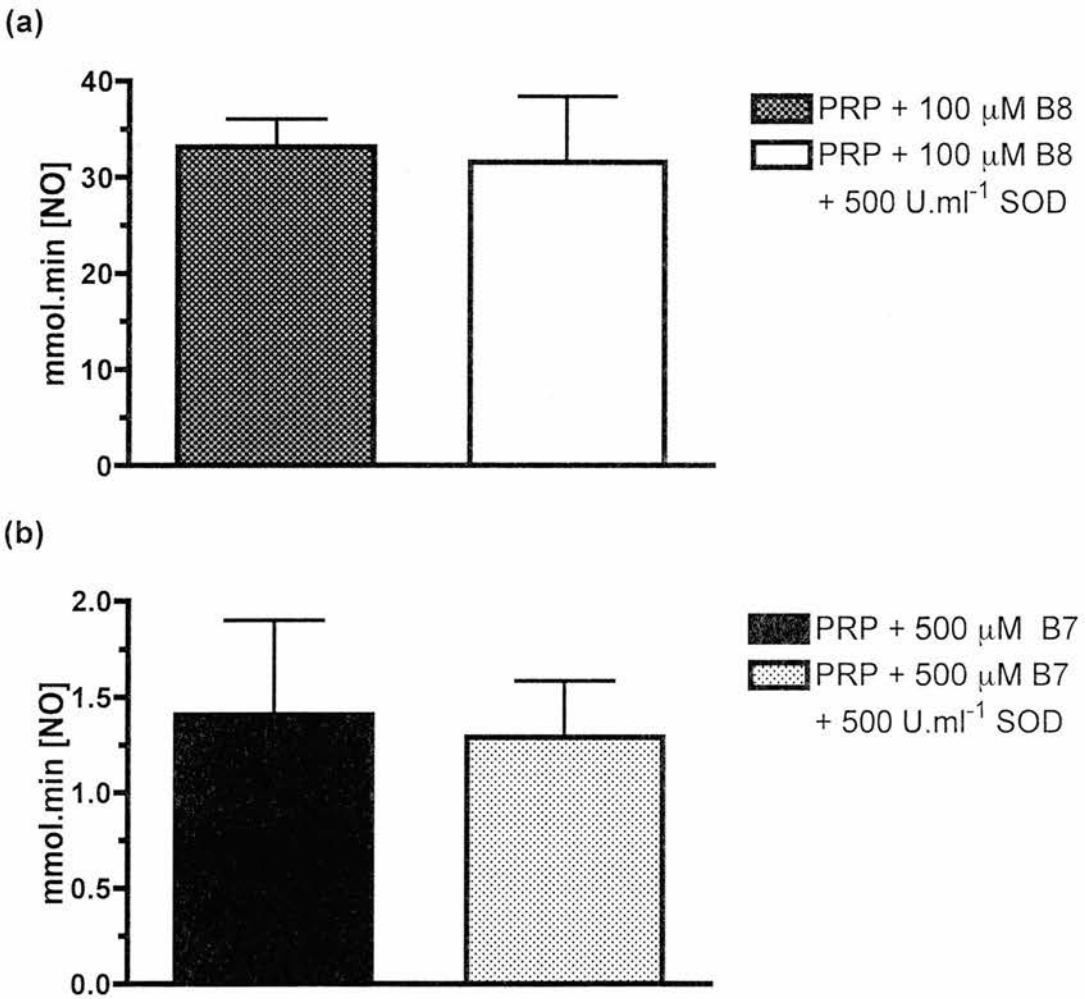


Fig. 6.6. NO release recorded over 10 min from **(a)** B8 (100 μM) and **(b)** B7 (500 μM) in PRP. NO release from the furoxan-aspirin compounds was detected in the presence and absence of the enzyme SOD to determine if there was any peroxynitrite release by the compounds. n = 6. Values are mean ± SEM. Results were deemed not significant as analysed by paired Students' t-test.

EPR was also used to investigate any possible peroxynitrite release by the furoxan NO-aspirins. The data showed no oxidation of the spin-trap by B7 in either PRP or PPP; levels were very similar to an aspirin control (Fig. 6.7; p = 0.32 in PRP and 0.39 in PPP. 2-way ANOVA). There was an apparent effect of B8 in both PRP and PPP but this was insensitive to 500 $U.ml^{-1}$ SOD (p = 0.89 in PRP and 0.91 in PPP. 2-way ANOVA).

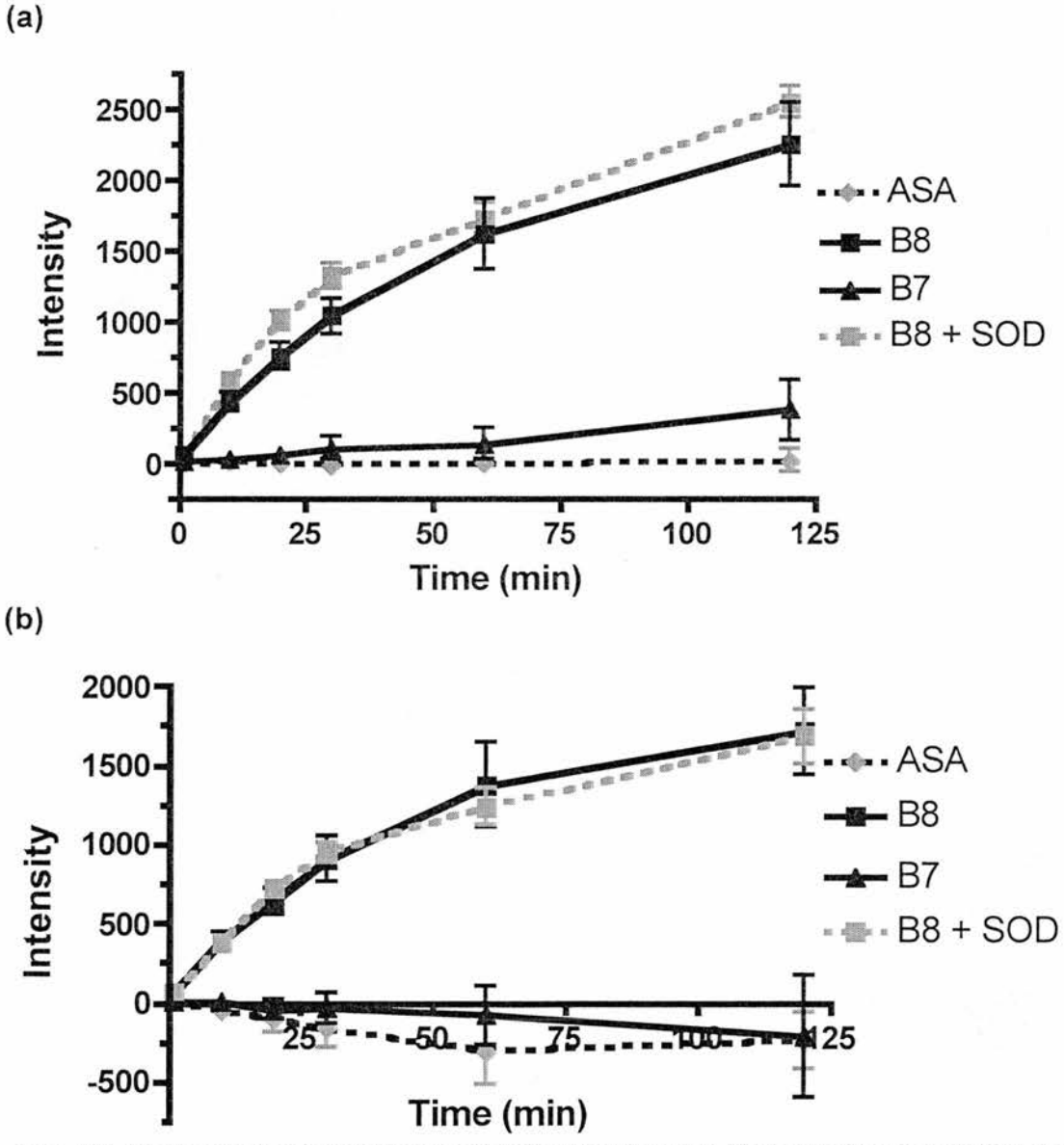


Fig. 6.7. Mean data for development of EPR signal intensity (AU) with time in **(a)** PRP and **(b)** PPP in the presence of aspirin, B8 or B7 (all 100 μM) and spin trap CPH (1 mM). EPR settings: B0-field, 3356 gauss; sweep width; 50 Gauss, sweep time, 30 sec; modulation amplitude, 1500 mGauss; microwave power, 20 mW. n = 6.

The known peroxynitrite generator, GEA 3162 (30 μM) caused an increase in EPR signal (Fig. 6.8) that was significantly greater than that caused by B8 and was sensitive to 500 U.ml $^{-1}$ SOD ($P<0.0001$; 2-way ANOVA).

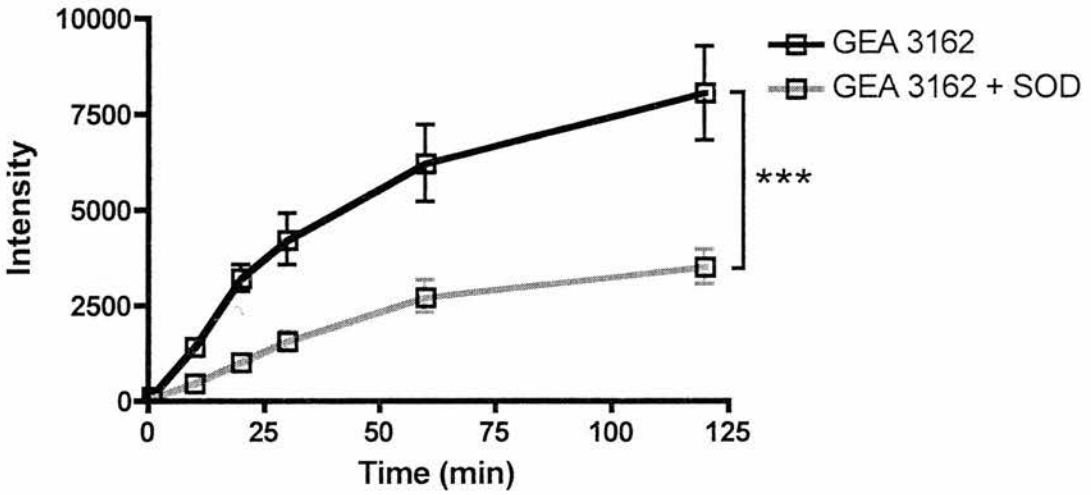


Fig. 6.8. Mean data for development of EPR signal intensity (AU) with time in PRP in the presence of GEA 3162 (30 μM) and spin trap CPH (1 mM). EPR settings: B0-field, 3356 gauss; sweep width; 50 Gauss, sweep time, 30 sec; modulation amplitude, 1500 mGauss; microwave power, 20 mW. n = 6.*** = $P<0.0001$; 2-way ANOVA.

6.4 Discussion

6.4.1 NO Release by B13

Significant release was achieved in platelet-containing samples (PRP and WP) compared to buffer alone. The plasma components, albumin, GSH and ascorbate, chosen for their reducing properties, were all found to have a limited capacity to stimulate furoxan decomposition to release NO from B13. At extracellular concentrations, these components individually stimulate small amounts of NO and in combination the effects are additive but do not reach the amount released by PPP. At intracellular concentrations, the antioxidant elements, GSH and ascorbate, play a role in accelerating the release of NO compared to buffer alone; combination of these elements accelerated release to beyond the levels recorded from platelet-containing samples, suggesting that NO release by furoxan B13 is stimulated intracellularly by a dual-effect of GSH and ascorbate. A minimal additional release of NO does occur in the plasma; another element further to the antioxidants studied may contribute to this release.

The significant stimulation of NO release in environments that mimic intracellular conditions indicates very specific NO release and could explain the ability of B13 to cause the vasorelaxation observed in chapter four at very low concentrations, as well as and its resistance to the scavenging effects of cPTIO.

The NO release data also shed some light on the results from the platelet studies in chapter five. The intracellular release indicated by the data in this chapter further confirm that the cGMP-dependent antiplatelet effects are a consequence of

intracellular release of NO (Crane *et al.*, 2005). Intracellular release by B13 may also explain the resistance of B13 to the scavenging effects of haemoglobin as demonstrated by the whole blood aggregation study.

6.4.2 NO Release from NO-Aspirin Hybrid Drugs

The inability to detect NO from NCX4016 in this study is perhaps not surprising. The release at NO from NCX4016 and GTN has been reported to occur through identical mechanisms (Grosser & Schroder, 2000). Platelets have a poor capability to release NO from organic nitrates (Weber *et al.*, 1996) and thus, NCX4016 would not be expected to release NO in the *in vitro* platelet samples investigated in this study.

The furoxan-aspirin hybrids B8 and B7, like the aspirin free equivalent, are very stable compounds that appear to only decompose to release measurable NO when they encounter appropriate media. There was no release of NO from the furoxans in Tyrode's buffer, but NO generation was detected in PPP, WP and PRP. These observations suggest that some elements of plasma can stimulate decomposition of the furoxans and that the platelets themselves enhance the effect to a greater extent for B8 than B7.

The plasma components, albumin, GSH and ascorbate were all found to have a limited capacity to stimulate furoxan decomposition to release NO. In the case of B7, these constituents could individually stimulate moderate NO release, and the additive effect of all three could fully emulate NO release in PPP. The effect of these constituents on B8 were similar, if less dramatic, for the individual reducing agents, but here there was no additive effect when co-incubated, suggesting that an as yet unidentified plasma constituent is responsible for a proportion of NO release from B8.

Reconstitution of Tyrode's buffer with intracellular levels of GSH and ascorbate demonstrated that both of these reducing agents were mildly effective in stimulating release of NO from the compounds. Interestingly, however, they acted synergistically in co-incubation experiments to massively increase NO generation. The separate experiments using platelet extracts established that there was a relationship between the concentration of platelet extract and amount of NO generated from the furoxan-aspirin hybrids. The ability of platelet extracts to generate NO from both furoxans was found to be dependent on the presence of reduced thiol groups and, in keeping with the other NO release data and the platelet aggregation experiments (chapter five), B8 generated considerably more NO in the 10 min incubation period than B7. Taken together, these results indicate that low-level decomposition of B7 in plasma can be explained by the additive effects of albumin, GSH and ascorbate but that there is also likely to be much greater NO release inside platelets mediated by the synergistic action of ascorbate and reduced intracellular thiols. B8 is more sensitive to the same agents, and its plasma decomposition is also affected by an as yet unidentified plasma component, again similar to its aspirin-free equivalent, B13. Given the reactive nature of NO, the apparent preferential release inside target cells is a likely advantage for successful NO delivery over compounds that can only generate NO remotely in cell types other than platelets.

The data here show that the compound B8 releases much more NO than B7 (~5-fold more despite being used at a 5-fold less concentration). Such high levels of NO explain the dominance of NO over aspirin in the antiplatelet studies (chapter five) and further suggest that B7 is a more promising hybrid drug target where the NO release is less dominant.

6.4.3 Peroxynitrite Release

The two sets of studies used to investigate possible peroxynitrite release by the hybrids revealed that neither B8 nor B7 releases significant amounts of peroxynitrite *in vitro*. However, the results do not preclude generation of peroxynitrite in situations where superoxide arises from a source other than the drugs themselves (e.g. oxidative stress).

Peroxynitrite is formed on the reaction of NO with superoxide (Saran *et al.*, 1990). The enzyme SOD breaks down peroxynitrite by removing superoxide and catalyzing its conversion to hydrogen peroxide. This yields the NO that the electrode detects and thus is an indirect method of recording peroxynitrite release. The results of this indirect recording revealed that in the case of both NO-aspirins, incubation with the enzyme SOD had no effect on NO release therefore ruling out any masking of NO as peroxynitrite.

Peroxynitrite releasewas also investigated using a direct method of detection. EPR utilising a spin-trap agent has previously been demonstrated as an efficient method of measuring peroxynitrite (Dikalov *et al.*, 1997; Taylor *et al.*, 2004). Whilst B7 generated no detectable signal in either PRP or PPP, was detected from B8 in both PRP and PPP. It is most likely that this signal is not due to generation of peroxynitrite but rather to a non-specific effect of NO in the system as indicated by the failure of SOD to decrease the signal. In contrast, in the same system, a signal corresponding to the formation of $CP^{\bullet}$ from the reduced form of the spin-trap was detected from the peroxynitrite generator, GEA 3162. In this case, incubation with SOD caused a significant depletion of the signal.

6.4.4 Summary and Future Directions

The results of this chapter reveal the furoxan and furoxan-aspirin compounds to release their NO primarily in the intracellular environment when they encounter antioxidants such as GSH and ascorbate. There is perhaps an as yet unidentified component which contributes to the release of NO from B8 and B13 for which cysteine is a possible candidate (Ferioli *et al.*, 1995). The predominantly intracellular release of NO by B13 is perhaps the reason behind its resistance to haemoglobin in the whole blood aggregometry (chapter five) and its remarkable ability to cause vasodilatation at picomolar concentrations (chapter four). The intracellular release by the hybrids gives them an advantage as antithrombotics over the existing nitroaspirin, NCX4016 which requires enzymatic breakdown not achievable by platelets (Grosser & Schroder, 2000; Weber *et al.*, 1996). Of the hybrids, B7 is the better drug candidate due to its lower NO release, providing opportunity for a more balanced action between the NO effect and thee COX inhibitory effect identified in chapter three.

The major limitation of these experiments was the solubility of the furoxans and B12 in particular, which failed to stay in solution to allow *in vitro* exploration of NO release. Chemical modification to the structure may improve the solubility of compounds more soluble, however, it is likely that with development of a more sensitive technique, data will be obtained for this compound.

CHAPTER SEVEN

Anti-Inflammatory Effects of Furoxan-Aspirin Hybrid Drugs in Human Monocytes and Monocyte-Derived Macrophages

7. Anti-Inflammatory Effects of Furoxan-Aspirin Hybrid Drugs in Human Monocytes and Monocyte-Derived Macrophages

7.1 Introduction

In order to further determine cardiovascular benefits of the NO-aspirin compounds, their anti-inflammatory actions in cell types relevant to atherosclerosis were examined. Due to their ability to retain an aspirin-like inhibition of COX (chapter three) and to release NO (chapter six), these drugs have the potential to be anti-inflammatory.

The anti-inflammatory effects of NO in the vasculature centre around its ability to regulate endothelial cell-mediated leukocyte recruitment at a site of injury, which is central to the atherogenesic process. NO reduces expression of P-selectin and thus leukocyte recruitment in a cGMP-dependent process (Ahluwalia *et al.*, 2004; Kubes *et al.*, 1991) and inhibits ICAM and VCAM expression (Berendji-Grun *et al.*, 2001; De Caterina *et al.*, 1995; Spiecker *et al.*, 1997). In addition, NO has anti-inflammatory effects in macrophages. Immunological or inflammatory stimuli, such as activation by cytokines and bacterial products, stimulate macrophages to express iNOS and generate large quantities of NO (Moncada & Higgs, 1995). NO inhibits production of inflammatory cytokines IL-1 and IL-12 (Huang *et al.*, 1998; Obermeier *et al.*, 1999; Thomassen *et al.*, 1997) and furthermore, *in vitro* and *in vivo* evidence show that L-NMMA treatment increases TNFα production whereas L-arginine

treatment reduces it (Iuvone *et al.*, 1996). Interestingly, it has recently been shown that a cGMP-mediated reaction is responsible for inhibition of peroxynitrite-induced programmed cell death in human monocyte-derived macrophages, implying a further role for NO during inflammation (Shaw, 2006).

COX inhibition is anti-inflammatory due to reduced production of prostaglandins involved in the inflammatory response. The anti-inflammatory effects of aspirin are more likely to be COX-2-mediated due to its tendency to be expressed in response to inflammatory stimuli. Aspirin also plays a role in the production of endogenous anti-inflammatory agents known as aspirin triggered lipoxins (ATL). These are short-lived eicosanoids whose appearance in inflammation signals its resolution. ATL are formed by aspirin by the 15-epi-LX pathway. In cytokine-rich environments, aspirin acetylates COX-2, prompting it to act as a lipoxygenase rather than an endoperoxide and thus, converting AA to 15-HETE which is subsequently transformed by 5-LO to the ATLs, 15-epi-LXA_4 or 15-epi-LXB_4 (Claria & Serhan, 1995).

ATLs are thought to bring about beneficial effects, particularly in the resolution of inflammation (McMahon *et al.*, 2001). Recent evidence has shown that LXA_4 downregulates the production of the inflammatory mediator, IL-12, (Machado *et al.*, 2006) which would otherwise persist, resulting in host damage. Furthermore, it has been demonstrated that a defect in the signalling process of ATL results in uncontrolled inflammation (Machado *et al.*, 2006). The ability of aspirin to inhibit cell accumulation during inflammation has also been attributed to the action of ATL. It is thought that this process is due to the stimulation of eNOS and iNOS to produce NO, which acts to provide anti-inflammatory effects such as inhibition of leukocyte-

endothelial interactions (Paul-Clark *et al.*, 2004; Taubert *et al.*, 2004).

Cytokines are polypeptide or glycoprotein factors that act in an autocrine and/or paracrine fashion to signal in a variety of biological processes. They are generally classed as either pro-inflammatory (e.g. TNFα and IL-8) or anti-inflammatory (e.g. TGFβ and IL-10), although they can have paradoxical actions. Cytokines act in various cell types and perform diverse functions. For example, the chemokine, IL-8, which is produced by IL-1- and TNFα-stimulated monocytes is involved in chemotaxis for various immune cells, including neutrophils (Harada *et al.*, 1994). It also plays a role in leukocyte adhesion to the endothelium (Graves & Jiang, 1995; Rot *et al.*, 1996). Other roles of IL-8 are in the inhibition of histamine release from activated basophils (Kuna *et al.*, 1991) and also in the mediation of pain (Cunha *et al.*, 1991). TNFα is secreted by monocytes, macrophages and neutrophils following their stimulation by bacterial lipopolysaccharides. The various activities of TNFα include mediation of CAM (Graves & Jiang, 1995), regulation of cell death in tumour cells (Chang & Wisnieski, 1990), enhancement of neutrophil phagocytosis (Klebanoff *et al.*, 1986), control of neutrophil adherence to the endothelium (Graves & Jiang, 1995) and synthesis of IL-1 production by macrophages (Dinarello *et al.*, 1986).

LPS is a bacterial endotoxin that stimulates the production of inflammatory mediators such as the cytokines (i.e. TNFα and IL-8) via the activation of NF-κB (Baldwin, 1996; Kubes & McCafferty, 2000; Totzke *et al.*, 2006). The gene expression of the pro-inflammatory cytokine, TNFα is controlled by the transcription factor, NF-κB. Normally NF-κB is kept in an inactive state in the cytoplasm by being

bound to a member of the IκB family. To date, seven such inhibitory subunits comprise the IκB family (May & Ghosh, 1998). NF-κB exists as a dimer, most commonly as a heterodimer composed of the p65/p50 subunits (Panwalkar *et al.*, 2004). The phosphorylation of IκB and its subsequent degradation leads to the activation of the NF-κB. IκB is phosphorylated by kinases known as IKKs (IκB-kinases) of which two subunits (α and β) exist. The subunits form homo- or hetero-dimers which, depending on their conformation, result in different patterns of NF-κB activation (May & Ghosh, 1998). Following activation, the NF-κB dimer containing the p65 subunit, the dominant factor in the induction of the TNFα gene then translocates from the cytoplasm to the nucleus, where it activates transcription of target genes, including TNFα. (Baldwin, 1996; Kaltschmidt *et al.*, 1999; Liu *et al.*, 2000; Totzke *et al.*, 2006). Glucocorticoids such as dexamethasone are commonly used anti-inflammatory agents. Glucocorticoids have been shown to effectively inhibit suppression of pro-inflammatory transcription factors such as NF-κB (Barnes, 2006). They act upon glucocorticoid receptors, which when stimulated, translocate to the nucleus preventing histone acetylation, a vital step in the NF-κB-induced gene transcription (Adcock *et al.*, 2004; Barnes, 2006). Other mechanisms by which the glucocorticoid receptor reduces TNFα expression include decreasing mRNA stability, inducing expression of the inhibitor IκB, and altering co-factor (AP-1) activity (Adcock *et al.*, 2006).

This chapter shall investigate the anti-inflammatory properties of the hybrid drugs in order to test the hypothesis that via their ability to inhibit inflammatory cytokine release from LPS-stimulated human monocytes and monocyte-derived macrophages the hybrids have an anti-inflammatory potential.

7.2 Methods

7.2.1 Preparation of Monocytes and Macrophages

Peripheral venous blood was drawn from the antecubital fossa of human volunteers (non-smokers; age 20-45). Mononuclear cells were isolated from human blood using dextran sedimentation and Percoll gradients as described in section 2.7 and plated out at a concentration of 2×10^6 per well (0.5 ml). Cells were grown for a week as described in section 2.8 in order to differentiate into macrophages. On day 7 (or immediately following their isolation for experiments on monocytes) the medium in each well was changed to that containing 10 μM (selected based on results from previous chapters) of either B8, B7, B16, B15, aspirin, NCX4016, DEA/NO or dexamethasone (1 μM) with and without LPS (10 $ng.ml^{-1}$). Cells were then incubated at 37 °C for 4 hours before removal of the cell supernatants, which were frozen at - 70 °C and later used for biochemical analysis.

7.2.2 Cytometric Bead Array

The bead array was used in a pilot study to determine whether the NO-aspirin compounds had any anti-inflammatory effects by inhibiting release of inflammatory mediators released by macrophages following LPS stimulation. It was also used to determine suitable drug and LPS concentrations for future studies. A commercial human inflammation CBA kit that detects levels of IL-8, IL-1β, IL-6, IL-10, TNFα and IL-12p70 was used (BD Biosciences). The protocol was carried out as described in chapter two (section 2.8.1) on supernatants from macrophages matured and treated as in section 7.2.1 with the approximate antiplatelet EC_{50} concentration (chapter five;

0.3 μM for B8/B16, 100 μM for B7/B15, 50 μM for aspirin and 10 μM for NCX4016) of furoxan, NCX4016 or aspirin or a DMSO (0.1 %) control. The furazan concentrations were determined by their furoxan-equivalent. Cells were stimulated with 1-100 $ng.ml^{-1}$ LPS. Levels of the cytokines were recorded by detecting PE-conjugated antibodies using flow cytometry. Results are expressed as a percentage of level recorded from an LPS-only control.

7.2.3 Enzyme-Linked Immunosorbent Assays

Supernatants from the cell treatments described above were assayed for levels of TNFα and IL-8 using commercially available ELISA kits (BD Biosciences). Experimental protocols were carried out as per kit instructions, as described in the methods chapter on monocytes and macrophages isolated and treated as in section 6.2.1. Levels of the cytokines were recorded by colourimetric analysis using a platereader. Results are expressed as a percentage of level recorded from an LPS-only control.

7.2.4 Lactate Dehydrogenase (LDH) Assay

LDH is a stable cytoplasmic enzyme present in all cells. As it is rapidly released following damage to the plasma membrane its quantification can be used to measure cell death. A commercially available kit (Roche) was utilised to quantify LDH and thus cell death. 100 μl supernatant from the monocyte / macrophage studies described above were mixed with 100 μl reaction mixture as per kit instructions and left to incubate in the dark for 30 min before reading in the plate reader at 492 nm. A calibration was performed by carrying out the assay on suspensions of varying cell

concentrations (4, 3, 2, 1, and 0.5 × 10^6 cells/ml) which had been incubated with an equal volume of 0.5 % Triton X for 1 hr at RT, homogenised for 15 min then centrifuged at 250 *g* for 10 min. 100 µl supernatant was used in the assay giving a final cell count of 2, 1.5, 1, 0.5 and 0.25 × 10^5 cells in the assay. Levels of the cell death were recorded by colourimetric analysis and results are expressed as absorbance units.

7.2.5 Immunofluorescnce for NF-κB p65 Subunit

Monocytes (1 ml of 4×10^6 cells.ml^{-1}) isolated as above were plated out onto glass coverslips within wells of a 6-well plate and left to adhere. Following washing, medium was then changed to ISCOVES DMEM (supplemented with 10 % autologous serum) containing either gliotoxin (0.1 µg/ml), B8 or B16 (20 µM) or no drug (DMSO 0.1 % control). Cells were left to incubate (37 °C) for 30 min. LPS (10 ng.ml^{-1}) or vehicle (ISCOVES DNEM supplemented with 10 % autologous serum) was then added on top of the coverslip and left to incubate for a further 45 min.

The immunofluorescence protocol detailed in section 2.8.4, was then carried out on the cell-covered coverslips before capturing the images using a camera connected to a Zeiss Axiovert S100 at a magnification of x100 (using oil-immersion).

7.2.6 Statistics

Statistical analysis was by 1-way ANOVA followed by Dunnett's post-test and was carried out using GraphPad Prism version 4. * is used to represent a p value between 0.01 and 0.05. P values greater than 0.05 were deemed to be not significant. Where expressed, data are in the form mean ± s.e.m.

7.3 Results

From the CBA pilot study data, the cytokines TNFα and IL-8 were selected for further exploration of any possible anti-inflammatory action of the compounds. 10 μM was identified as a suitable concentration at which to observe an effect of the drugs.

7.3.1 Enzyme-Linked Immunosorbent Assay

None of the treatments had a significant effect on IL-8 release in either monocytes or macrophages (Fig. 7.1. $p > 0.05$; 1-Way ANOVA; n =8 -10). Basal IL-8 levels were 21.3 ± 7 ng.ml^{-1} for monocytes and 685 ± 67 pg.ml^{-1} for macrophages. After LPS-treatment these rose to 127 ± 11 and 34 ± 5 ng.ml^{-1} respectively.

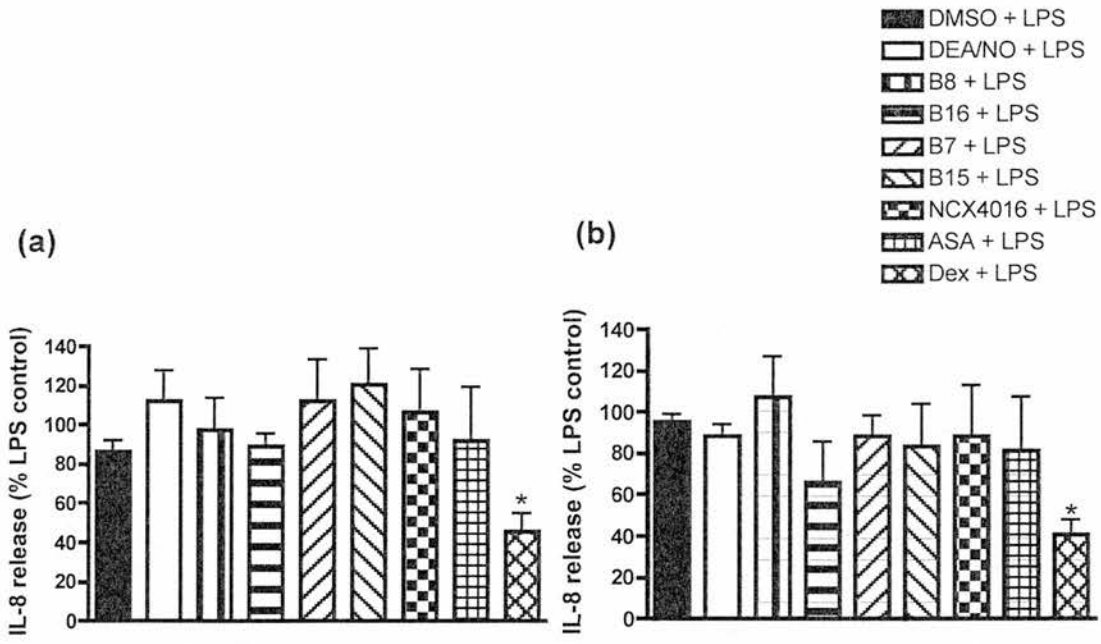


Fig. 7.1. Effect of potential anti-inflammatory agents on LPS (10 ng.ml^{-1}) -induced IL-8 release in human (a) monocytes and (b) monocyte-derived macrophages after 4 h treatment with 10 μM of either DEA/NO, B8, B16, B7, B15, NCX4016 or aspirin or 1 μM dexamethasone (Dex). n = 8-10.* indicates $P < 0.05$ as assessed by 1-way ANOVA.

B8 had a significant inhibitory effect on TNF-α release in human monocyte-derived macrophages treated with LPS (Fig 7.2; p <0.05, 1-Way ANOVA followed by Dunnett's test; n = 8-10, 36 ± 10 % of LPS control) but not in macrophages without LPS. The effect was equivalent in magnitude to that of dexamethasone, but was not shared by DEA/NO, B7, the furazans, aspirin or NCX4016. In monocytes, B8, and to a lesser extent, its NO-free equivalent, B16, significantly inhibited TNF-α release (to 28 ± 5, and 49 ± 9 % of control respectively). Basal TNF-α levels were 1.6 ± 0.4 $ng.ml^{-1}$ for monocytes and 0.9 ± 0.2 $pg.ml^{-1}$ for macrophages. After LPS-treatment these rose to 5.5 ± 0.5 and 9.6 ± 0.3 $ng.ml^{-1}$ respectively.

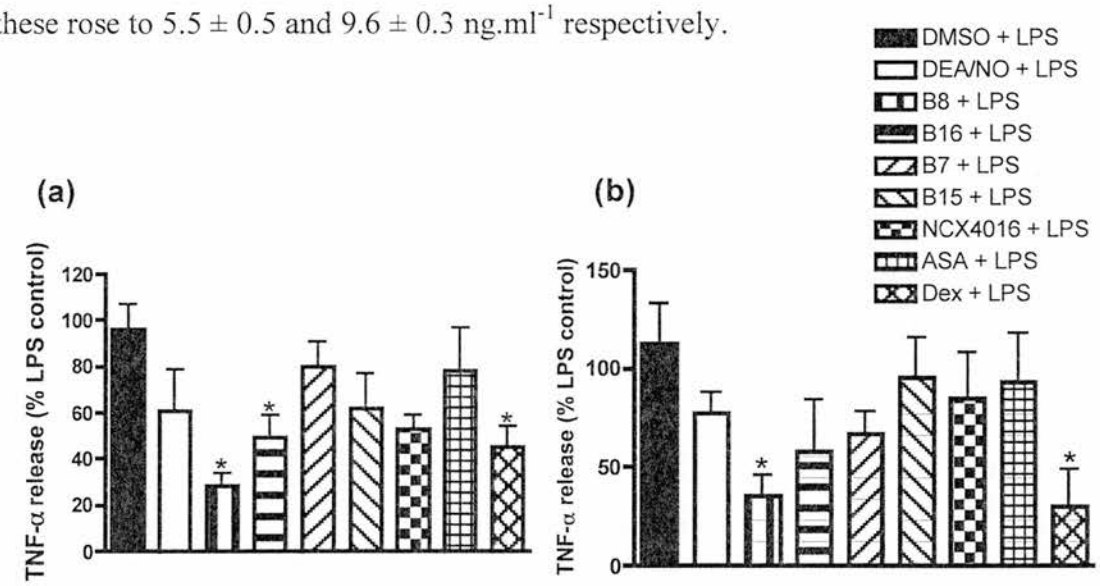


Fig. 7.2. Effect of potential anti-inflammatory agents on LPS (10 $ng.ml^{-1}$)-induced TNF-α release in human (a) monocytes and (b) monocyte-derived macrophages after 4 h treatment with 10 μM of either DEA/NO, B8, B16, B7, B15, NCX4016 or aspirin or 1 μM of dexamethasone (Dex). n = 8-10. * = $p < 0.05$ 1-Way ANOVA followed by Dunnett's test; n=8-10.

7.3.2 Lactate Dehydrogenase (LDH) Assay

None of the treatments studied caused significant cell death (Fig. 7.3) compared to untreated monocytes and macrophages. Levels of LDH released following the treatments were comparable with that from ~ 0.5 x 10^5 cells, indicating an approximate 13 % cell death in untreated cells.

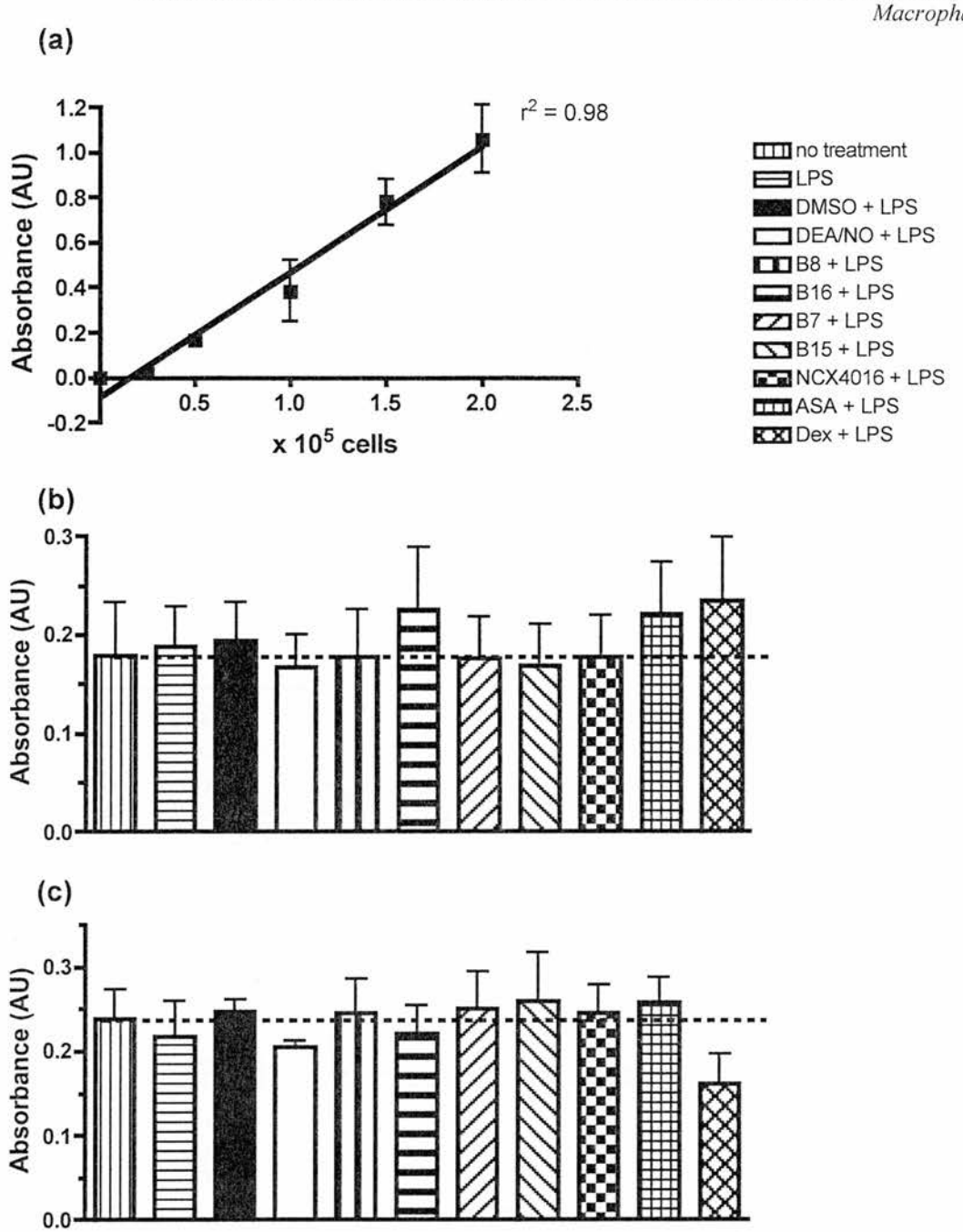


Fig. 7.3. (a) Calibration curve for the LDH assay. Bar graphs show LDH measurement in (b) monocyte and (c) monocyte-derived macrophage supernatants after treatment with 10 μM of either DEA/NO, B8, B16, B7, B15, NCX4016 or aspirin or 1 μM of dexamethasone (Dex) and stimulated with LPS (10 $ng.ml^{-1}$). n = 8-10. 1-way ANOVA revealed that there were no significant differences between groups in either cell type.

7.3.3 Immunofluorescence for NF-κB p65 Subunit

NF-κB p65 immunofluorescence revealed that the subunit location varied with the drug treatment. Control cells displayed even staining throughout the cytoplasm, but following stimulus with LPS, strong staining was observed in the nucleus and much less in the cytoplasm (Fig. 7.4).

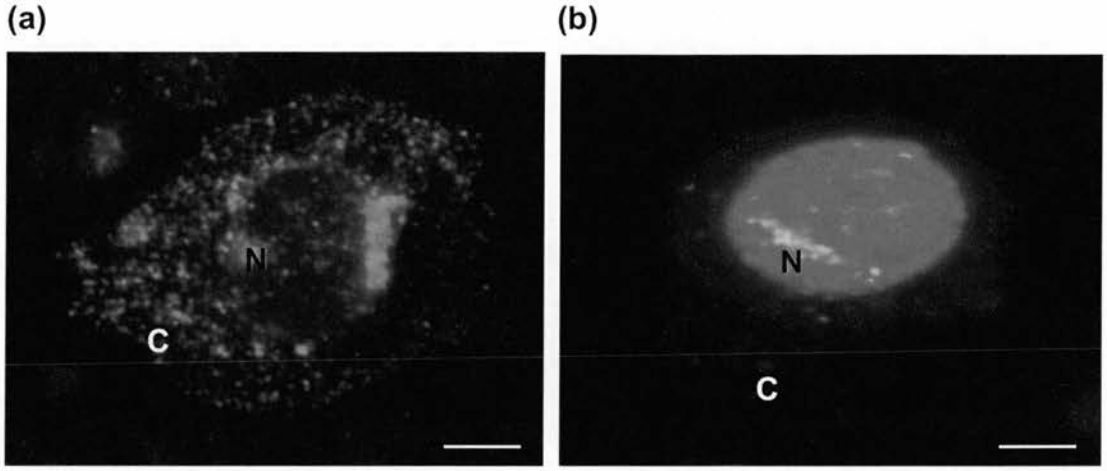


Fig. 7.4. Representative immunofluorescent images from an n=3 (each of 4×10^6 cells). (a) shows a control treated monocyte and (b) a monocyte following a 45 min LPS stimulus. C = cytoplasm. N = nucleus. Scale bar represents 100 μm.

Incubation with gliotoxin before the LPS stimulus inhibited the nuclear translocation of p65 as demonstrated by the presence of cytoplasmic staining (Fig. 7.5).

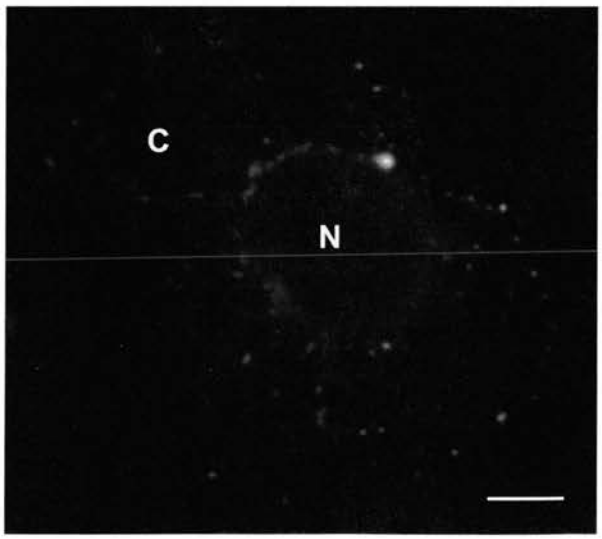


Fig. 7.5. Representative immunofluorescent image from an n=3 (each of 4×10^6 cells). Image shows a monocyte treated with gliotoxin (0.1 μg/ml, 30 min) and stimulated with LPS (10 ng/ml, 45 min). C = cytoplasm. N = nucleus. Scale bar represents 100 μm.

Preincubation with the NO-aspirin B8 gave a dramatic shift in the staining compared to the LPS control. Location of the NF-κB p65 subunit was now revealed to be cytoplasmic (Fig. 7.6).

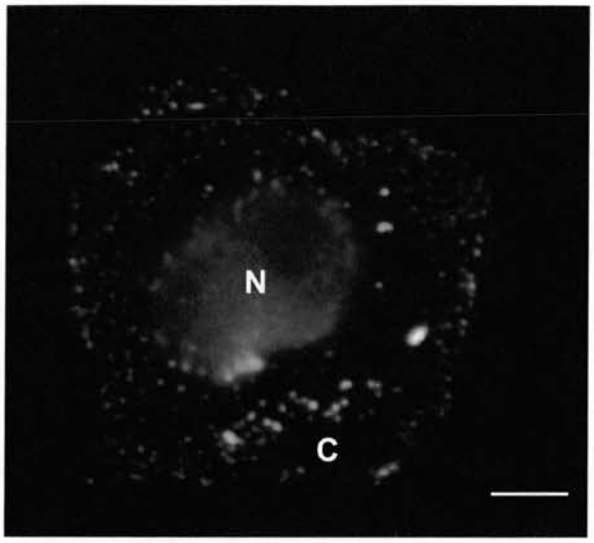


Fig. 7.6. Representative immunofluorescent image from an n=3 (each of 4×10^6 cells). Image shows a monocyte treated with B8 (20 μM, 30 min) and stimulated with LPS (10 ng/ml, 45 min). C = cytoplasm. N = nucleus. Scale bar represents 100 μm.

Cells treated with the NO-free compound, B16, displayed cytoplasmic staining but with a greater amount of nuclear staining than B8 treated cells (Fig. 7.7).

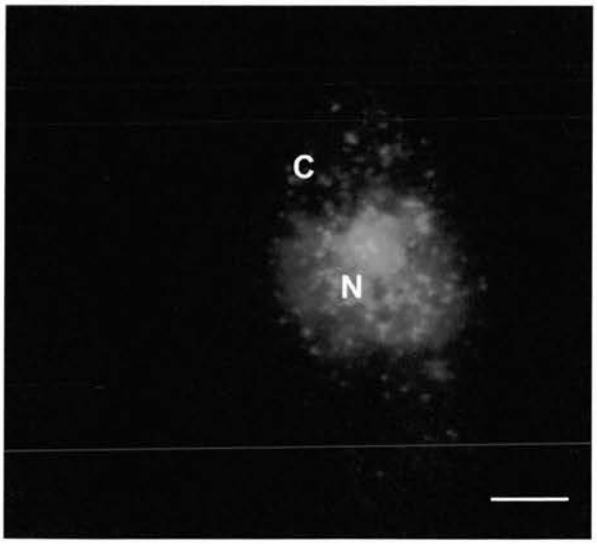


Fig. 7.7. Representative immunofluorescent image from an n=3 (each of 4×10^6 cells). Image shows a monocyte treated with B16 (20 μm, 30 min) and stimulated with LPS (10 ng/ml, 45 min). C = cytoplasm. N = nucleus. Scale bar represents 100 μm.

7.4 Discussion

Taken together, these studies provide evidence that whilst no treatment significantly altered IL-8 release, treatment with NO-aspirin B8, significantly reduced TNFα release from both monocytes and monocyte-derived macrophages. A possible mechanism for this anti-inflammatory action is through the inhibition of its transcription factor, NF-κB.

The CBA pilot studies revealed that treatment by the furoxan and furazan aspirin compounds had some impact on the release of various cytokines, in particular IL-8 and TNFα, from LPS-stimulated monocyte-derived macrophages *in vitro*. For this reason the effect on these two cytokines were selected for further study.

7.4.1 Impact on TNFα-Release by Monocytes and Macrophages

In monocyte-derived macrophages, the lack of effects of B16 and aspirin suggest that the inhibitory effect of B8 on TNFα release is NO-mediated. However, as this effect is not mimicked by the NO donor, DEA/NO, it is apparently a specific property of B8 that is possibly related to amount, duration or site of NO release. The possibility of the diminished release of TNFα by B8-treated cells was simply due to a cytotoxic effect of the compound was ruled not supported by results from the LDH assay. None of the treatments caused a significantly different amount of cell death when compared to untreated cells.

In monocytes, TNFα release was inhibited by the NO-aspirin, B8, but also by its NO-free furazan counterpart, B16. Aspirin showed no significant difference from the LPS control. Similar to the macrophage results, DEA/NO did not cause significant inhibition. Again, B8 could be acting via an NO-mediated mechanism specific to the to amount, duration or site of NO release. However, the interesting observation that NO-free, B16 also causes a significant inhibition suggests a possible further mechanism. As discussed in previous chapters, the acetyl group of these compounds is lost in plasma (Cena *et al.*, 2003), leaving SA, through which, inhibition of cytokine release has been previously reported (Feng *et al.*, 2005; Mitchell *et al.*, 1997; Osnes *et al.*, 1996). It may therefore be possible that under these experimental conditions, a SA-mediated mechanism is responsible for the inhibition of TNFα release observed with B8 and B16 treated cells.

In this study aspirin did not significantly reduce TNFα release from LPS-stimulated monocytes or macrophages. This is consistent with a similar study in which aspirin failed to have an effect even at a 30 x higher dose than was used in the present study (Minuz *et al.*, 2001). A further study did report an inhibitory effect of aspirin on TNFα release from LPS-stimulated monocytes but this was at doses of 5-10 mM (Osnes *et al.*, 1996).

This study showed that NCX4016 did not significantly reduce the release of TNFα. In a study carried out by others, NCX4016 did not inhibit TNFα release at the same concentration used here (10 μM), but was shown to inhibit the release of TNFα and IL-6 from LPS-stimulated macrophages at higher (100 and 300 μM) concentrations and following a 6 h incubation (Minuz *et al.*, 2001). Despite not being affected by

ODQ, the authors suggest that the inhibitory effect of NCX4016 is NO-mediated due to the failure of aspirin to inhibit cytokine release. A further study also showed that NCX4016 (again at concentrations 10-fold higher than used in this thesis), inhibited the release of IL-1β and IL-18 from LPS-stimulated monocytes, via NO-mediated inhibition of the enzyme required for intracellular processing and maturation of IL-1 and IL-18 (caspase-1) activity (Fiorucci *et al.*, 2000).

It is likely that the differing outcomes observed between this thesis and previous studies are purely due to drug incubation time or concentrations. As the concentrations studied in this thesis are more physiologically relevant, they are more demonstrative of the true therapeutic potential of the drug. The results here show that at a concentration at which the furoxan compound, B8, causes a significant 72% reduction in TNFα release from monocytes and a 64% reduction from macrophages, its organic nitrate counterpart does not. Such results indicate that further to its lack of NO-mediated effects in the platelet studies (chapter five), NCX4016 also has no impact on IL-8 or TNFα release from either monocytes or macrophages.

The differential effects of the same treatments observed between monocytes and macrophages may indicate the necessity for different drug concentrations and incubation times for the individual cell types. Possible explanations for this effect may be the differential expression and activity of receptors and signalling pathways between the two cell types. It has previously been reported that the anti-inflammatory effect of IL-4 on the release of TNFα and other cytokines, varies between LPS-stimulated monocytes and macrophages (Hart *et al.*, 1999). This effect is due to loss of a receptor for IL-4 during monocyte differentiation (Hart *et al.*,

1999). Other differences reported between monocytes and macrophages include those showing that LPS activates cytosolic PLA_2 in monocytes but not in macrophages. A similar activation in monocytes, but not macrophages, is seen after LPS-stimulation in the following signalling pathways: the MAP kinase, ERK, phosphatidylinositol-3 kinase and p70S6 kinase (Barbour *et al.*, 1998; Rao, 2001). Further variations include increased expression of Ca^{2+}-dependent protein kinase C isoforms in monocytes when compared to macrophages (Monick *et al.*, 1998) and also that maturation into macrophages results in slower production of the cytokine, IL-1β (Herzyk *et al.*, 1992). It is, therefore, possible that changes such as these to receptor expression, signalling pathways and to the biosynthesis of cytokines, which normally occur during the maturation of monocytes to macrophages, may impact on the ability of the studied compounds to have a significant effect.

7.4.2 Impact on IL-8-Release by Monocytes and Macrophages

The IL-8 data revealed the hybrid compounds caused no significant enhancement or reduction of IL-8 release from LPS-stimulated monocytes or macrophages at the same drug concentrations and incubation times as for the TNFα experiments, despite a significant reduction being induced by the anti-inflammatory glucocorticoid, dexamethasone. The lack of effect of aspirin on IL-8-release is consistent with previous studies where aspirin, even at ten-fold higher concentrations failed to affect IL-8 release from LPS-stimulated monocytes (Fiorucci *et al.*, 2000). Although the possibility for an inhibitory effect of aspirin occurs in a cytokine-rich milieu through the action of ATL to inhibit IL-8 mRNA expression by leukocytes (Jozsef *et al.*, 2002), failure of the NO-aspirins to have an effect is possibly due to the

activation/release processes for IL-8 differing from that of TNFα. Whilst it is generally accepted that IL-8, like TNFα, is stimulated by the transcription factor NF-κB (Liu & Malik, 2006), it has been demonstrated that IL-8 gene transcription also requires cooperative activity of an additional transcription factor, AP-1 (McKay & Cidlowski, 1999; Mukaida & Murayama, 1998). Such complexities in the pathways for the transcription of the gene give multiple points at which B8 could act to inhibit TNFα release and not IL-8. Indeed, the action of B8 could even be further downstream in the release of IL-8 protein or the lack of observed effect on IL-8 might possibly be due to insufficient drug being used. NCX4016 has previously been demonstrated to inhibit IL-8 release from LPS-stimulated monocytes (Fiorucci *et al.*, 2000). However, they did use an unrealistically high concentration, whereas the one used in this thesis was more physiologically relevant.

7.4.3 Impact of B8 on NF-κB Translocation in Monocytes

The immunofluorescence experiments further suggested a possible mechanism for the B8-mediated inhibition of TNFα release. The stimulant used in these experiments, LPS, is known to activate TNFα via the activation of the transcription factor NF-κB (Baldwin, 1996; Kubes & McCafferty, 2000; Totzke *et al.*, 2006). Following stimulation with LPS, the p65-subunit containing NF-κB normally translocates from the cytoplasm to the nucleus where it induces transcription of the TNFα gene (Baldwin, 1996; Kaltschmidt *et al.*, 1999; Liu *et al.*, 2000; Totzke *et al.*, 2006). Control experiments involving a pre-treatment with the epipolythiodioxoperazine, gliotoxin, demonstrated clear cytoplasmic staining and reduced nuclear staining. Gliotoxin is known to exhibit immune suppressive activity

in vitro and to selectively inhibit NF-κB activation (Pahl *et al.*, 1996). The immunofluoresence staining showed that pre-treatment with the NO-aspirin B8 inhibited the translocation of NF-κB, providing a similar level of cytoplasmic staining as the gliotoxin control. The inhibition of NF-κB translocation to the nucleus provides a plausible explanation for the B8-induced reduction in TNFα release as observed in the ELISA studies. It has previously been shown that NO inhibits LPS-induced IκB-phosphorylation, thus inhibiting the activation of NF-κB (Hattori *et al.*, 2004).

B16-treated monocytes also displayed some evidence of cytoplasmic staining, but with more nuclear staining than its NO counterpart. This result is consistent with the ELISA data, where significant inhibition of TNFα release, albeit lesser than B8-treated cells, was observed following B16 treatment.

7.4.4 Future Directions

The ELISA studies were carried out with equal concentrations of each of the drugs studied (with the exception of dexamethasone). It is therefore possible that the optimum concentration to observe any anti-inflammatory effects was not utilised. Time-permitting, full concentration-response curves to each drug would have been carried out to establish such information.

Whilst these studies reveal that the effect of B8 on the release of TNFα by monocytes is due to an inhibitory effect on NF-κB translocation, the mechanism for this effect is as yet unknown. Normally, NF-κB is kept in an inactive state in the cytoplasm, bound to an inhibitory subunit named IκB (Ghosh *et al.*, 1998). The phosphorylation of IκB by IKK and the subsequent degradation of IκB leads to the

activation of the NF-κB (Yamamoto & Gaynor, 2004). The degradation of IκB can be studied by Western blotting using antibodies for IκB (Fujihara *et al.*, 2002). A possible future study could investigate the impact of the NO-aspirin compounds on IκB to determine whether they activate NF-κB by causing IκB degradation.

7.4.5 Summary and Therapeutic Implications

The data here show that the furoxan-aspirin compound B8 has anti-inflammatory effects in LPS-stimulated monocytes and macrophages through its reduction in NF-κB-mediated TNFα release.

This action by B8 may provide clinical benefit in inflammatory diseases where anti-TNFα therapy has been shown to be of therapeutic benefit such as arthritis, Crohn's disease and asthma (Carroccio *et al.*, 2006; Christodoulou & Choy, 2006; Russo & Polosa, 2005; Siddiqui & Scott, 2006). The dual action of the COX-mediated aspirin action of B8 (chapter three) and the anti-TNFα demonstrated here along with its resistance to gastrotoxic effects (Cena *et al.*, 2003), could indicate B8 to be a promising antiarthritic drug.

It is now widely accepted that inflammation is a key element in atherogenesis and atherosclerotic plaque rupture which leads to acute cardiovascular events such as myocardial infarction or stroke (Ross, 1999). The release of TNFα by monocytes and macrophages causes various effects involved in destabilising the atherosclerotic plaque. TNFα plays a role in inflammatory cell recruitment to the plaque (Pober & Cotran, 1990). It can also regulate the production of monocyte chemoattractant protein-1, which is a potential signal for directed migration of monocytes into the

intima (Libby *et al.*, 1995). TNFα can regulate genes that encode other growth factors and cytokines themselves (Libby *et al.*, 1995). Furthermore, TNFα has been demonstrated to promote adverse remodelling of vascular smooth muscle cells (Galis *et al.*, 1994; Geng *et al.*, 1996; Jovinge *et al.*, 1997) and to play a pro-inflammatory role in plaque rupture (Galis *et al.*, 1994).

CHAPTER EIGHT

General Discussion

8. General Discussion

8.1 Introduction

Aspirin is one of the most commonly used drugs; over 40 billion aspirin are taken every year in the United States (Schiffmann *et al.*, 2005). Its use ranges from an anti-inflammatory and analgesic for arthritis sufferers to a prophylactic against thrombosis in those at risk of a coronary event. Aspirin has been used for over 100 years and its mechanism of action has been understood for over 35 years. Despite this, however, its use is still severely limited by its toxic effects in the gastro-intestinal tract.

Studies have revealed that gastrointestinal incidences associated with aspirin use cause 16,000 deaths each year in the USA (Keeble & Moore, 2002). In the UK 12,000 hospital admissions and 2,230 deaths are attributable to NSAID use (Blower *et al.*, 1997). Furthermore, a recent study indicated that over 12% of all gastrointestinal incidences and deaths could be attributed to low-dose aspirin use (Lanas *et al.*, 2005). It is therefore apparent that there is an urgent clinical need to resolve the issue of gastrotoxicity related to aspirin use.

There have been several approaches to overcome the gastric side-effects of aspirin, with mixed success. The recently developed selective COX-2 inhibitors, hailed to be the new "wonder aspirins", have unfortunately now been withdrawn due to mounting evidence of an increased risk of stroke. However, the newest candidate in the search for a gastric-friendly aspirin, the NO-aspirin, appears to be more promising. The

well-documented cytoprotective effects of NO led to the development of the NO-releasing aspirin esters in the hope of overcoming the gastrotoxicity of aspirin. However, the multifunction nature of NO has given these esters a broad range of beneficial effects beyond their intended purpose.

8.2 Summary

The experiments in this thesis have explored a range of effects relevant to the cardiovascular system of a novel series of furoxan derivatives of aspirin.

The experiments in chapter three were carried out in order to ensure that the hybridisation process had not impacted on the activity of the aspirin moiety. If the drugs are to be a suitable successor for aspirin, then it is imperative that they retain its ability to inhibit COX-1 and thus have potential for an aspirin-mediated antithrombotic (Patrignani *et al.*, 1982; Roth *et al.*, 1975), anti-inflammatory (Amann & Peskar, 2002; Vane & Botting, 1998) and analgesic (Ferreira, 1980) effect. In the absence of a simple and reliable commercially available COX assay, a novel assay was first developed and verified in order to allow effective screening of the ability of the NO-aspirins to inhibit COX-1.

The assay results were demonstrated to be comparable to those of other assay techniques (Corazzi *et al.*, 2005; Noreen *et al.*, 1998; Young *et al.*, 1996). All NO-aspirins along with their NO-free counterparts were able to inhibit COX-1 to a similar, if not greater extent than aspirin. The furoxan compounds were also demonstrated to have a similar capability to the existing nitroaspirin, NCX4016, at inhibiting COX (Corazzi *et al.*, 2005). However, as plasma-mediated decomposition

is not tested by this assay, it is important to recognise that the effect might be lost *in vivo* depending on how these compounds are hydrolysed. As the furoxan–aspirin acetyl group has been previously demonstrated to undergo complete hydrolysis in serum (Cena *et al.*, 2003), a partial loss of the acetyl group in plasma was only to be expected. How active the compound remains in biological media could be further elucidated by *in vivo* studies.

The studies in chapter four explored the activity of the NO element of NO-aspirin in vascular tissue. The native furoxan compounds were found to have remarkable potency; the reversible vasodilatory effects of B13 in the picomolar range revealed it to be approximately 300-fold more potent than DEA/NO (Sausbier *et al.*, 2000). Such data show potential for the furoxan compound as a possible stand-alone donor. The effect of the hybridisation of the furoxans to their aspirin derivatives on their ability to cause vasodilatation appeared to vary with the chemical structure, but both hybrids were revealed to cause concentration-dependent vasodilatation of phenylephrine-constricted rat aortic rings. The vasodilatation caused by the hybrids was determined to be NO:sGC-dependent as revealed by the cGMP-dependent nature of the response and the lack of effect displayed by their NO-free equivalents, making them comparable with the existing nitroaspirin, NCX4016 (Rossoni *et al.*, 2002).

The combined data from chapters three and four indicated that both elements of the furoxan hybrids were active, as would be a pre-requisite for an effective hybrid drug. The remainder of the studies investigated properties of the drugs in cell types relevant to atherothrombotic disease.

As NO-hybrids were originally developed in order to overcome the gastric side-

effects of aspirin associated with its long-term use or high dose, the potential for these hybrids to succeed aspirin as prophylactics against thrombotic coronary events was investigated in chapter five. Firstly, it was demonstrated with the furoxan compound, B13, that furoxans are capable of overcoming the NO-scavenging effect of endogenous haemoglobin (Gladwin *et al.*, 2004; Kosaka *et al.*, 1989). The hybrids were demonstrated to be potent antiplatelet agents, causing inhibition of collagen-induced aggregation at doses up to 100-fold lower than aspirin. This suggests that the NO element is active and contributing to the antiplatelet action (Pasqui *et al.*, 1991; Radomski *et al.*, 1990; Radomski *et al.*, 1987c). Studies with ODQ revealed that the effect of inhibition of platelet aggregation was mainly cGMP-dependent in PRP, indicating dominance of the NO moiety in the antiplatelet effect. In WP, the aspirin moiety was more active, especially in the case of B7, confirming that the COX-inhibitory action revealed in chapter three could be retained in biological media, but suggesting that plasma impacted on the acetyl stability (Cena *et al.*, 2003). The studies in chapter five perhaps reveal the furoxan hybrids to be a better option than the nitrooxy ester, NCX4016, which was shown to have no antiplatelet action in PRP and, in WP, it failed to demonstrate aspirin-independent effects. These studies extended work carried out by others with NCX4016 (Lechi *et al.*, 1996) in WP. The data in chapter five show the WP antiplatelet effect is lost in PRP, possibly due to sequestration by a plasma constituent such as albumin or due to breakdown in the plasma to the inactive salicylic acid. Unlike NCX4016, the furoxans demonstrated aspirin-independent effects on platelet aggregation in both PRP and WP *in vitro.* The advantage the furoxans have over NCX40116 is likely due to their lack of reliance on enzymatic cleavage to release their NO (Grosser & Schroder, 2000).

The experiments in chapter six were carried out to explore the NO-release mechanism for the furoxans and their hybrids. Results from previous chapters suggested that release was likely to occur in very close proximity to sGC in order to explain the effects seen at very low doses. The lack of effect of the NO scavenger, cPTIO in chapter four also indicated release in a compartment inaccessible to the scavenger. Furthermore, the hypothesis by Crane *et al* that cGMP-dependent NO effects correspond with intracellular release of NO (Crane *et al.*, 2005) prompted investigation into the effects of intracellular antioxidant species on the release of NO from the furoxan compounds. Studies revealed that the furoxan-aspirin hybrids, B8 and B7, like their aspirin-free equivalents, are very stable compounds that appear only to decompose to release significant NO when they encounter appropriate media. There was no release of NO from the furoxans in Tyrode's buffer, but NO generation was detected in PPP, WP and PRP. The observations indicated that some elements of plasma can stimulate decomposition of the furoxans, but that a major release of NO from furoxans and furoxan-aspirin hybrids occurs on encountering intracellular concentrations of antioxidants, such as GSH and ascorbate. As the decomposition is catalysed by endogenous agents, it may instil a potential for primarily intracellular delivery of NO, on account of the differential distribution of such agents; concentrations of glutathione and ascorbate are approximately 100 - 1000-fold higher within cells compared to plasma and extracellular fluid. This may be of clinical benefit in ensuring that following administration, the drug is not sequestered by cell free haemoglobin. Experiments within chapter six further revealed that under *in vitro* conditions, neither B8 nor B7 release significant amounts of peroxynitrite in addition

to NO, indicating they can be classified as pure NO-donors and are likely to avoid the deleterious effects associated with peroxynitrite (Shaw, 2006).

The final chapter investigated the anti-inflammatory effects in monocytes and macrophages. Data in chapter seven indicated that, whilst the furoxan-hybrids had no significant impact on the release of IL-8, an anti-inflammatory action was demonstrated via inhibition of TNFα release from LPS-stimulated human monocytes and monocyte-derived macrophages by B8. Further investigation using by immunofluorescence suggested a possible mechanism for this action to be through impedance of the nuclear translocation of the TNFα transcription factor, NF-κB, which NO has been previously shown to impact upon (Hattori *et al.*, 2004).

8.3 Clinical Applications

Further to their intended use as a gastric-safe aspirin, the furoxan-aspirins have been demonstrated throughout this thesis to have further beneficial effect due to the actions of the drug-derived NO and this gives potential for various clinical applications.

8.3.1 Cardiovascular Drugs

The multiple actions of the furoxan-hybrids instil the potential for them to be beneficial in different aspects of cardiovascular disease.

8.3.1.1 Antithrombotic Effects

The most likely application for these drugs is in the replacements of low-dose aspirin for the prevention of coronary events in patients prone to gastric events. The drugs

have demonstrated both aspirin and NO-mediated inhibition of platelet aggregation *in vitro*. The furoxan NO-aspirins are predicted to have a better antithrombotic profile than NCX4016 due to the stability of the compounds, their targeted intracellular release, but most importantly their lack of requirement for enzymatic degradation, which platelets cannot perform.

The COX-2-specific inhibitors were recently withdrawn due to their actions causing an imbalance between vascular PGI_2 and platelet TXA_2, resulting in an increased risk of stroke. The increased risk of thrombus is thought to be due to inhibition of COX-2 in endothelial cells, leading to reduced generation of the antithrombotic and vasodilator agent, PGI_2, in relation to the unaffected COX-1 derived, TXA_2 (Fitzgerald, 2004). It is predicted that, since aspirin is approximately 170-fold more selective for inhibiting COX-1 than COX-2 (Vane *et al.*, 1998), low-dose furoxan-aspirins can be administered to achieve inhibition of platelet TXA_2 without affecting vascular PGI_2, mirroring prophylactic aspirin therapy (Forster & Parratt, 1997; Vane *et al.*, 1998).

8.3.1.2 Anti-Inflammatory Effects

As it is now generally accepted that atherosclerosis is an inflammatory disease (Ross, 1999), the ability of B8 to inhibit the release of inflammatory cytokine, TNFα, as presented in chapter seven, may reveal a further cardiovascular target for the furoxan NO-aspirins. As discussed in chapter seven, the release of TNFα by monocytes and macrophages during atherosclerosis causes various effects involved in both formation and destabilisation of the plaque.

With regard to utilising the drugs to treat atherosclerosis, caution must be applied. Whilst they have an anti-inflammatory action which may decrease the inflammatory load in a plaque, the role of NO-related species in necrosis of vascular smooth muscle cells (Shaw, 2006) may result in destabilisation of the plaque cap, promoting plaque rupture.

As the atherosclerotic lesion increases in size it can impact on blood flow through the lumen resulting in ischaemia or angina, and this is where the furoxans may have benefit through their vasodilatory action (discussed below).

8.3.1.3 Vasodilators?

Despite showing great potency in the myography experiments, the application of these drugs as vasodilators is likely to be unsuccessful in the setting of angina. The nitrate compounds, GTN and ISDN are effective at providing symptomatic therapy for angina. They bring about their effect by rapidly dilating veins and coronary arteries, thus reducing pre-load and cardiac work and improving blood supply to the heart (Abrams, 1885). GTN predominantly dilates the larger coronary arteries while having a minimal effect on small coronary resistance vessels as they lack the metabolic pathway needed to convert them to their active form (Harrison & Bates, 1993). It is possible that the furoxans may not be as selective and also produce vasodilation of the coronary resistance vessels, thus resulting in the phenomenon of coronary steal.

NO-mediated vasodilators such as nitroprusside are not commonly used as antihypertensive agents except in medical emergencies (Williams *et al.*, 2004). As

the selectivity of furoxan NO-aspirin drugs has not been studied, the remarkable potency of the drugs indicates that, unless the release can be specifically targeted to capacitance vessels, in order to prevent dilation of both arterial and venous smooth muscle, it may be necessary administer it intravenously as occurs for nitroprusside (Mann *et al.*, 1978).

8.3.2 Anti-Arthritic Agents

The most likely application for these drugs apart from antithrombotic therapy is in the treatment or relief from arthritic symptoms. NO-aspirins have the potential to be anti-inflammatory through the actions of both the NO-moiety and the parent compound. The ability to inhibit COX-1, as demonstrated in chapter three highlights the potential that the NO-aspirins could retain the anti-inflammatory, as well as analgesic actions of the parent compound, aspirin, providing benefit in arthritis.

The analgesic effect of aspirin is brought about through inhibition of COX-mediated prostaglandin E_2 and I_2 production. These prostaglandins, whether synthesized during inflammation or in the spinal cord, play a role in firing of nociceptors (Ferreira, 1980). NO-aspirins have the potential to be analgesic through COX-mediated inhibition of prostaglandin production, but the impact of their NO moiety is harder to predict due to the role of NO apparently varying with the type, location and cause of pain (Hoheisel *et al.*, 2005; Luo & Cizkova, 2000; Semos & Headley, 1994).

The carrageenan-induced hind paw oedema model is used experimentally as an acute model of inflammation. This model has demonstrated the furoxan derivatives to cause at least similar anti-inflammatory effects to the aspirin (Cena *et al.*, 2003).

The data in chapter seven show the furoxan NO-aspirin, B8, to inhibit the release of inflammatory cytokine, TNFα but not IL-8. This anti-TNFα action by NO-aspirins may provide clinical benefit in inflammatory diseases such as arthritis (Christodoulou & Choy, 2006; Siddiqui & Scott, 2006). Rheumatoid arthritis is a chronic inflammatory autoimmune disorder characterised by inflammation of the lining, or synovium, of the joints. The joint destruction and pain associated with the synovial inflammation can lead to substantial loss of mobility. Pro-inflammatory cytokines are abundant in the joints of sufferers (Christodoulou & Choy, 2006). Anti-TNFα drugs have been recently licensed for use in arthritis to limit the contribution of the cytokine to inflammation (Siddiqui & Scott, 2006).

Treatment of arthritis with conventional NSAIDs is severely limited due to their gastric side-effects. However, the furoxan NO-aspirins could offer a preferable alternative to NSAID therapy. The dual action of the COX-mediated aspirin action and the anti-TNFα response along with their resistance to gastrotoxic effects (Cena *et al.*, 2003) show a promising antiarthritic profile of the furoxan NO-aspirins.

In contrast, the nitroaspirin, NCX4016, despite having an equivalent anti-inflammatory potential to the furoxans, is unlikely to be a successful antiarthritic drug due to problems of tolerance. As the NO-donating group of NCX4016 is an organic nitrate it will be just as susceptible to the tolerance problems associated with

long-term or high dosage use as nitrates such as GTN (Artz *et al.*, 2002; Chen *et al.*, 2002; Munzel *et al.*, 2005; Munzel *et al.*, 1995; Stewart, 1888; Sydow *et al.*, 2004).

8.3.3 Cancer Therapy

Another possible clinical application for the NO-aspirins is in cancer therapy. The use of aspirin has been reported to reduce the risk of several cancers particularly colorectal, but also oesophageal, breast, lung, and bladder (Baron, 1995; DuBois & Smalley, 1996; Marnett, 1995; Wang & Dubois, 2006).

Colon cancers have been demonstrated to produce high levels of COX-derived prostaglandins, particularly PGE_2 (Karim & Rao, 1976). Such prostaglandins act to enhance tumour growth (Lupulescu, 1996). Aspirin has a beneficial effect in preventing tumour growth due to its ability to inhibit synthesis of prostaglandins (Rosenberg *et al.*, 1991; Sano *et al.*, 1995). The involvement of PGE_2 in tumour growth is attributed to stimulation of its EP_4 receptor, leading to phosphorylation of extracellular signal-regulated kinases (ERKs). Such activation of ERK signalling by the EP_4 receptors induces the functional expression of early growth response factor-1 which promotes tumour growth (Fujino *et al.*, 2003; Pozzi *et al.*, 2004). Studies have shown the prostaglandins involved in cancer growth to be derived from COX-2 (Lim *et al.*, 2001; Sano *et al.*, 1995) and thus the COX-2 inhibitors were proposed to have a potential use in cancer prevention (Lim *et al.*, 2001; Sinicrope, 2006; Yona & Arber, 2006). However, with the discovery of the cardiovascular risk related to use of the specific COX-2 inhibitors (Fitzgerald, 2004; Yona & Arber, 2006), there appears to be a further clinical opportunity for the NO-aspirins.

It is hoped that the furoxan NO-aspirins could ideally inhibit the cancer-promoting prostaglandins to the same extent as aspirin, but would avoid the gastrotoxicity of aspirin and the cardiovascular risk of the specific COX-2 inhibitors. The nitrooxy-ester has already been shown to be beneficial in an *in vitro* model using colon cancer cell lines, where it was up to 250-fold more potent than aspirin at inhibiting the growth of cancer cells (Kashfi & Rigas, 2005). The NO-aspirin was also determined to be more potent than the parent compound in pancreatic, prostate, lung, skin, leukaemia and breast cancer cell lines, in some cases up to 6000-fold more potent (Kashfi & Rigas, 2005). Studies have also demonstrated beneficial effects of NCX4016 *in vivo*, where it reduced cancer growth in a rat model of colonic adenocarcinoma to a greater extent than aspirin in an NO-dependent manner (Bak *et al.*, 1998). It is therefore hoped that drugs of the furoxan-aspirin class could mirror the positive effects of NCX4016 in cancer.

8.4 Limitation of Compounds & Future Directions

Future studies with this drug class must include screening of a wide range of furoxan NO-aspirins (Cena *et al.*, 2003) in order to eliminate the limitations highlighted in this thesis. Such limitations include instability of the acetyl group in plasma resulting in diminished aspirin-mediated actions in the antiplatelet studies. As more promising results were achieved with B7 over B8 it is likely that the acetyl stability is related to the structure and thus screening may reveal a more favourable candidate.

Further issues exist related to structure and these may be resolved by screening of other furoxan NO-aspirins (Cena *et al.*, 2003). As demonstrated by the furoxans B12 and B13 in chapters five and six, the effects of these compounds are hindered by

their solubility in aqueous media. However, as an effect was observed in the *ex vivo* studies in chapter four, the solubility may not impact upon their therapeutic potential. Chemical modification is also required to rectify the balance issue of B8 in order that in can act equally through both moieties as should be expected of a true 'hybrid'. B7 is the better prototype hybrid due to its lower NO release but it still requires chemical modification in order to retain its aspirin-like action in PRP.

8.4.1 In Vivo Effects

The aim of the experiments performed within this thesis was to characterise the NO-release and COX-inhibitory actions of a novel class of NO-aspirins and to determine their actions in cell types relevant to atherosclerosis. While these experiments have provided valuable information on the actions of the furoxan aspirin compounds within *in vitro* situations, the next logical step is to study their potential effects in an *in vivo* setting.

As discussed, the most likely clinical application for these drugs is as antithrombotic or anti-arthritic therapies and future *in vivo* studies would further elucidate their suitability for such applications.

8.4.1.1 Ex Vivo Studies - Antithrombotic Effect

Ex vivo studies can be utilised to study the antithrombotic effect of drugs administered *in vivo*. Such studies have been carried out with the existing NO-aspirin, NCX4016, following oral administration in both pigs (Wainwright *et al.*, 2002) and humans (Fiorucci *et al.*, 2004). Commonly, following administration of the test drug, blood is drawn and whole blood aggregation is carried out as in chapter

five. Such protocol allows any impact of the *in vivo* metabolism of the drug on its antiplatelet activity to be determined. These studies would be of particular importance in investigating how stable the acetyl group is *in vivo* and if an aspirin-mediated effect is observed as a consequence.

Another *ex vivo* technique commonly used to study platelets is the Badimon perfusion chamber (Badimon *et al.*, 1999; Badimon *et al.*, 1987; Fernandez-Ortiz *et al.*, 1994). It can be utilised to simulate blood flow through tissue segments in order to study platelet deposition and thrombus formation.

8.4.1.2 In Vivo Studies - Anti-Arthritic Effect

As the original purpose of the drugs was to avoid the gastrotoxicity associated with long-term NSAID use for conditions such as arthritis, investigations into the application of the drugs as anti-arthritic compounds should be tested in a chronic model of arthritis. The adjuvant-induced model of arthritis in either mice or rats (Donaldson *et al.*, 1993; Gauldie *et al.*, 2004) would enable study of the long-term effects of the compounds could be investigated. Such studies would also be useful in revealing the furoxans to be devoid of the tolerance problems expected to limit the application of NCX4016.

8.4.1.3 In Vivo Studies - Anti-Atherosclerotic Effect

The complexity of the disease makes atherosclerosis difficult to treat. However, through their antiplatelet and anti-inflammatory actions, the furoxans may provide some clinical benefit. In order to investigate such a possibility the Apo-E model could be used for *in vivo* studies.

The Apo-E deficient mouse is often used as a model of atherosclerosis (Nakashima *et al.*, 1994; Piedrahita *et al.*, 1992; Reddick *et al.*, 1994; Zhang *et al.*, 1994). Mice deficient in the amphipathic protein, Apo-E, suffer from a lack of receptor-mediated clearance of chylomicrons, very low- and high-density lipoproteins, resulting in plasma cholesterol levels 4-5 times higher than normal (Ishibashi *et al.*, 1994). These mice develop complex atherosclerotic lesions before they are 20 weeks old.

The Apo-E model could be a means of further investigating the anti-inflammatory properties of the furoxan NO-aspirins in cardiovascular tissue. As previously suggested, without knowing whether the NO-aspirins contribute to smooth muscle cell necrosis (Shaw, 2006), it is difficult to predict their actions in an established plaque. However, the effects on smooth muscle cell necrosis could be established first using an *in vitro* assay that monitors uptake of bromodeoxyuridine into proliferating cultured smooth muscle cells (such as bovine aortic smooth muscle cells). Bromodeoxyuridine is a thymidine analogue which becomes incorporated into the newly synthesised DNA of actively proliferating cells. Anti-bromodeoxyuridine antibodies are commercially available (Calbiochem) which allow detection and quantification of incorporation of bromodeoxyuridine and thus cell proliferation. Such techniques would allow the impact of the furoxan NO-aspirins on the proliferation of smooth muscle cells to be determined *in vitro* and may act as an indicator of their affect on the stability of the atherosclerotic plaque cap. Cell viability of the cultured smooth muscle cells could be assessed by LDH assay as in chapter seven.

The Apo-E knockout model could be utilised to investigate the possible beneficial effects of the furoxan NO-aspirins *in vivo* during atherosclerosis by experiments

involving chronic subcutaneous implantation of NO-aspirin-eluting osmotic pumps and post-mortem analysis of atheroma using conventional histological and biochemical techniques at specific animal ages.

The Apo-E model may also be utilised to further explore anti-inflammatory effects of the furoxan NO-aspirins. The induction of iNOS is common under inflammatory conditions such as atherosclerosis, where inflammatory cytokines are associated with its stimulus (Behr-Roussel *et al.*, 2000; Buttery *et al.*, 1996; MacNaul & Hutchinson, 1993). Experiments could be undertaken to determine whether the anti-inflammatory nature of the furoxan NO-aspirin drugs have any effect on iNOS function through the anti-cytokine effect revealed in chapter seven, in aortic rings from Apo-E knockout mice.

A further use of the Apo-E model could be in a study to determine the effects that the furoxan NO-aspirins have on blood pressure in both the atherosclerotic-prone and normal mouse. Such a study would determine if the vasodilator action is retained *in vivo* and would also clarify if the drugs are likely to suffer from the coronary steal phenomenon.

However, if as predicted, the beneficial effect of these compounds is due to their antithrombotic effect demonstrated in chapter five, it is imperative to study a murine colony in which plaque rupture occurs (Johnson & Jackson, 2001) in order to observe any beneficial effect in reducing the incidence of clinical manifestations of thrombus formation such as MI or stroke.

It is to be expected that the *in vivo* application of these drugs will raise further issues which commonly limit the use of NO-donor drugs. Such limitations include

unwanted vasodilatation in patients prescribed the drugs for their antithrombotic actions. However, as the COX-mediated inhibition gives an antiplatelet action for the lifetime of the platelet, any problems related to hypotension evoked by these drugs are likely to be shorter acting than the antiplatelet effect and although still potentially problematic, it may be resolved by altering the dosing regime.

8.5 Conclusions

Data presented within this thesis have provided insight into the *in vitro* vascular actions of a novel series of furoxan derivatives of aspirin. The drugs have been demonstrated to have potent antiplatelet, vasodilatory and anti-inflammatory actions contributed to by both moieties of the hybrid. It is hoped that such actions, along with their favourable gastric profile, will enable them to replace aspirin as a prophylactic against thrombotic events or perhaps even as an anti-inflammatory agent in patients with arthritis, with further more speculative applications as anti-cancer or anti-atherosclerotic drugs.

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APPENDIX

Publications

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Mechanism of action of novel NO-releasing furoxan derivatives of aspirin in human platelets

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1 Incorporation of a nitric oxide (NO)-releasing moiety in aspirin can overcome its gastric side effects.

2 We investigated the NO-release patterns and antiplatelet effects of novel furoxan derivatives of aspirin (B8 and B7) in comparison to existing antiplatelet agents.

3 Cyclooxygenase (COX) activity was investigated in purified enzyme using an electron paramagnetic resonance-based technique. Concentration–response curves for antiplatelet agents ± the soluble guanylate cyclase inhibitor, ODQ (50 μM) were generated in platelet-rich plasma (PRP) and washed platelets (WP) activated with collagen using turbidometric aggregometry. NO was detected using an isolated NO electrode.

4 The furoxan derivatives of aspirin (B8, B7) and their NO-free furazan equivalents (B16, B15; all 100 μM) significantly inhibited COX activity ($P<0.01$; $n=6$) *in vitro* and caused aspirin-independent, cGMP-dependent inhibition of collagen-induced platelet aggregation in WP. B8 was more potent than B7 (PRP $IC_{50}=0.62\pm0.1\,\mu$M for B8; $400\pm89\,\mu$M for B7; $P<0.0001$. WP IC_{50}s $=0.6\pm0.1$ and $62\pm10\,\mu$M, respectively). The NO-free furazan counterparts were less potent antiplatelet agents (WP IC_{50}s $=54\pm3\,\mu$M and $62\pm10\,\mu$M, respectively; $P<0.0001$, B8 vs B16). Of the hybrids investigated, only B8 retained antiplatelet activity in PRP.

5 NO release from furoxan–aspirin hybrids was undetectable in buffer alone, but was accelerated in the presence of either plasma or plasma components, albumin (4%), glutathione (GSH; 3 μM) and ascorbate (50 μM), the effects of which were additive for B7 but not B8. NO generation from furoxans was greatly enhanced by platelet extract, an effect that could largely be explained by the synergistic effect of intracellular concentrations of GSH (3 mM) and ascorbate (1 mM).

6 We conclude that the decomposition of furoxan–aspirin hybrids to generate biologically active NO is catalysed by endogenous agents which may instil a potential for primarily intracellular delivery of NO. The blunting of the aspirin effects of furoxan hybrids is likely to be due to loss of the acetyl moiety in plasma; the observed antiplatelet effects are thereby primarily mediated *via* NO release. Compounds of this class might represent a novel means of inhibiting platelet aggregation by a combination of NO generation and COX inhibition.

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Keywords: Nitric oxide; platelets; furoxan; nitroaspirin; thrombosis; antithrombotic

Abbreviations: AA, arachidonic acid; AUC, area under the curve; cGMP, cyclic 5′-guanosine monophosphate; COX-1, cyclooxygenase-1; CPH, 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine.HCl; DEA/NO, 2-(*N*,*N*-diethyamino)-diazenolate-2-oxide; DMSO, dimethylsulphoxide; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); EPR, electron paramagnetic resonance; GSH, glutathione; NO, nitric oxide; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one; PPP, platelet poor plasma; PRP, platelet-rich plasma; sGC, soluble guanylate cyclase; TFA, trifluoroacetic acid; WP, washed platelets

Introduction

Aspirin is a nonsteroidal anti-inflammatory drug that has long been used as a prophylactic against thrombotic coronary events (ISIS-2, 1988; PHS-Committee, 1989; Manson *et al.*, 1991). It reduces cardiovascular risk, primarily through irreversible inhibition of prostaglandin H synthase-1 (also termed cyclooxygenase-1, COX-1)-mediated platelet aggregation (for review see, Patrono, 1994). Aspirin selectively and irreversibly acetylates a serine residue (Ser 530) of COX-1 (Roth & Majerus, 1975; Roth *et al.*, 1975; DeWitt & Smith, 1988). COX-1 inhibition results in reduced production of thromboxane A_2, a vital element in the induction of irreversible platelet aggregation in response to stimuli such as collagen (FitzGerald, 1991; Hamberg *et al.*, 1975). The anucleate nature of platelets makes them unable to synthesize new proteins and replace inhibited enzyme; recovery of full platelet activity only takes place as a function of platelet turnover (Burch *et al.*, 1978). Aspirin also acts to inhibit the

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formation of thrombin (Kessels *et al.*, 1994; Szczeklik *et al.*, 1992), a unique action that also prevents platelet aggregation and impacts on the coagulation pathway. Taken together, these properties offer a degree of platelet selectivity in the action of aspirin.

Unfortunately, gastrointestinal disorders, including ulceration, are a common side effect of aspirin, limiting its use (Cameron, 1975; Wallace, 1997; Tramer *et al.*, 2000; Seager & Hawkey, 2001). The effect is due to inhibition of prostaglandins that normally protect the gastric mucosa (Whittle, 1977; Robert *et al.*, 1979; Schoen & Vender, 1989; Wallace, 1997). Aspirin esters containing a nitric oxide (NO)-donor nitrooxy function (e.g. NCX4016) are thought to overcome the gastric side effects through the protective actions of drug-derived NO. NO increases blood flow in the gastric mucosa, promoting repair and removal of toxins (Wallace & Miller, 2000). NO also increases secretion of protective gastric mucus (Brown *et al.*, 1993) and is thought to promote healing of gastric ulcers by promoting angiogenesis (Ma & Wallace, 2000). Alternatively, the protective effects of NO aspirin could be due to masking of the aspirin carboxylic acid moiety by the ester function (Rainsford & Whitehouse, 1976; Cena *et al.*, 2003).

While NO hybrids of aspirin were primarily designed to protect against damage to the gastric mucosa, there may be additional benefits of drug-derived NO through its many protective effects in the vascular system. NO is a powerful endogenous vasodilator (Palmer *et al.*, 1987) which acts to keep the vasculature in an active state of dilatation by stimulating cGMP-mediated relaxation of vascular smooth muscle cells. NO also opposes the adherence of monocytes to the vessel wall (Tsao *et al.*, 1997) and displays antithrombotic actions through its ability to inhibit platelet adhesion (Radomski *et al.*, 1987b,c) and aggregation (Radomski *et al.*, 1987a; 1990; Pasqui *et al.*, 1991). Inhibition of platelet aggregation occurs primarily *via* stimulation of cGMP; the platelet aggregation response to sodium nitroprusside has been shown to be entirely cGMP dependent (Sogo *et al.*, 2000). However, cGMP-independent signalling mechanisms have also been identified (Gordge *et al.*, 1998; Trepakova *et al.*, 1999; Sogo *et al.*, 2000; Homer & Wanstall, 2002; Crane *et al.*, 2005). Reduced NO synthesis or availability is heavily implicated as a key factor in the initiation and progression of atherogenesis (Anderson *et al.*, 1995; Maxwell, 2002; Shaul, 2003).

The release at NO from NCX4016 and glyceryl trinitrate has been reported to occur through identical mechanisms (Grosser & Schroder, 2000). Platelets have a poor capability to release NO from organic nitrates (Weber *et al.*, 1996) and it is possible that compounds such as NCX4016 will fail to show additional, NO-mediated effects in platelets, at least *in vitro*. A new range of drugs, in which a NO-donating moiety (furoxan group) is joined by an ester linkage to the aspirin molecule (B8, B7; Figure 1) appear to overcome the problem of gastric lesions (Cena *et al.*, 2003). However, the NO-release mechanism is unlikely to require the same cellular machinery as that for organic nitrates, suggesting that they might offer a degree of NO-mediated antiplatelet effects to complement those of the aspirin moiety.

In the present study, we investigated the mechanism of action of two examples (3-cyanofuroxan-4-yl)methyl 2-acetoxybenzoate (B8) and (3-carbamoylfuroxan-4-yl)methyl 2-acetoxybenzoate (B7); Figure 1), with different NO-releasing properties (Cena *et al.*, 2003) in human platelets *in vitro*. Here, we characterize the NO-releasing and COX-inhibiting effects of these novel furoxan derivatives of aspirin and compare their actions to related antiplatelet agents.

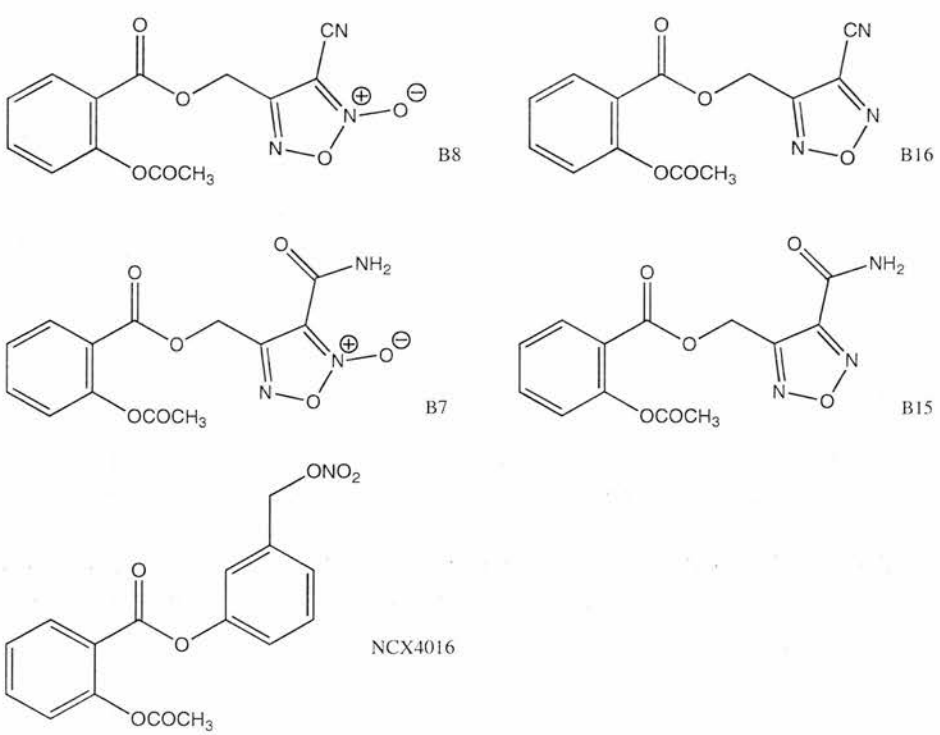

Figure 1 Structural formulae of furoxan hybrids (B8 and B7), furazan hybrids (B16 and B15) and the nitrooxy-ester NCX4016.

Methods

Materials

2-(*N*,*N*-diethyamino)-diazenolate-2-oxide (DEA/NO; Axxora, Nottingham, U.K.) was dissolved in 0.01 M NaOH. 1*H*-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ; Cookson, Langford, Bristol, U.K.) was dissolved in dimethylsulphoxide (DMSO) and stored at −20°C. Collagen was purchased from Labmedics (Stockport, U.K.). 1-Hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine. HCl (CPH) was purchased from Axxora. All other chemicals were purchased from Sigma (Poole, Dorset, U.K.). All nitro-aspirins were synthesized at the Università degli Studi di Torino, as described (Cena *et al.*, 2003). Compound purity was assessed by high-performance liquid chromatography before biological assays (over 98% for all compounds; Merck Purosphere RP-18 column (Merck, Darmstadt, Germany; 250 × 4 mm^2, 5 μm particle size) eluting with flow rate of 1 ml min^{-1}. Mobile phase consisted of 0.1% aqueous trifluoroacetic acid (TFA) and acetonitrile containing 0.1% TFA in different ratios according with compounds properties. The column effluent was monitored at 224/254 nm). Compound identity was chemically confirmed by nuclear magnetic resonance.

The nitro-aspirins were dissolved in DMSO, then diluted in PBS to give a final concentration of 0.1% DMSO, which pilot studies had determined not to affect platelet aggregation. No precipitation of any drug was observed following dilution.

COX activity assay

The ability of novel and established compounds to inhibit COX-1 was measured using an adaptation of a published method (Schreiber *et al.*, 1989). The assay relies on detection of oxidizing free radicals produced as a by-product by the peroxidase element of the COX enzyme. A spin-trapping agent, CPH, was utilized to trap the short-lived radicals, forming a stable adduct, 3-carboxy-proxyl. The assay makes use of commercially available purified COX-1 from ovine seminal vesicles (Sigma), pretreated with haematin for 5 min.

The COX activity assay was performed at 37°C in 1 ml of Tyrode's buffer. In all, 100 U ml^{-1} purified COX-1 was incubated with 3 nM haematin (5 min, 37°C) prior to the assay. Aspirin, salicylic acid, a furoxan, a furazan, NCX4016 (100 μM) or a vehicle control (DMSO, 1%) was added and left to incubate for a further 10 min prior to addition of the spin-trap, CPH (1 mM). At this point (time zero), a baseline EPR measurement was taken (MS200, Magnettech, Germany. Instrument settings: B0-field, 3356 G; sweep width; 50 G, sweep time, 30 s; modulation amplitude, 1500 mG; microwave power, 20 mW; microwave frequency, 9.3 GHz). After 2 min, 0.5 mM AA (sodium salt) was added; EPR measurements were made (50 μl samples in micropipette tubes) after a further 1.5 min incubation. The suicidal nature of COX-1 activation means that the period of activation is complete within ~1 min of AA addition (as confirmed by pilot experiments).

The EPR results were corrected for any auto-oxidation of spin-trap by subtraction of values recorded from a duplicate sample run in the absence of AA. The intensity scale on the *y*-axis of all graphs is an arbitrary scale based upon the area under the curve (AUC) of the first derivative traces generated. Results graphs show the timepoint 1.5 min after the addition of AA, as this was determined in pilot experiments to be the most appropriate point at which to compare free radical generation between control and furoxan-treated COX-1, given that spin-adduct generation in response to AA had peaked – subsequent adduct formation was at an equivalent rate in control and AA-treated samples and was likely to be due to nonspecific auto-oxidation of CPH.

Blood preparation

Peripheral venous blood was drawn from the antecubital fossa of human volunteers aged 20–45 years who were non-smokers and had not taken any platelet-active agents during the previous 10 days. Blood was collected into tubes containing 3.8% sodium citrate and centrifuged at 200 × *g* for 10 min at room temperature to obtain platelet-rich plasma (PRP). An aliquot of PRP was further centrifuged at 1200 × *g* for 10 min to obtain platelet-poor plasma (PPP). Washed platelets (WP) samples were obtained by adding 300 ng ml^{-1} prostacyclin to a 2 ml PRP sample before centrifuging at 1200 × *g* for 10 min. Prostacyclin is commonly used at this concentration in this type of study (Giuliano & Warner, 1999; Kobzar *et al.*, 2001; Crane *et al.*, 2002). The effect of the prostacyclin is only temporary due to its short half-life. The supernatant was then discarded and the pellet resuspended in Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 1.05 mM $MgSO_4$, 0.4 mM NaH_2PO_4, 12.5 mM $NaHCO_3$, 5.6 mM Glucose, 10 mM HEPES and 0.8 mM CaCl in dH_2O at pH 7.4). Prostacyclin (300 ng ml^{-1}) was again added before a second 10 min centrifugation (1200 × *g*). Finally, the pellet was resuspended in 2 ml Tyrode's buffer. For aggregation, platelet counts were determined using a Coulter Ac.T 8 Hematology Analyzer (Coulter Electronics, Luton, U.K.) and standardized to 250 × 10^9 l^{-1} *via* dilution with PPP (PRP) or Tyrode's buffer (WP).

Aggregation

Inhibition of platelet aggregation was measured using optical platelet aggregometry in a 4-channel aggregometer (Chrono-Log, Labmedics, Stockport, U.K.) and data captured *via* an analogue-digital converter (Maclab 4e, AD Instruments, Sussex, U.K.). The instrument was calibrated such that the difference in light transmission between test (PRP or WP) and reference (PPP or Tyrode's buffer, respectively) samples was set to generate an 80 mV signal. Typically, maximal aggregation caused ~60 mV change in signal. Briefly, PRP or WP samples were equilibrated at 37°C and stirred continuously at 1000 r.p.m. The samples were then treated with either B8 (10 nM–3 μM), B7 (10 μM–1 mM), their respective NO-free structurally related furazan derivatives, (4-cyanofurazan-3-yl)methyl 2-acetoxybenzoate (B16; 10 nM–3 μM) or (4-carbamoylfurazan-3-yl)methyl 2-acetoxybenzoate (B15; 10 μM–1 mM), NCX4016 (3 μM–0.3 mM) or aspirin (acetylsalicylic acid; 3 μM–1 mM) for 10 min before induction of aggregation with supramaximal concentrations of collagen (2.5 μg ml^{-1}). The aggregation was then recorded turbidometrically over 5 min against a reference PPP sample. Experiments were also performed with the addition of 50 μM (supramaximal concentration (Crane *et al.*, 2005)) of the cGMP inhibitor ODQ 15 min before addition of the drug. Aggregation was expressed

as a percentage inhibition of control aggregation obtained with 2.5 μg ml^{-1} collagen.

NO release

Samples of PRP, PPP or Tyrode's buffer (2 ml) were incubated in the aggregometer at 37°C and stirred continuously at 1000 r.p.m. An isolated NO electrode (ISO-NO MARKII, World Precision Instruments, Stevenage, U.K.), calibrated using DEA/NO (0.1–1.6 μM), was introduced into the cuvette and allowed to stabilize for 10 min. B8 (100 μM) or B7 (500 μM) was then added to the cuvette and the release of NO recorded for 10 min before addition of haemoglobin (10 μM) to scavenge any generated NO. Experiments were also carried out in Tyrode's buffer $\pm$4% albumin, with the addition of glutathione (3 μM or 3 mM), $\pm$50 μM ascorbate before B8 or B7. Ascorbate (1 mM) was also used, but only in the presence of 10 mM HEPES to buffer its acidic pH. The concentrations of albumin, ascorbate and glutathione studied are all physiologically relevant. The detection limit for the electrode under the conditions of the experiment was found to be $\sim$10 nM NO.

A platelet extract was also prepared from samples of PRP and platelet count was determined using the Coulter Ac.T 8 Hematology Analyzer. The platelets were washed as above in the presence of prostacyclin to obtain a platelet pellet. The total platelet volume of the pellet was calculated by multiplying the number of platelets by the average platelet volume (5.14×10^{-15} l; obtained from the hematology analyzer). The pellet was resuspended in 1 ml of 0.5% Triton X in Tyrode's buffer for 1 h and the dilution of cell contents estimated using the total platelet volume from the above calculation. This was then homogenized by hand for 15 min and centrifuged at 12,000 $\times$ *g* for 10 min to give a cell extract. B8 (100 μM) or B7 (500 μM) was added to 1 ml of this supernatant in the assay as described above.

A further set of experiments was carried out following a 10 min preincubation of the platelet extract with the thiol alkylator, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB; 500 μM). The NO release from B8 (100 μM) or B7 (500 μM) was then recorded as above. The samples were collected and frozen (−20°C) and later used for thiol determination by colorimetric analysis at 405 nm. Thiol levels of the extract samples were determined from a standard curve created using 10 μM–30 mM glutathione in 500 μM DTNB.

Statistics

All results are expressed as the mean $\pm$ s.e.m. Concentration–response curves were analysed by two-way analysis of variance (two-way ANOVA). EPR data were analysed by 1-way ANOVA and NO electrode data by two-tailed paired Student's *t*-test. Dunnett's or Bonferroni post-tests were carried out where appropriate. $P<0.05$ was considered to be statistically significant.

Results

COX activity

Aspirin (100 μM) significantly reduced the EPR signal generated by AA-stimulated purified COX-1 in Tyrode's buffer compared to a vehicle control (Figure 2; $P<0.01$; $n=8$). A significant inhibition of COX activity was also observed with the furoxan–aspirins, B8 and B7 (100 μM; $P<0.05$ and $P<0.01$ respectively, $n=6$). Even greater inhibition was demonstrated with the NO-free furazan equivalents, B16 and B15 (100 μM, $P<0.01$ for both, $n=6$). The furazan compounds demonstrated a significantly greater inhibition of COX than aspirin ($P<0.01$). The nitrooxy-ester, NCX4016 (100 μM) also abolished COX activity ($P<0.01$, $n=6$). Salicylic acid (100 μM, $n=6$) failed to significantly inhibit generation of the spin-adduct, as did the NO donor DEA/NO (100 μM, $n=6$; Figure 2b).

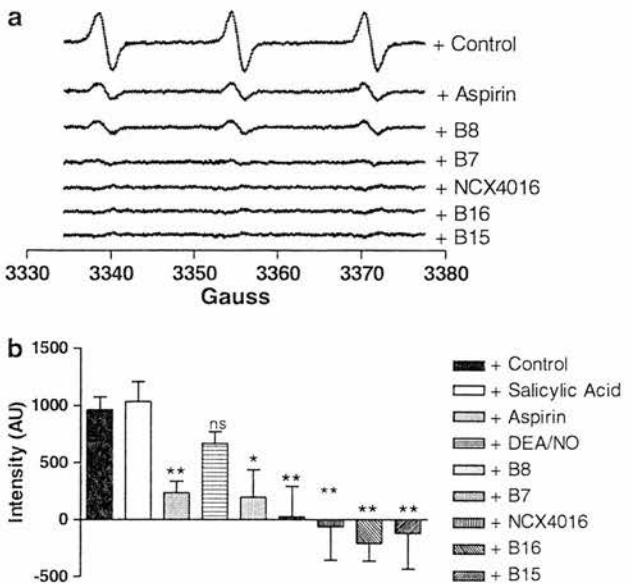

Figure 2 EPR-based COX-activity assay. (a) Typical 3-peak EPR spectra obtained in the absence (control; COX + AA) and presence of aspirin or hybrid compound (all 100 μM) after correction for background autoxidation. EPR settings: B0-field, 3356 G; sweep width; 50 G, microwave frequency, 9.3 GHz; sweep time, 30 s; modulation amplitude, 1500 mG; microwave power, 20 mW. (b) Effect of aspirin, salicylic acid, furoxans, furazans and NCX4016 (all 100 μM) on EPR signals generated from COX-1 after treatment with substrate (AA). In each case, drug incubations were for 10 min prior to the baseline EPR reading. Readings shown were taken 1.5 min after the addition of AA. $^*P<0.05$, $^{**}P<0.01$; one-way ANOVA with Dunnet's *post hoc* test vs control: $n=6$–10. Values are mean $\pm$ s.e.m.

Effect of aspirin, salicylic acid and NCX4016 on platelet aggregation

Aspirin (3–300 μM) caused concentration-dependent inhibition of collagen-induced platelet aggregation in PRP and the effect was enhanced in WP, but salicylic acid failed to show an inhibitory effect, even at concentrations of 300 μM (Figure 3; $n=6$–8). NCX4016 (3–300 μM) had no effect on collagen-induced platelet aggregation in PRP (Figure 3a; $n=6$), but did cause concentration-dependent inhibition of platelet aggregation in WP that was significantly more potent than aspirin (Figure 3b; $P=0.002$; $n=6$). Responses to NCX4016 were insensitive to the soluble guanylate cyclase inhibitor, ODQ (15 min preincubation; $P=0.88$, NCX4016 + ODQ vs NCX4016 alone in WP; Figure 3b).

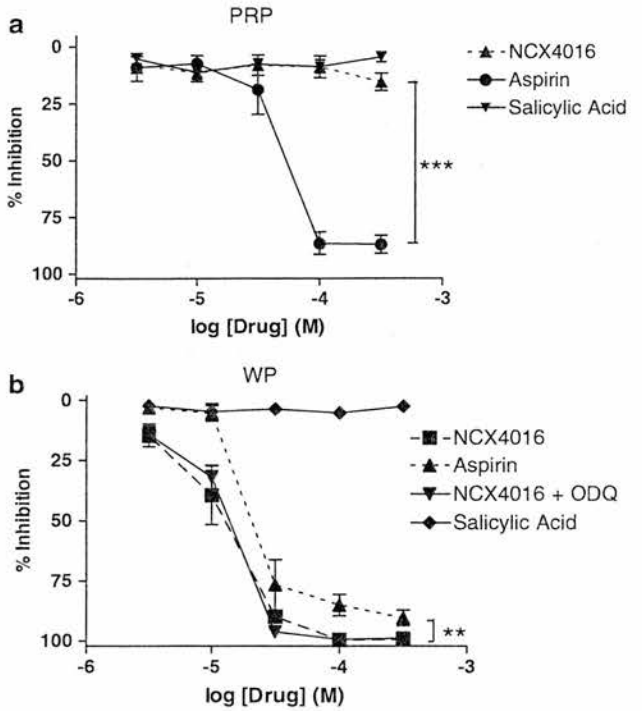

Figure 3 Collagen ($2.5\,\mu g\,ml^{-1}$)-induced platelet aggregation in PRP and WP. (a) Effect of NCX4016, salicylic acid and aspirin on collagen-induced platelet aggregation in PRP. $^{***}P<0.0001$, $n=6$–7. (b) The effect of the guanylate cyclase inhibitor ODQ ($50\,\mu M$; 15 min preincubation) on responses to NCX4016 in WP. $^{**}P=0.002$, $n=6$. Values are mean ± s.e.m. Statistical analysis by two-way ANOVA.

Effect of B8 and B7 on platelet aggregation

B8 (10 nM–3 μM) caused concentration-dependent inhibition of collagen-induced platelet aggregation in PRP at concentrations ~100-fold lower than for aspirin (B8 IC_{50} in PRP = 0.62 ± 0.1 μM; Figure 4a, (see Figure 3a for aspirin effect)). ODQ significantly inhibited the responses to B8 in PRP (Figure 4a; $n=6$–8; $P<0.0001$), while the NO-free, structurally related furazan derivative of B8 (B16; 10 nM–1 mM) was considerably less effective at inhibiting platelet aggregation than B8 in PRP (Figure 4a; $P<0.0001$; $n=8$).

The effects of B8 in PRP were largely mirrored in WP: B8 was found to be a powerful inhibitor of collagen-induced aggregation ($IC_{50}=0.6\pm0.1\,\mu M$) and its actions were significantly inhibited by ODQ (Figure 4b; $P<0.0001$; $n=6$–8). B16 had a significantly greater inhibitory effect on platelet aggregation in WP compared to PRP ($IC_{50}=54\pm3\,\mu M$; Figure 4b; $P<0.0001$; PRP B16 vs WP B16; $n=6$–8).

B7 (10 μM–1 mM) was less effective than B8 and aspirin at inhibiting collagen-induced platelet aggregation in PRP (B7 $IC_{50}=0.36\pm0.1$ mM). ODQ significantly inhibited the response of B7 in PRP (Figure 4c; $n=6$–7; $P<0.0001$). B15 was ineffective in PRP.

In order to make a direct comparison, concentrations studied with WP were dictated by the PRP response curve. B7 (10 μM–1 mM) was considerably more effective in WP ($IC_{50}=24\pm8\,\mu M$) than PRP. The inhibitory effects of B7 in WP were significantly attenuated by ODQ ($P=0.002$, two-way ANOVA; $n=6$–7). In stark contrast to the findings in PRP, B15 was found to be a powerful inhibitor of platelet

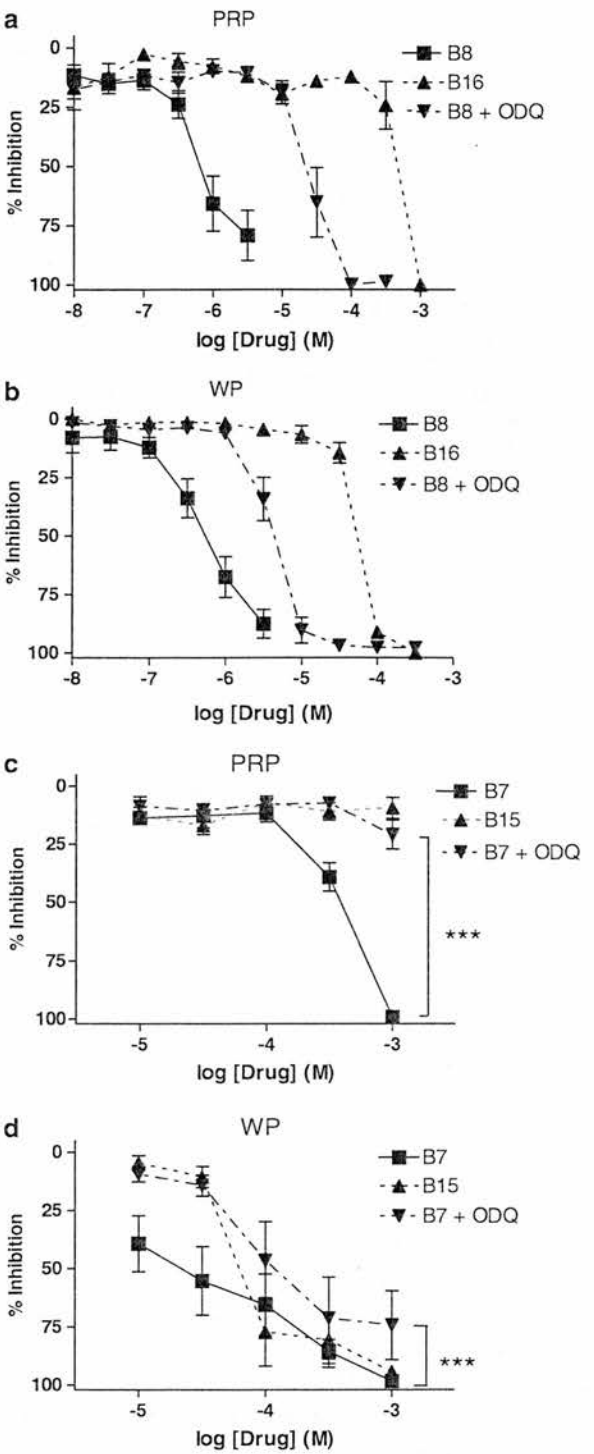

Figure 4 Collagen ($2.5\,\mu g\,ml^{-1}$)-induced platelet aggregation in PRP and WP. (a) Effect of B8 (±ODQ; $50\,\mu M$, 15 min preincubation) and its NO-free equivalent, B16, on collagen-induced platelet aggregation in PRP. $P<0.0001$ (B8 + ODQ vs B8 alone, $n=6$–9). (b) The effect of ODQ ($50\,\mu M$) on responses to B8 in WP. $P<0.0001$ (+ODQ vs B8 alone), $n=6$–9. (c) Effect of B7 (±ODQ; $50\,\mu M$) and its NO-free equivalent B15 on collagen-induced platelet aggregation in PRP. $^{***}P<0.0001$, $n=6$–7. (d) The effect of ODQ ($50\,\mu M$) on the B7 response in WP. $^{***}P<0.0001$, $n=6$–7. Values are mean ± s.e.m. Statistical analysis by two-way ANOVA.

aggregation in WP (B15 IC_{50} in WP = 62 ± 10 μM); indeed, at concentrations of 100 μM or more, it was as effective as B7 under these conditions.

NO release from furoxan–aspirin hybrids: effect of endogenous antioxidants

Sample recordings of NO generation from B7 and B8 in the presence and absence of GSH and ascorbate are shown in the inserts to Figure 5a and c. Owing to the different profile of NO release by the different drugs, it was established that AUC was more representative of NO release than peak concentration in these experiments; subsequent values quoted are all AUC over the 10 min incubation (mmol min), but sample peak values are also given for information.

NO release from NCX4016 (100 μM) was undetectable in Tyrode's buffer, PRP or WP (n = 6–8 for each). Likewise, NO release from the furoxans B8 and B7, was undetectable in

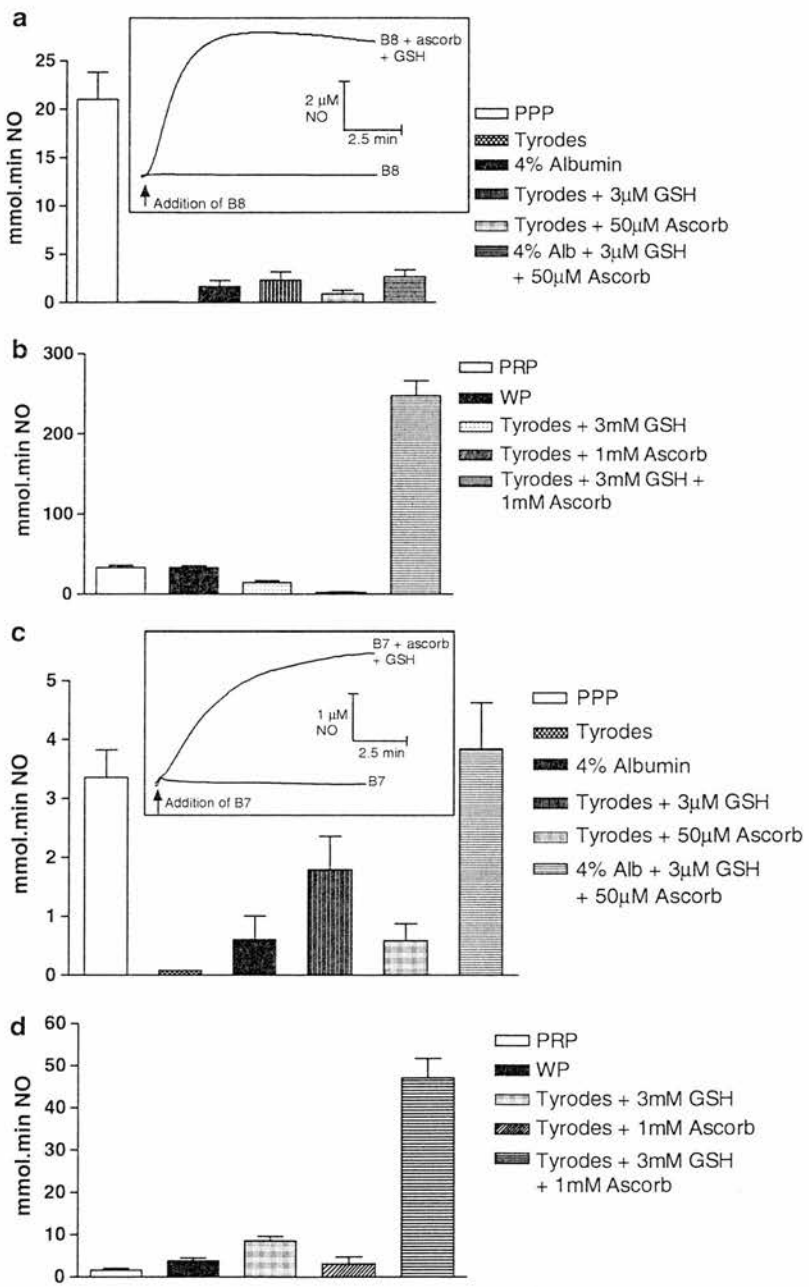

Figure 5 NO release recorded over 10 min from B8 (100 μM) or B7 (500 μM) in various media. (a) NO release from B8 in media related to plasma conditions: Tyrode's buffer was reconstituted with approximate plasma concentrations of the plasma constituents, albumin (4%), GSH (3 μM) and ascorbate (50 μM). Inset shows typical 10 min traces of NO release recorded *via* the NO electrode in Tyrode's buffer with or without ascorbate (1 mM) and GSH (3 mM). (b) Shows typical NO release from B8 in media related to platelet conditions: Tyrode's buffer 3 and 1 mM are approximate intracellular concentrations of glutathione and ascorbate, respectively; n = 6–7. GSH = Glutathione. Ascorb = ascorbate. Values are mean ± s.e.m. (c) Shows NO release from B7 in media related to plasma conditions. Inset shows typical 10 min traces of NO release from B7 recorded *via* the NO electrode in Tyrode's buffer with or without ascorbate (1 mM) and GSH (3 mM) (d) shows NO release from B7 in media related to platelet conditions.

Tyrode's buffer alone (Figure 5a and c). However, it was detected in samples of PRP, WP, PPP or Tyrode's buffer in the presence of approximate plasma concentrations of albumin (4%), GSH (3 μM) or ascorbate (50 μM; Figure 5b and d).

NO was released from B8 in platelet-containing samples (PRP and WP; mean NO release = 33.2 ± 2.9 and 33.0 ± 2.5 mmol min, respectively, with both peaking at ~1.25 μM NO; Figure 5b), and in PPP (mean NO release for B8 = 21.0 ± 2.8 mmol min, peak at ~0.6 μM NO; Figure 5a).

Reconstitution of Tyrode's buffer with approximate plasma concentrations of either albumin (4%), glutathione (3 μM) or ascorbate (50 μM) enhanced NO generation from B8. A combination of these three constituents failed to enhance NO release from B8 beyond the effects seen with the individual components alone (2.7 ± 0.7 mmol min, peak at ~0.040 μM for GSH alone and ~0.038 μM NO in the presence of plasma concentrations of albumin, GSH and ascorbate; Figure 5a; $P = 0.78$ vs GSH alone, $P = 0.13$ vs ascorbate alone, both unpaired Student's t-test).

Reconstitution of Tyrode's buffer with approximately intracellular concentrations of GSH (3 mM) generated marginally less NO from B8 (14.4 ± 2.5 mmol min; Figure 5b; peak at ~0.124 μM NO) compared with samples containing platelets. Ascorbate (1 mM) in Tyrode's buffer caused minimal NO release from B8 (= 2.2 ± 0.9 mmol min; Figure 5b; peak at ~0.004 μM NO), but a combination of GSH and ascorbate caused a considerable increase in NO generation from B8 (247 ± 19 mmol min, $n = 6$, Figure 5b, $P < 0.0001$ vs individual components alone, unpaired Student's t-test; peak at ~2.31 μM NO).

Relatively low levels of NO were released from B7 in PRP and WP (1.6 ± 0.4 and 3.8 ± 0.7 mmol min, respectively; $n = 6$, Figure 5d. Peaks at ~0.085 and ~0.120 μM NO, respectively) despite the higher drug concentration used. Reconstitution of the Tyrode's buffer with approximate plasma concentrations of either albumin (4%), glutathione (3 μM) or ascorbate (50 μM) enhanced NO generation from B7 and a combination of these three constituents had an additive effect on the release of NO from B7 (3.8 ± 0.8 mmol min; peak at ~0.295 μM NO), where release was equivalent to that seen in PPP (unpaired Student's t-test $P = 0.628$; Figure 5c).

Reconstitution of Tyrode's buffer with approximately intracellular concentrations of GSH (3 mM) increased NO release from B7 (8.5 ± 1.1 mmol min; peak at ~0.504 μM NO). Ascorbate (1 mM) in Tyrode's buffer caused minimal NO release from B7 (3.1 ± 1.6 mmol min; peak at ~0.004 μM NO), but a combination of GSH and ascorbate caused a considerable increase in NO generation from B7 (47 ± 4 mmol min $n = 6$, Figure 5d; peak at ~0.642 μM NO).

Effect of platelet extract on NO generation from B8 and B7

There was a relationship between the dilution factor of platelet extract and the amount of NO released from both B8 and B7 (Figure 6a and b). NO generation by B8 (100 μM) failed to increase at platelet extract dilutions below ~1 in 30, with a maximum NO generation of ~90 mmol min. Pretreatment of platelet extracts with the thiol alkylator, DTNB (500 μM), all but abolished NO generation.

A similar pattern was observed with B7 (500 μM), but the amount of NO generated in the presence of platelet extracts was >10-fold lower than from B8, despite the higher drug concentration. In the case of B7, dilutions of platelet extract lower than ~1 in 75 failed to generate detectable NO, but there was a direct relationship between NO generation and dilution factor at dilutions of 1 in 75 to 1 in 25 (Figure 6b). DTNB again showed a marked inhibitory effect on NO generation in the presence of platelet extract. Colorimetric analysis of DTNB-treated samples yielded intracellular total reduced thiol concentrations of 22 ± 4 mM (corrected for dilution; $n = 5$).

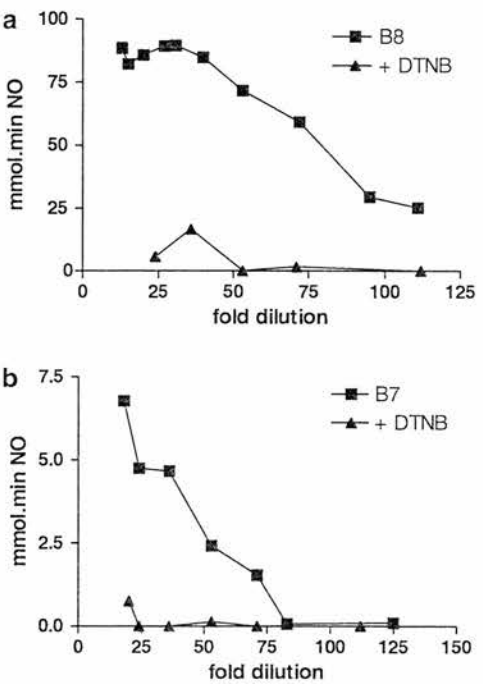

Figure 6 A sample of WP was suspended in 1 ml 0.5% Triton X and then homogenized to release platelet extract before centrifugation to remove membrane fraction. The dilution factor on x-axis was calculated by volume Triton X (1 ml)/(number of platelets × average platelet volume). (a) NO release from 16 platelet extracts treated with 100 μM B8. The triangles show samples treated in the same way but with a 10 min preincubation with 500 μM DTNB before addition of B8. (b) NO release from 13 platelet extracts treated with 500 μM B7. The triangles show samples treated in the same way but with a 10 min preincubation with 500 μM DTNB before addition of B7.

Discussion

Our results show that two novel furoxan–aspirin hybrids drugs effectively inhibit collagen-induced platelet aggregation and the relative contribution of NO to the inhibitory effect was dependent on the characteristics of the specific furoxan involved. The potent effects of B8 were considered to be largely NO dependent on account of the fact that the closely related NO-free furazan, B16, had only a very weak antiplatelet effect. B7 was a less potent inhibitor of aggregation than B8 but the effects were also primarily NO mediated in PRP, where the furazan counterpart was largely ineffectual. However, in WP, it was apparent that the antiplatelet activity of B7 comprised both NO-dependent and -independent components. The existing nitrooxy ester, nitro-aspirin (NCX4016), was also an effective antiplatelet agent in WP but the effects were

entirely sGC independent and were altogether lost in PRP. The release of NO from the furoxans was found to be critically dependent on the presence of endogenous reducing agents. NO generation was most striking in the presence of intracellular levels of ascorbate + GSH, which acted synergistically to release NO. By contrast, the nitrooxy ester, NCX4016, failed to generate detectable NO in samples containing either platelets or plasma.

Both the nitrooxy ester, NCX4016, and the furoxan–aspirin hybrids were demonstrated to significantly inhibit COX-1. The alterations to the chemical structure in the hybrids perhaps play a role in bringing about the enhanced inhibition that was observed compared to the unaltered aspirin compound. The powerful inhibitory effect of the furoxan–aspirin hybrids in the purified COX-1 assay, together with the equally powerful effects of the furazan equivalents and the lack of significant inhibition observed with the NO donor DEA/NO, suggests that the COX-1-inhibitory activity of B8 and B7 is more likely to be aspirin-mediated than NO-mediated. This assay demonstrates that, in addition to their ability to inhibit platelet aggregation *via* NO, the furoxan–aspirin hybrids retain an aspirin-like action *in vitro*. At face value, these data would indicate that any antiplatelet effects of the furoxan–aspirin hybrids should be at least partially mediated by aspirin-mediated inhibition of COX-1. However, it is important to recognize that this effect might be lost in biological media or *in vivo*, depending on how these compounds are hydrolysed under physiologically relevant conditions. Previous studies (Cena *et al.*, 2003) have demonstrated complete hydrolysis of the furoxan–aspirin acetyl group in serum. This feature would suggest that in plasma, there is at least a partial loss of the acetyl group, resulting in formation of the salicylic acid equivalent. As salicylic acid was demonstrated to have no effect in this assay of COX activity and failed to affect platelet aggregation, modifications to the chemical structure to improve retention of the acetyl group in plasma is likely to be necessary to avoid loss of the COX-inhibiting effects of the compounds. Other groups have also demonstrated that COX inhibition by the hybrid NCX4016 has a similar requirement for the acetyl group but not the NO moiety (Corazzi *et al.*, 2005).

The present study extends findings from preliminary aggregation studies which have been published previously (Cena *et al.*, 2003). Here, we further investigated the relative contribution of the NO- and aspirin moieties to the antiplatelet effect. The furoxan–aspirin hybrid drugs inhibited platelet aggregation in both WP and PRP, although the effects of both compounds, and B7 in particular, were attenuated in PRP. The comparatively weak inhibitory effects of the NO-free furazan counterparts is indicative of a major role for NO in platelet inhibition, while the inhibitory effect of ODQ confirmed that a major component of the effects is sGC dependent, especially in PRP. The impact of ODQ on responses was less pronounced in WP, particularly in the case of B7, due in part to the greater influence of NO-independent effects, as illustrated by increased sensitivity of WP to the furazan counterparts of B7 and B8. The effects of the furazan derivatives were weak compared to aspirin, but were nevertheless more potent than salicylic acid under the conditions of these experiments. The lower activity of these agents in PRP compared to WP is in keeping with the complete hydrolysis of the furoxan–aspirin acetyl group in serum (Cena *et al.*, 2003), resulting in formation of relatively ineffectual salicylic acid. These hybrid molecules are likely to undergo hydrolysis at two positions: firstly, at the acetyl group, converting aspirin to salicylic acid and secondly at the other ester linkage to release the furoxan. The order and rates at which hydrolysis occurs will greatly influence the ability of these compounds to retain aspirin activity.

The high levels of NO release by B8 and the loss of its aspirin effect in PRP imply that its antiplatelet actions are mainly NO mediated. By comparison, B7, which releases NO more slowly, has an IC_{50} closer to that of aspirin. These characteristics are more suitable for a NO–aspirin hybrid drug because they provide potential for a more balanced action between NO and aspirin antiplatelet effects. The unfortunate loss of the aspirin effect in PRP, however, is an issue that needs to be addressed by appropriate modification of the chemical structure.

The finding that NCX4016 is an effective inhibitor of platelet aggregation in WP is in keeping with previous *in vitro* studies using NCX4016 (Lechi *et al.*, 1996) and the related drug, NCX4125 (Minuz *et al.*, 1995; Wallace *et al.*, 1995). However, our results go on to show that the effect is lost in PRP, possibly due to sequestration by a plasma constituent such as albumin or due to breakdown in the plasma to the inactive salicylic acid. Interestingly, the inhibitory effect of NCX4016 was significantly enhanced compared to aspirin but was not affected by the sGC inhibitor, ODQ. While a cGMP-independent effect of NCX4016-derived NO cannot be ruled out, our inability to detect NO generation from this compound, coupled with the known inability of platelets to effect NO release from organic nitrates (Weber *et al.*, 1996), and the COX assay data demonstrating powerful anti-COX activity of NCX4016, suggests that the antiplatelet action of NCX4016 is most likely NO independent. Although our study shows NCX4016 to be a poor inhibitor of platelet function, its effects in other cell types may still make it a useful cardiovascular drug. Antiplatelet effects may still be retained *in vivo* through remote nitrooxy-ester activation in cells other than platelets (e.g. smooth muscle cells), although this would appear to be a rather inefficient method of NO delivery specifically to platelets. Nevertheless, antiplatelet effects of NCX4016 have been demonstrated *ex vivo*, in animals and humans (Wainwright *et al.*, 2002; Fiorucci *et al.*, 2003; 2004; Momi *et al.*, 2005). Furthermore, NCX4016 has beneficial effects *in vivo*, where it has been demonstrated to protect the vascular endothelium in diabetic rats (Pieper *et al.*, 2002), to reduce blood pressure in hypertensive rats (Muscara *et al.*, 2001), to prevent restenosis in hypercholesterolemic mice (Napoli *et al.*, 2001) and to reduce infarct size in a model of cardiac ischaemia in pigs (Wainwright *et al.*, 2002).

The furoxan drugs are very stable compounds that appear only to decompose to release significant NO when they encounter appropriate media. There was no release of NO from the furoxans in Tyrode's buffer, but NO generation was detected in PPP, WP and PRP. These observations suggest that some elements of plasma can stimulate decomposition of the furoxans and that the platelets themselves enhance the effect to a greater extent for B8 than B7.

The plasma components, albumin, glutathione and ascorbate, chosen for their reducing properties, were all found to have a limited capacity to stimulate furoxan decomposition to release NO. In the case of B7, these constituents could

individually stimulate moderate NO release, and the additive effect of all three could fully emulate NO release in PPP. The effect of these constituents on B8 were similar, if less dramatic, for the individual reducing agents, but here there was no additive effect when coincubated, suggesting that an as yet unidentified plasma constituent is responsible for a proportion of NO release from B8.

Reconstitution of Tyrode's buffer with intracellular levels of GSH and ascorbate demonstrated that both these reducing agents were mildly effective in stimulating release of NO from the compounds. Interestingly, however, they acted synergistically in coincubation experiments to massively increase NO generation. The separate experiments using platelet extracts established that there was a relationship between the concentration of platelet extract and amount of NO generated from the furoxans. The ability of platelet extracts to generate NO from both furoxans was found to be dependent on the presence of reduced thiol groups and, in keeping with our other NO release data and the platelet aggregation experiments, B8 generated considerably more NO in the 10 min incubation period than B7. Taken together, these results indicate that low-level decomposition of B7 in plasma can be explained by the additive effects of albumin, GSH and ascorbate but that there is also likely to be much greater NO release inside platelets mediated by the synergistic action of ascorbate and reduced intracellular thiols. B8 is more sensitive to the same agents, and its plasma decomposition is also affected by an as yet unidentified plasma component. Given the reactive nature of NO, the apparent preferential release inside target cells is a likely advantage for successful NO delivery over compounds that can only generate NO remotely in cell types other than platelets.

Previous studies have shown that both furoxan–aspirins (Cena *et al.*, 2003) and NCX4016 (Fiorucci *et al.*, 1999; 2003; Fiorucci & Del Soldato, 2003) have a preferential gastrotoxicity profile over aspirin, suggesting either a masking of the toxic aspirin effect or an active NO element in the gastrointestinal tract. However, the activity of both the NO and aspirin elements of the hybrids in platelets is questionable. NCX4016 has no antiplatelet action in PRP, and in WP it fails to demonstrate aspirin-independent effects, possibly due to sequestration by a plasma constituent. Unlike NCX4016, the furoxans demonstrate aspirin-independent effects on platelet aggregation in both PRP and WP *in vitro*, but their ability to retain the action of aspirin is compromised, particularly in PRP. The furoxans appear to display a more effective antiplatelet activity over NCX4016, but further molecular modifications are necessary in an effort to retain the aspirin actions, in addition to achieving the added benefits of NO.

In summary, furoxan–aspirin hybrids are stable antiplatelet compounds, the decomposition of which is catalysed by endogenous agents which may instil a potential for primarily intracellular delivery of NO, on account of the differential distribution of glutathione and ascorbate within cells compared to plasma and extracellular fluid. While the furoxan hybrid examples tested in these experiments carry a number of limitations, they also highlight the therapeutic potential of future exponents of this class of drugs.

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A novel electron paramagnetic resonance-based assay for prostaglandin H synthase-1 activity

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Abstract

Background: Prostaglandin H_2 synthase (PGHS) is the enzyme that catalyses the two-stage conversion of arachidonic acid to prostaglandin H_2 (PGH_2) prior to formation of prostanoids that are important in inflammation. PGHS isozymes (-1 and -2) are the target for nonsteroidal anti-inflammatory drugs (NSAIDs).

Given the rekindled interest in specific anti-inflammatory PGHS inhibitors with reduced unwanted side effects, it is of paramount importance that there are reliable and efficient techniques to test new inhibitors. Here, we describe a novel *in vitro* electron paramagnetic resonance (EPR)-based assay for measuring the activity of PGHS-1.

Methods: We validated a novel *in vitro* PGHS-1 activity assay based on the oxidation of spin-trap agent, 1-hydroxy-3-carboxy-pyrrolidine (CPH) to 3-carboxy-proxy (CP) under the action of the peroxidase element of PGHS-1. This quantifiable spin-adduct, CP, yields a characteristic 3-line electron paramagnetic (EPR) spectrum.

Results: The assay is simple, reproducible and facilitates rapid screening of inhibitors of PGHS-1. Aspirin (100 μM, 1 mM) caused significant inhibition of spin-adduct formation (72 ± 11 and 100 ± 16% inhibition of control respectively; $P < 0.05$). Indomethacin (100 μM) also abolished the signal (114 ± 10% inhibition of control; $P < 0.01$). SA and the PGHS-2-selective inhibitor, NS398, failed to significantly inhibit spin-adduct generation ($P > 0.05$).

Conclusion: We have demonstrated and validated a simple, reproducible, quick and specific assay for detecting PGHS-1 activity and inhibition. The EPR-based assay described represents a novel approach to measuring PGHS activity and provides a viable and competitive alternative to existing assays.

Background

Prostaglandins are derived from arachidonic acid (AA) in a pathway dependent on the PGHS (EC 1.14.99.1) family of enzymes, which are commonly known as cyclooxygenase (COX), referring to the first step of enzymatic activity. PGHS converts AA to prostaglandin H_2 (PGH_2), the precursor of all prostanoids. The enzyme contains two active sites: a COX site, where AA is converted into the hydroperoxy endoperoxide, prostaglandin G_2 (PGG_2), and a haem with peroxidase activity that reduces PGG_2 to PGH_2 (For review see [1]). The reduction of PGG_2 by the peroxidase element generates the corresponding alcohol. This reaction has previously been demonstrated to concurrently oxidise aminopyrine molecules to aminopyrine free radicals [2]. Here, a spin-trapping agent, 1-hydroxy-3-carboxy-pyrrolidine (CPH) is oxidised to 3-carboxy-proxy (CP), probably under the action of the peroxidase, in a similar fashion to that previously seen with aminopyrine (Fig. 1).

Two structurally similar PGHS isoforms exist (PGHS-1 and PGHS-2) which are encoded by different genes and the expression of which varies between tissues [3]. PGHS-1 is often referred to as the 'house-keeping' isoform due to its regulatory functions in many tissues. PGHS-2 is virtually undetectable under normal conditions in most tissues and is often referred to as the 'inducible' isoform due to its tendency to be expressed in response to inflammatory stimuli. The exception to this is in the brain and spinal cord, where PGHS-2 is constitutively expressed and plays a role in nociception signaling [4].

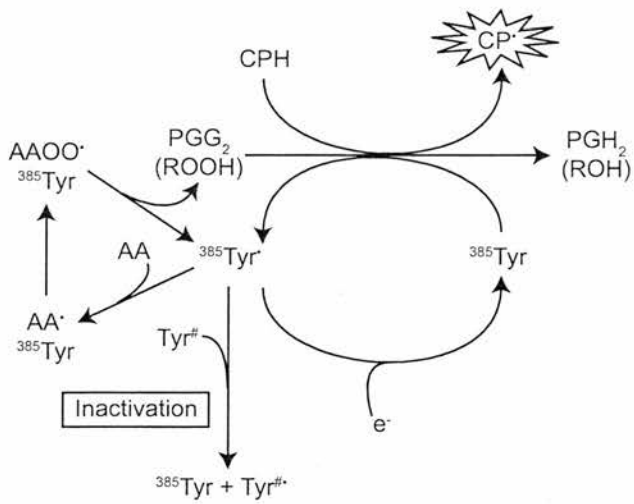

Figure 1
Schematic diagram showing the peroxidase activity of PGHS. The process requires prior formation of a tyrosine radical from a tyrosine residue in close proximity to the haem group (Tyr 385). The tyrosyl radical is either recycled or participates in the suicide inactivation of the enzyme (for review of this process see [39]. Following incorporation of oxygen and formation of PGG_2, the peroxidase reduces the peroxyl moiety to the equivalent alcohol. The process allows for the concomitant oxidation of spin-trap CPH to CP which is detected by EPR.

The importance of PGHS as a therapeutic target has long been highlighted by the actions of aspirin, [5,6] the first drug of the family of nonsteroidal anti-inflammatory drugs (NSAIDs) for use as analgesics, anti-inflammatory agents and antithrombotic agents. In contrast to other NSAIDs, such as indomethacin, which reversibly bind at the COX active site [7], aspirin causes an irreversible inhibition of PGHS by rapidly and selectively acetylating the hydroxyl group of a serine residue (Ser 530) near the C-terminus of the enzyme, forming an impediment to the binding of AA [8-10]. The ensuing irreversible PGHS inhibition requires *de novo* synthesis of the enzyme for subsequent production of prostaglandins.

Interest in PGHS has been re-ignited recently on account of two advances in the development of novel NSAIDs. Firstly, nitroaspirins [11-14] are being developed in an effort to overcome the gastrotoxic side-effects of aspirin that represent the major limitation to its therapeutic use [15-17]. Nitroaspirins make use of the protective effects of nitric oxide (NO) to compensate for the potentially damaging impact of aspirin-mediated depletion of protective prostaglandins in the gastric mucosa. Secondly, the suggestion that the gastrotoxic side effects of aspirin are due to the inhibition of housekeeping PGHS-1, whereas its anti-inflammatory effects are due to inhibition of PGHS-2, led to the development of selective inhibitors of the COX-2 activity of PGHS-2, in the hope that the beneficial effects could be retained without injury to the gastric mucosa [18]. However, recently, several of these new selective PGHS-2 inhibitors have been withdrawn due to mounting evidence of an increased risk of stroke. The increased risk of thrombus is thought to be due to inhibition of PGHS-2 in endothelial cells leading to down regulation of anti-thrombotic prostaglandins (such as prostacyclin) in relation to the unaffected PGHS-1 derived thromboxanes [19-21].

Given the intense interest in PGHS activity and inhibition, it is perhaps surprising that relatively few assays for the activity of this enzyme have been developed. Amongst the most popular techniques is the measurement of thromboxane B_2 (TXB_2), the stable metabolite of PGH_2-derived TXA_2, as a marker of PGHS activity [22]. Alternatively, measurement of oxygen consumption using an oxygen electrode [23,24], assays using radio-labelled substrate [25] and immunoassays for the prostaglandin products [23] can also be applied. More recently, a commercial chemiluminescent assay (Axxora, Nottingham, UK) has

been developed for use on purified enzyme. The assay involves the use of labelled substrate that generates a luminescent product under the action of the hydroperoxidase element of PGHS, most likely via the generation of oxidising free radicals.

Electron paramagnetic resonance (EPR) has been previously utilized to measure free radicals generated by PGHS as a measure of its activity [26,27]. However these techniques can involve complex mechanisms to trap the short-lived radical species. For instance in an *in vitro* assay using purified PGHS, liquid nitrogen was utilised to stop the reaction and was required to stabilise the tyrosyl radical species generated, in order that it could be recorded by EPR [26]. Another technique which recorded PGHS activity in mouse keratinocytes relied on the use of the antioxidant glutathione to stabilize the generated radical before trapping it with DMPO [27].

Here, we present a novel method for assaying the activity of PGHS-1 in which EPR is used to detect the stable adduct CP, formed from oxidation of commonly available spin-trap, CPH [28], under the action of the peroxidase element of PGHS-1. The aim of the studies described was to validate this technique for assaying PGHS-1 activity *in vitro*, to determine its effectiveness at establishing the inhibitory effects of conventional NSAIDs and to confirm that a COX-2-selective inhibitor was ineffective in this assay.

Methods

Unless otherwise stated, all drugs and chemicals were purchased from Sigma, Dorset, UK. The assay was performed at 37°C in 1 ml of Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 1.05 mM $MgSO_4$, 0.4 mM NaH_2PO_4, 12.5 mM $NaHCO_3$, 5.6 mM Glucose, 10 mM HEPES and 0.8 mM $CaCl_2$ in deionised water at pH 7.4. 100 units/ml ovine seminal PGHS-1 was incubated with 1.5 μM haematin (5 min, 37°C) prior to the assay. Data from the manufacturers of the PGHS-1 reveals that 1 unit of enzyme consumes 1 nanomole of oxygen at 37°C in the presence of 1 μM haematin and 100 μM AA. Aspirin, salicylic acid, indomethacin, NS398 (Merck Biosciences, Nottingham, UK) or vehicle control (DMSO, 1%) was added and left to incubate for a further 10 min prior to addition of the spin-trap, CPH (1 mM; Axxora). At this point (t = -2 min), a baseline EPR measurement was taken (MS200, Magnettech, Germany. Instrument settings: B0-field, 3356 gauss; sweep width; 50 Gauss, sweep time, 30 sec; modulation amplitude, 1500 mGauss; microwave power, 20 mW). 2 min later, 100 μM AA (as sodium salt) was added (t = 0). Further EPR readings were taken at t = 1.5 (the earliest timepoint at which readings could consistently be taken after addition of AA), 4 and 6 min. The suicidal nature of PGHS-1 activation means that the period of activation is anticipated to be complete within ~1 min of AA addition [3].

The results are corrected for any auto-oxidation of spin-trap by subtraction of values recorded from a duplicate sample run in the absence of AA. The intensity scale on the y-axis of all graphs is an arbitrary scale based upon the area under the curve of the first derivative traces generated.

Statistical analyses

All statistical tests were performed using GraphPad Prism version 4. $P < 0.05$ was considered to be statistically significant. Tests performed were either 1-way ANOVA with Dunnett's post-test or 2-way ANOVA, as indicated in the text.

Results

Time-dependent adduct generation by PGHS-1

Addition of AA caused a time-dependent increase in the characteristic 3-line EPR spectrum for a spin-adduct with the unpaired electron in the vicinity of a nitrogen atom (Fig 2a). The majority of the reaction was complete by the time the first reading was taken (t = 1.5 min). The signal developed rapidly before the first reading (244 intensity units.min^{-1}), but subsequently slowed to a relatively constant rate (65 intensity units.min^{-1}) over the following 4.5 min of the assay (Fig 2b, n = 9–12); the equivalent experiment without AA failed to show the initial rapid rise and was significantly lower than the AA-treated sample throughout ($p = 0.02$, 2-way ANOVA, repeated measures). An inter-sample coefficient of variation of 0.18 (18%) was calculated from the control data obtained. From these data, it was determined that t = 1.5 min was an appropriate point at which to compare free radical generation between control and NSAID-treated PGHS-1, given that spin-adduct generation in response to AA had peaked – subsequent adduct formation was at an equivalent rate in control and AA-treated samples and was likely to be due to non-specific auto-oxidation of CPH.

Inhibitory effect of aspirin and salicylic acid

The impact of pre-incubation of PGHS-1 with different concentrations of aspirin that spanned the known therapeutic range (10 μM – 1 mM) is shown in Fig 3. Our results indicate that aspirin concentrations of 100 μM and 1 mM caused significant inhibition of spin-adduct formation at the 1.5 min time-point (to 72 ± 11 and 100 ± 16% of control respectively; $P < 0.05$ for both compared to control, 1-way ANOVA followed by Dunnet's post-hoc analysis).

Parallel experiments with salicylic acid (SA, 10 μM – 1 mM; n = 6) showed that a 10 min pre-incubation with SA failed to significantly inhibit generation of the spin-adduct, even at the highest concentration ($P > 0.05$).

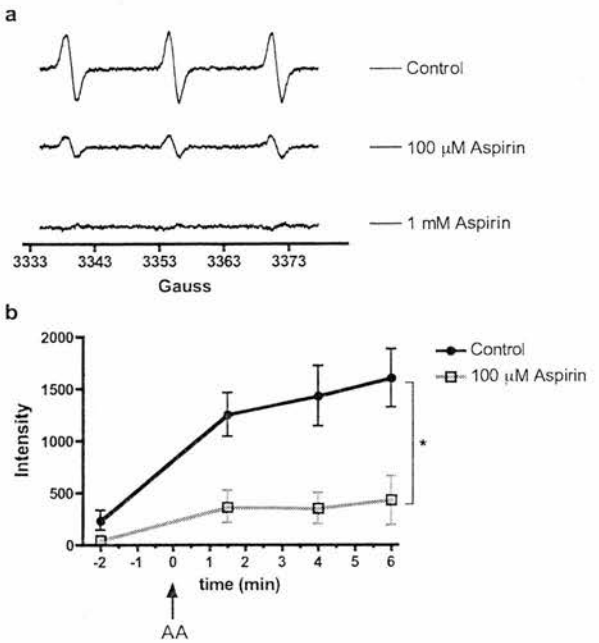

Figure 2
(a) Sample EPR spectra obtained in the absence (control; PGHS + AA) and presence of aspirin (100 μM or 1 mM) after correction for background autoxidation. EPR settings: B0-field, 3356 gauss; sweep width; 50 Gauss, sweep time, 30 sec; modulation amplitude, 1500 mGauss; microwave power, 20 mW. (b) Mean data for development of EPR signal intensity (AU) with time in the absence (control; PGHS + AA) and presence of aspirin (100 μM). In both cases, substrate (AA) was added at t = 0 min. P = 0.02, 2-way ANOVA, repeated measures: n = 9–12.

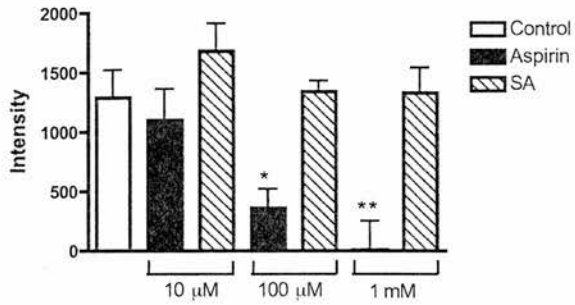

Figure 3
Effect of aspirin and salicylic acid (10 μM – 1 mM) on EPR signals generated from PGHS-1 after treatment with substrate (AA). In each case, incubations with aspirin or SA were for 10 min prior to the baseline EPR reading (t = -2 min, not shown). AA was added at t = 0 min and readings shown were taken at t = 1.5 min. *P < 0.05, **P < 0.01; 1-way ANOVA with Dunnett's post-hoc test vs. control: n = 8–10.

Comparative pharmacology of PGHS-1 and PGHS-2 inhibitors

Equivalent concentrations (100 μM) of the recognized non-selective PGHS inhibitors, aspirin and indomethacin both significantly (P < 0.05 and P < 0.01 respectively) inhibited generation of the EPR-detectable spin adduct at 1.5 min (72 ± 11 and 114 ± 10% inhibition of control response respectively), but the PGHS-2-selective inhibitor, NS398 had no effect (P > 0.05) on this assay of PGHS-1, despite its use at a concentration which is in excess of that required to significantly inhibit PGHS-2 [29] (Fig 4).

Discussion

Here we have validated a new, quick, simple and reproducible method for detecting PGHS-1 activity and inhibition *in vitro*. The principle of the assay was based on the concomitant oxidation which occurs with the reduction of PGG_2 by the peroxidase element of PGHS [2]. This oxidation can be exploited in the absence of antioxidant glutathione to oxidise spin-trapping agent CPH to the stable adduct, CP which generates a characteristic 3-line EPR spectrum. The amplitude of the EPR signal is proportional to the amount of adduct generated.

Our results indicated that oxidation of CPH by the isolated PGHS-1 enzyme upon addition of the enzyme substrate, AA, was sufficient to be easily detectable by EPR. The initial peak in the detected signal recorded at the first reading, had by the subsequent time-points slowed to a rate that was equivalent to the signal generation from substrate-free enzyme, most likely due to autoxidation of the spin trap. The loss of specific enzyme-mediated radical generation is unsurprising, given the well-recognised suicidal nature of activated PGHS-1 [3]. From these time-course experiments, we selected a 1.5 min time-point for subsequent comparative studies because by this time the

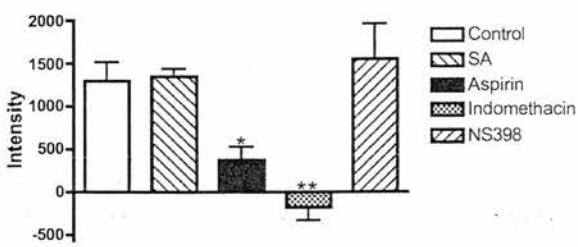

Figure 4
Comparative effects of SA and recognized NSAIDs (all 100 μM) on EPR signal intensity measured at t = 1.5 min. *P < 0.05, **P < 0.01; 1-way ANOVA with Dunnett's post-hoc test vs. control: n = 6–10.

AA-dependent free radical generation was complete but the signal was not significantly enhanced by autoxidation of the spin trap.

Aspirin pretreatment (10 min) was shown to have a concentration-dependent inhibitory effect in the published range [30], but the acetyl-free counterpart, SA, failed to significantly inhibit enzyme activity. The lack of effect of SA indicates that aspirin-mediated inhibition of the enzyme is dependent on the acetyl group, the moiety involved in PGHS inhibition, and not due to a non-specific antioxidant effect. SA was not expected to have any effect in this assay because it is known not to affect PGHS-1 or 2 activity [31] except in intact cells [30], possibly by suppressing PGHS-2 transcription in response to exogenous stimuli [32]. Furthermore, the reversible, non-specific PGHS inhibitor, indomethacin [33] was demonstrated to have a powerful inhibitory effect, whilst the PGHS-2 specific inhibitor, NS398 at a concentration known to inhibit PGHS-2 [29] failed to do so. Signal intensities shown in the figures are after subtraction of an AA-free control from a parallel experiment to account for autooxidation of the spin trap. A negative signal (as observed with indomethacin for example) therefore indicates that the control value was greater than the AA-treated sample, which might suggest that AA has a slight antioxidant effect on its own.

These results confirm that the assay is relevant for NSAIDs with different modes of inhibitory action. As purified PGHS-2 is now available commercially (Sigma), it may be possible to adapt this assay system to help determine the specificity of novel PGHS-2 inhibitors.

This assay provides a convenient screening method for inhibitors of the PGHS enzyme. Whilst various techniques exist to assay the activity of PGHS isoforms, each has its own disadvantages. For instance, recording the oxygen uptake by PGHS is an option to measure its activity but this requires high enzyme concentrations and also accurate control of the initial oxygen concentrations [24]. Optical techniques are prone to interference from coloured assay constituents (such as haematin) and require high AA concentrations. Techniques recording uptake of radiolabelled substrate [25] can be complex and expensive. Our technique provides quick inexpensive results that give real-time determination of COX-inhibition. Data obtained, as demonstrated by percentage inhibition of control response achieved with aspirin, is comparable to other *in vitro* techniques [34-36].

The technique described in the present study also offers a simpler alternative than previous EPR techniques where generated radicals are detected following complex radical stabilization steps [26,27] and thereby providing potential for less loss of signal. Tsai *et al.* [26] used dry ice and liquid nitrogen to stop the reaction and trap the radical. The recording of spectra was then carried out under liquid nitrogen. By comparison, our method uses direct oxidation of a spin-trap to generate an adduct that is sufficiently stable to allow successive time-point readings to be taken without the need to freeze the sample at the required time-point. The method by Schreiber *et al.* [27] did use a spin-trap, but required the use of glutathione to reduce an amine radical to the parent amine, liberating a thiyl radical which was trapped by DMPO. In our method, the spin-trap is oxidised directly, thus reducing the potential for loss of signal. Furthermore, there is some evidence of rapid signal decay in the Schreiber method; the spectra show a decrease of the DMPO-thiyl signal during the course of the measurement, whereby the spectral lines no longer conform to the expected 1:2:2:1 ratio. By comparison, the EPR signal generated in the present technique is much more resilient and does not decay during the time-course of experiments.

It is important to recognize the potential limitations of this assay. Its reliance on the oxidation of CPH might preclude its use in cells, given that the cellular environment is usually very rich in antioxidants such as GSH, and its presence at intracellular concentrations (~5 mM) could effectively compete out the spin-trap and nullify the assay. The susceptibility of polyunsaturated fatty acids such as AA to peroxidative attack by reactive oxygen species may impact on the recorded level of radical if radicals are consumed by free AA in the samples [37,38]. Furthermore, it is important to recognize that the *in vitro* nature of the assay does not account for any absorption, metabolic or availability issues that might relate to applied NSAIDs.

Conclusion

In summary, we have validated a new, simple, EPR-based assay for detecting PGHS-1 activity and inhibition. We have demonstrated it to be sensitive to the inhibitory effects of conventional NSAIDs (aspirin and indomethacin) and not to SA or a PGHS-2 specific inhibitor. In principle, this assay should be equally applicable to measuring PGHS-2 activity in isolated enzyme. As such, this assay might prove to be a useful research tool in the ongoing search for novel PGHS inhibitors of both isoforms of the enzyme.

Abbreviations

AA, arachidonic acid; **COX**, cyclooxygenase; **CPH**, 1-hydroxy-3-carboxy-pyrrolidine; **EPR**, electron paramagnetic resonance; **NO**, nitric oxide; **NSAID**, nonsteroidal anti-inflammatory drugs; **PGG_2**, prostaglandin G_2; **PGH_2**, prostaglandin H_2; **SA**, salicylic acid; **TXB_2**, thromboxane B_2.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

Experimental design and procedures were performed by CMT and assisted by DM. AGR and ILM supervised experimental design and procedures. AGR and ILM oversaw manuscript construction, revising it critically for important intellectual content. All authors have given final approval of the version to be published.

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Therapeutic effects of nitric oxide-aspirin hybrid drugs

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This review examines the therapeutic potential and mechanisms of action of drugs known as nitric oxide (NO)-aspirins. Drugs of this class have an NO-releasing moiety joined by ester linkage to the aspirin molecule. NO-aspirins have the capability to release NO in addition to retaining the cyclooxygenase-inhibitory action of aspirin. The protective nature of NO led to the development of NO-aspirins in the hope that they might avoid the gastric side effects associated with aspirin. However, it has become apparent that the drug-derived NO instils potential for a wide range of added beneficial effects over the parent compound. In this review, the authors focus on the analgesic, anti-inflammatory, cardiovascular and chemopreventative actions of compounds of this emerging drug class.

Keywords: analgesia, anti-inflammatory, aspirin, cancer, cardiovascular, gastrotoxicity, nitric oxide, NO-aspirin, NSAID, platelet

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1. Introduction

1.1 Nitric oxide

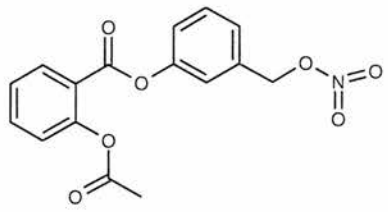
Nitric oxide (NO) is an ubiquitous signalling messenger molecule involved in diverse physiological and pathophysiological processes, including vasodilatation, inhibition of platelet activation and inflammatory cell adhesion [1-6]. Furthermore, NO functions as a neurotransmitter at non-adrenergic, non-cholinergic neurons [7].

The enzyme soluble guanylate cyclase (sGC) is the primary target for NO. NO binds sGC with high affinity, prompting it to undergo a conformational change, resulting in a 400-fold increase in the rate of enzyme catalytic activity [8]. Activated sGC converts guanosine-5′-triphosphate (GTP) to 3′, 5′-cyclic guanosine monophoshate (cGMP) [9-11]. There are a number of cGMP-dependent effector pathways that involve cGMP-dependent protein kinases, cGMP-regulated phosphodiesterases and cGMP-gated ion channels. Low levels of NO acting primarily via these pathways are responsible for several protective physiological processes including vasodilation, antithrombotic effects and anti-inflammatory actions.

1.2 Aspirin

Aspirin (acetylsalicylic acid) was first synthesised in 1899 and was the first example of the family of NSAIDs. Its therapeutic uses include the treatment of headache, rheumatic pain and inflammation; low-dose aspirin is also an effective prophylactic against thrombotic events in the cardiovascular system.

Aspirin causes irreversible inhibition of the cyclooxygenase (COX) family of enzymes by selectively and rapidly acetylating a serine residue (Ser 530) near the C-terminus of the protein, forming an impediment to the binding of AA [12-14]. The acetylation evokes a requirement for new COX to be synthesised for subsequent production of prostaglandins, which are derived from arachidonic acid in a COX-mediated process. There are two structurally similar COX isoforms named COX-1 and COX-2, which are encoded by different genes. The expression of the COX isozymes varies between tissues and aspirin is more selective for COX-1 than COX-2. Other



NCX4016

Figure 1. Structural formula of NO-aspirin, NCX4016.

NSAIDs, such as naproxen and ibuprofen, reversibly bind at the active site of COX and have different selectivity profiles to aspirin for the different COX isoforms [15].

1.3 Why make an NO-aspirin?

The long-term use of aspirin for pain and inflammation associated with conditions such as rheumatism and arthritis is limited due to its serious side effects in the gastrointestinal tract, which are reported to cause 16,000 deaths each year in the US [16]. The first evidence for gastric damage caused by aspirin was presented in 1938 [17] and there is now clear evidence for an association between gastric and duodenal ulcers and NSAID use [18-22].

NSAIDs cause gastric mucosal injury through their topical irritant effect and, more importantly, through suppression of gastric prostaglandin synthesis [23,24]. Many components of mucosal defense rely on prostaglandins, including mucus and bicarbonate secretion, blood flow [25,26], epithelial cell turnover and repair, as well as mucosal immunocyte function. It is believed, therefore, that inhibition of prostaglandin synthesis by aspirin depresses these defences, providing an opportunity for further damage by endogenous agents, such as acid, pepsin and bile salts [22].

1.4 Overcoming gastrotoxicity

NO-aspirins are hybrid drugs that have been designed to combine the cytoprotective effects of NO with aspirin, with a view to their use as 'gastric-friendly' NSAIDs [27,28].

NO has various effects on the gastric mucosa which might contribute to enhanced mucosal defense. It increases blood flow in the gastric mucosa, promoting repair and removal of toxins [29]. It also increases secretion of protective gastric mucus [30] and is thought to promote healing of gastric ulcers by promoting angiogenesis [31]. NO from NO-aspirins can inhibit neutrophil adhesion to the blood vessel wall in a similar fashion to prostacyclin (PGI_2) [32]. It is, therefore, plausible that NO could replace aspirin-inhibited PGI_2 as an inhibitor of neutrophil adherence and also of mucosal vasodilatation [16]. Wallace *et al.* documented the role of neutrophils in gastropathy [33]. They reported neutrophil adherence to the vascular endothelium of the gastric microcirculation as one of the earliest events following NSAID administration to laboratory animals [34]. Furthermore, they showed, with the use of antibodies, that by preventing neutrophil adherence, mucosal injury was avoided [35]. As NO is known to prevent neutrophil adhesion, the idea of combining an NO group with an NSAID was suggested to prevent gastropathy [33].

Given that some of the gastrotoxic effects of aspirin have been suggested to be due to actions of the carboxylic acid moiety [36], it would be expected that esterification at this group might reduce such an outcome. NO-aspirin hybrids have been shown to have reduced acute gastric toxicity, thought to be primarily via prevention of the carboxylic acid effect, rather than a direct protective effect of NO, although the latter may contribute [27]. It is worth noting, however, that under the acid conditions of the stomach, aspirin will exist primarily in the non-ionised form and would not, therefore, be expected to significantly affect the pH through ionisation at the carboxylic acid.

1.5 NO-aspirins

Two main subtypes of NO-aspirins have so far been developed: the nitro-oxy ester derivatives and the furoxan derivatives.

The first NO-aspirin hybrid drugs to be released were the NicOx compounds, NCX4016 (2 acetoxy-benzoate 2-(2-nitroxymethyl)-phenyl ester; **Figure 1**) and the related, NCX4215 [37]. Both are nitro-oxy ester (organic nitrate) derivatives of aspirin, often referred to as nitroaspirins.

NCX4016 consists of an aspirin molecule linked by an ester bond to a molecular spacer, which, in turn, is linked to a nitro-oxy ester group [38]. NCX4016 has been demonstrated not to be ulcerogenic at equivalent concentrations to those of aspirin that are capable of inducing ulcers. Furthermore, it causes a dose-dependent protection against gastric lesions induced by hydrochloric acid/ethanol mix in rats [39].

More recently, another series of NO-aspirin hybrid drugs have been developed with a furoxan NO-donor moiety [27,40]. These drugs link the NO-donating furoxan group by ester linkage to the aspirin molecule (**Figure 2**). These compounds also appear to overcome the problem of gastric lesions [27], although the precise mechanism and the possible role of NO has not yet been fully determined.

The NO release mechanism of the different NO-aspirin drugs varies. The ester linkage of NCX4016 requires enzymatic cleavage, as has been identified for other organic nitrates, such as glyceryl trinitrate [41]. In contrast, the furoxan derivatives appear to release NO intracellularly on encountering antioxidant species glutathione and ascorbate [40].

In addition to their intended use as gastric-sparing NSAIDs, NO-aspirins have been demonstrated to bring about various therapeutic effects, which are explored throughout this review.

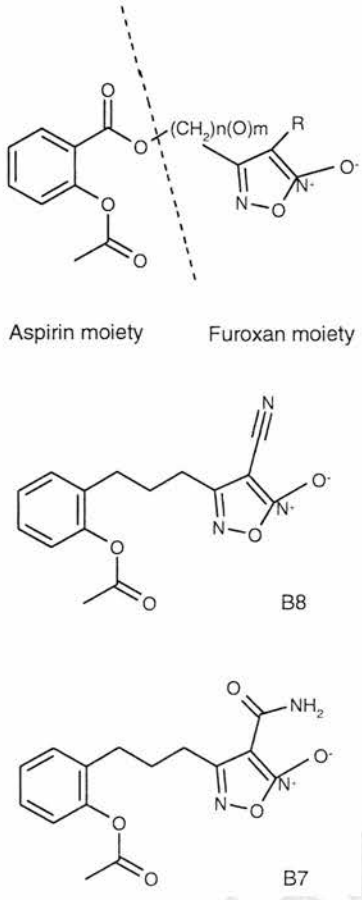


Figure 2. General structural formula of a furoxan–aspirin hybrid drug and structural formulae of two typical examples (B8 and B7).

2. Anti-inflammatory and analgesic effects

NO-aspirins have the potential to be anti-inflammatory through the actions of both the NO-moiety and the parent compound. The anti-inflammatory effects of aspirin are well covered in the literature (see [42-47] for reviews). Both major types of NO-aspirin have been shown to retain an inhibitory action on COX-1 *in vitro* [40,48] and, thus, have the potential to retain the anti-inflammatory, analgesic and antithrombotic actions of the parent compound, aspirin.

The analgesic effect of aspirin is brought about through suppression of prostaglandin E2 and I2 production via inhibition of COX. These prostaglandins, whether synthesised during inflammation or in the spinal cord, play a role in firing of nociceptors (for review see [49]). NO-aspirins have the potential to be analgesic through COX-mediated inhibition of prostaglandin production, but the impact of the NO moiety is more difficult to predict due to the impact of NO on pain pathways apparently varying with the type, location and cause of pain [50-52]. A study into the antinociceptive properties of NCX4016 revealed that in models of inflammatory pain, such as carrageenan-induced hindpaw hyperalgesia and acetic acid-induced abdominal constrictions, as well as acute pain (tail flick), NCX4016 demonstrated a very similar antinociceptive profile to aspirin itself [53]. Both furoxan–aspirin hybrids and NCX4016 cause similar anti-inflammatory effects to aspirin in the carrageenan model of inflammation [27,53,54].

NO derived from NCX4016 inhibits the action of caspase 1 (also known as IL-1β converting enzyme) in lipopolysaccharide (LPS)-stimulated monocytes *in vitro* [55]. Caspases are a family of proteases that are involved in cytokine release and apoptosis [56]. Caspase 1 is upregulated in inflammatory diseases and is responsible for the generation of IL-1β and IL-18, which themselves are responsible for stimulating formation of further pro-inflammatory cytokines such as TNF-α and IL-8. The anti-inflammatory effects of NCX4016 are due to inhibition of the formation of these pro-inflammatory cytokines through *S*-nitrosylation of a cysteine residue in caspase 1 [57]. A similar effect on caspase 1 has also been suggested to be responsible for the gastric-sparing property of NCX4016. Inhibition of gastric caspase activity in rats was determined to occur through both cGMP-dependent and -independent pathways [58]. Furthermore, NO-aspirins have also been reported to inhibit the release of inflammatory cytokines, such as TNF from LPS-stimulated macrophages [59,60]. This anti-TNF-α action by the NO-aspirins may provide clinical benefit in inflammatory diseases such as arthritis, Crohn's disease and asthma [61-64].

Rheumatoid arthritis is a chronic inflammatory autoimmune disorder characterised by inflammation of the lining (synovium) of joints. Joint deterioration, together with the pain associated with synovial inflammation can lead to substantial loss of mobility. Pro-inflammatory cytokines are abundant in the joints of sufferers [64]. Anti-TNF-α drugs have been recently licensed for use in arthritis to limit the contribution of the cytokine to inflammation [62]. Treatment of arthritis with drugs of the NSAID class is severely limited due to the gastric side effects associated with the high doses and chronic nature of the treatment required. However, it is hoped that NO-aspirins might offer a preferable alternative to therapy with conventional NSAIDs; the dual action of the COX-mediated aspirin action and the anti-TNF-α response, together with their resistance to gastrotoxic effects mediated by NO, is a promising antiarthritic profile for this target.

It is now widely accepted that inflammation is a key element in atherogenesis and atherosclerotic plaque rupture that leads to acute cardiovascular events (myocardial infarction or stroke) [65]. Given its potential to prevent the gastrotoxicity associated with aspirin, NO-aspirin hybrids might be a useful means of chronic delivery of aspirin, not only to help prevent thrombosis associated with vascular disease, but also to limit progression of disease itself. The potential of NO-aspirins in cardiovascular disease is explored in the following section.

3. Cardiovascular effects

NO-aspirins have the potential to bring about cardiovascular benefit through both the aspirin and the NO moieties. There are extensive protective effects of NO in the vascular system to complement those of aspirin. NO is a powerful endogenous vasodilator [66], which acts to keep the vasculature in an active state of dilatation by stimulating cGMP-mediated relaxation of vascular smooth muscle cells. NO also opposes the adherence of monocytes to the vessel wall [67]. The reduction of endogenous NO synthesis or availability is heavily implicated as a key factor in the initiation and progression of atherogenesis [68-71]. Furthermore, NO displays antithrombotic actions through its ability to inhibit platelet adhesion [4,72] and aggregation [3,73,74].

3.1 Antiplatelet action

The antithrombotic action of aspirin comes about through its ability to inhibit the action of platelet COX-1-derived thomboxane A_2 (TxA_2). TxA_2 exerts its effect by action on G-protein-coupled TP receptors, which activate phospholipase C to bring about Ca^{2+}-mediated platelet shape change and aggregation [75].

The inhibition of platelet COX-1 by aspirin occurs through a selective and rapid acetylation as outlined above. The acetylation causes irreversible COX inhibition and a requirement for new COX to be synthesised for subsequent production of prostaglandins. As platelets are widely thought to lack the necessary cellular machinery to synthesise new proteins, the effect of aspirin will last for the lifetime of the platelet (~ 10 days). An overall recovery of COX activity by 10% per day has been observed, in line with platelet turnover [76]. Following aspirin-mediated COX inhibition, there is a cumulative reduction in platelet TxA_2 production [77], leading to a prolonged antithrombotic effect.

The NO element of the hybrids instils potential for a secondary antithrombotic effect. NO-mediated inhibition of platelet aggregation occurs primarily via stimulation of cGMP, although cGMP-independent signalling mechanisms have also been identified [71,78-81].

The mechanism of NO release can impact on the ability of the clinical effectiveness of the drug. For instance, NCX4016 is a nitro-oxy ester (organic nitrate) that relies upon enzymatic breakdown to yield NO [82]. The metabolism of both NCX4016, and the organic nitrate compound, glyceryl trinitrate, has been reported to occur through identical mechanisms [41]. Due to the poor capability of platelets to release NO from organic nitrates [83], an NO-mediated antiplatelet effect of NO is not detected *in vitro* [40]. This lack of ability of platelets to release NO from NCX4016 is demonstrated in its lack of antiplatelet effects in plasma [40]. Antiplatelet effects with organic nitrate hybrids have been demonstrated in washed platelets [37,84,85], but it is likely that these occur through the aspirin moiety [40]. Furthermore, antiplatelet effects of NCX4016 have been demonstrated *ex vivo*, in animals and humans [86-89], presumably through remote nitro-oxy ester activation in vascular cells other than platelets (e.g., smooth muscle cells); a rather inefficient method of NO delivery specifically to platelets.

In contrast to NCX4016, the NO release mechanism of furoxans is dependent on the presence of intracellular antioxidants glutathione and acorbate [40] and, therefore, furoxans release NO without the requirement for the same cellular machinery as that for organic nitrates. The reliance of furoxan–hybrids upon such endogenous agents to catalyse decomposition may instil potential for primarily intracellular delivery of NO, on account of the differential distribution of glutathione and ascorbate within cells compared with plasma and extracellular fluid. The antiplatelet effects of the furoxan compounds have been demonstrated *in vitro* and have been shown to display a degree of NO-mediated antiplatelet effects to complement those of the aspirin moiety [40].

3.2 Vasodilatory effects

In healthy blood vessels, NO is synthesised by endothelial NO synthase in response to shear stress, hypoxia and endogenous mediators [90-92], whereupon it acts directly up on the adjacent smooth muscle cells to bring about local vasodilatation. NO-induced vasorelaxation is almost exclusively cGMP-dependent; NO acts on soluble guanylate cyclase to increase cGMP, which results in a reduction of intracellular calcium in smooth muscle cells and thereby relaxation [1,2,92-95]. NO also has a range of effects on COX activity (see [96] for review). Although it has no impact on purified COX-1 and can suppress LPS-induced COX-2 expression [97,98], it has been demonstrated to stimulate PGI_2, production in endothelial cells possibly by a cGMP-independent mechanism [99]. Given that PGI_2 is a potent vasodilator, this may be another means by which NO causes vasodilation.

An interesting situation arises with regard to the role of the NO-aspirin moiety in the vasculature. The dual inhibition by aspirin in the COX-generation of the vasoconstricting agent, TXA_2 [100], and the vasodilatory substance, PGI_2 [101,102], raises uncertainties as to whether aspirin will cause vasodilatation or vasoconstriction. TXA_2 acts through specific TP receptors leading to phospholipase C activation, release of inositol triphosphate and an increase in the intracellular Ca^{2+} level, thus triggering smooth muscle contraction [103,104]. The source of vasoconstricting TXA_2 is platelet COX-1 [105] and, due to their anucleate nature, full TXA_2 recovery will only take place as a function of platelet turnover following aspirin inhibition [106]. In contrast, in the vasculature, PGI_2 is predominantly COX-2-derived and the main source is endothelial cells [107]. PGI_2 brings about endothelium-dependent vasodilatation through activation of IP receptors [108] and the consequent stimulation of adenylyl cyclase to generate cAMP [108,109]. Interestingly, studies with cultured vascular smooth muscle cells demonstrate that the vasodilator PGI_2 is produced only on stimulation by thrombin [110,111] or when in co-culture with platelets [112], suggesting that PGI_2 is

produced endogenously to overcome the vasoconstrictive effects of TXA_2. It was further shown that following treatment with aspirin, PGI_2 is restored within only a couple of hours [111]. The reason behind the recent withdrawal of the COX-2-specific inhibitors valdecoxib and rofecoxib [107,113] is their instigation of an inbalance between vascular PGI_2 and platelet TXA_2, ultimately resulting in an increased risk of stroke. This is most likely due to inhibition of COX-2 in endothelial cells and the resultant loss of the antithrombotic and vasodilator agent, PGI_2, whereas COX-1-derived TXA_2 remains unaffected [107]. It is generally accepted that *in vivo*, aspirin treatment will result in a vasodilatory effect due to the predominant inhibition of platelet-derived TXA_2 over that of endothelium-derived PGI_2. Due to platelets only possessing COX-1 [114] and aspirin being nearly 170-fold more selective for COX-1 than COX-2 [43], low-dose aspirin that is effective at inhibiting platelet COX-1-derived TXA_2 without affecting endothelial COX-2-derived PGI_2 [115,116] can be administered. These facts, combined with the ability of endothelial cells to restore PGI_2 production quickly [110,117], suggest that aspirin will bring about a vasodilatory action. It is worth noting, however, that some reports suggest that very high concentrations of PGI_2 can paradoxically act as a vasoconstrictor [118,119].

The vasorelaxant effects of the nitro-oxy ester, NCX4016, have been investigated *in vitro*. Unlike platelets, vascular smooth muscle cells are able to metabolise NCX4016 to release NO and thus this class of compound has the potential for NO-mediated vasodilatory effects. NCX4016 causes vasodilatation in noradrenaline-preconstricted rat tail arteries in a process that relies on cGMP and not the aspirin moiety [120]. The lack of platelets in the isolated preparation likely excludes a vasodilatory role for the aspirin moiety in such a system. Interestingly, NCX4016 does not have a significant impact on blood pressure at therapeutically relevant concentrations on account of the slow release of NO [121] avoiding rapid vasodilation [122]. The furoxan–aspirin hybrid drugs have also been demonstrated to cause NO-mediated, cGMP-dependent vasodilatation in isolated rat aortae (unpublished data).

3.3 Restenosis

Restenosis is the reocclusion through vascular remodelling of a blood vessel after a procedure such as angioplasty. Aspirin is commonly given following angioplasty to prevent thrombotic events [123], but it carries the risk of gastric ulceration outlined above. The lack of endothelium-derived NO has been implicated in restenosis [124] and administration of NO or its precursor, L-arginine, can help prevent its progression [125-128]. NCX4016, but not aspirin, reduced experimental restenosis in aged rats, an effect attributed to a reduction in vascular smooth muscle cell proliferation [129]. The use of NCX4016 was demonstrated to significantly reduce restenosis when compared with aspirin in hypercholesterolaemic mice [130]. The effect was also shown to be greater than that achieved with an NO-donor [130].

3.4 Myocardial ischaemia and infarction

NCX4016 has been shown to reduce damage to the myocardium in an NO-dependent manner following ischaemia–reperfusion in a rabbit model [131]. It was further shown to reduce infarct size caused by myocardial ischaemia–reperfusion in pig [89] and anesthetised rat models [132]. The beneficial effects have been attributed to the release of NO preventing inflammation, obstruction of the coronary microcirculation, arrhythmias and myocardial necrosis [132].

4. Cancer therapy

The use of aspirin is reported to reduce the risk of several cancers, including colorectal, oesophageal, breast, lung and bladder cancer (for review see [133-136]) via inhibition of prostaglandin synthesis [137,138]. Colon cancer has been associated with high COX-derived prostaglandin output, particularly PGE_2 [139], which enhance tumour growth [140]. It is thought that these prostaglandins are derived from COX-2 [137,141], giving rise to the perceived benefits of COX-2 inhibitors in cancer prevention [141-143].

However, the use of NO-aspirin compounds would ideally inhibit the cancer-promoting prostaglandins to the same extent as aspirin, but would avoid gastrotoxicity and the cardiovascular risk of the specific COX-2 inhibitors [107,142]. Indeed, in an *in vitro* model using colon cancer cell lines, an NO-aspirin that shares many of the molecular characteristics of NCX4016 was up to 250-fold more potent than aspirin at inhibiting the growth of cancer cells [144]. The NO-aspirin was also determined to be more potent than the parent compound in pancreatic, prostate, lung, skin, leukaemia and breast cancer cell lines, in some cases up to 6000-fold more effective [144]. The increase in potency over the parent compound is likely an effect of the structural change [145]. Interestingly, a further nitro-oxy ester hybrid, NCX 4060, has been shown to have benefit in human prostate cancer cell systems [146], an area where the effect of the parent compound is unclear [147,148].

In vivo, NCX4016 has been demonstrated to reduce cancer growth in a rat model of colonic adenocarcinoma to a greater extent than aspirin [149]. The beneficial effect of the NCX4016 appears not to be related to COX-inhibition [149] and, thus, may be due to as yet undetermined effects of the NO and not the aspirin moiety.

5. Type 2 diabetes

NiCox are soon to initiate Phase II trials on NCX4016 in Type 2 diabetes. The company claims that previous clinical studies with the drug show an increased sensitivity to insulin in patients with Type 2 diabetes [201]. Interestingly, it is intimated that the effect is due to release of salicylic acid with high dose treatment, perhaps acting synergistically with NO to increase insulin sensitivity. These data do not appear to have yet been published, although some of them were

presented at the American Heart Association meeting in 2005. This approach represents an interesting new angle in development of this compound in particular; here it is being used as a vector for delivery of high concentrations of salicylic acid rather than aspirin. The protective effects of NO facilitate delivery of sufficient salicylic acid to have a therapeutic effect without inducing gastric side effects.

6. Expert opinion and conclusion

It has become apparent that compounds of the so-called NO-aspirin drug class have beneficial effects that exceed their intended use as 'gastric-friendly' alternatives to aspirin. The drugs have been demonstrated to retain the COX-inhibitory effect of the aspirin moiety, thus providing analgesic, anti-inflammatory and antithrombotic effects on a par with the parent compound. The introduction of the NO moiety does not prevent these actions and provides at least the potential for a beneficial range of anti-inflammatory and cardiovascular effects to complement those of the aspirin moiety.

Despite being the most commonly used drugs over the past century, there have been few advances in measures to reduce the gastroenteropathy associated with NSAIDs (for review see [33]). The search for gastric-friendly NSAIDs has recently encountered a major setback with the withdrawal of some COX-2-specific inhibitors due to mounting evidence of increased risk of stroke [107]. Clearly, there is still a market for a safer alternative to aspirin and it is possible that NO-aspirin drugs may fulfil such a requirement.

NO-aspirins have been demonstrated to have potent antiplatelet, vasodilatory and anti-inflammatory actions contributed to by both moieties of the hybrid. It is hoped that such actions, along with their favourable gastric profile, will enable them to replace aspirin as a prophylactic against thrombotic events or for use as an anti-inflammatory agent in patients with arthritis. Furthermore, data obtained with NO-aspirins are leading to more speculative applications to those envisaged for them and it is likely yet more will arise as future studies are carried out. Such applications include anticancer therapy where the nitro-aspirin family has been demonstrated to have beneficial effects in vitro [144] and Type 2 diabetes, where NCX4016 is soon to enter Phase II trials. The latter is a particularly interesting divergence for this compound because, in this setting, it is believed to be acting as a precursor for salicylic acid rather than aspirin per se. Nevertheless, the protective properties of NO in the gastric mucosa remain important and the developments in this setting will be watched with interest.

A further possible application is in atherosclerosis and the associated thrombosis, although the complexity of the disease poses a number of challenges. NO-aspirins may provide some clinical benefit through their antiplatelet and anti-inflammatory actions, but as NO-related species have been linked to smooth muscle cell necrosis [150], it is difficult to predict the actions of the NO-aspirins in an established plaque. Future studies utilising the Apo-E model [151] could help to clarify whether or not the drugs will be of benefit in this setting.

The *in vitro* experimental data using drugs of this class should be viewed with a degree of caution. The results of some studies may not translate into clinical effects on the basis that the drug concentrations used are unrealistically high and if such doses were to be used *in vivo*, the high plasma concentrations may result in toxic effects such as liver and kidney damage, inhibition of *de novo* protein synthesis associated with high aspirin dose [152,153], or the mutagenic effects associated with high doses of NO [154].

Both families of NO-aspirin hybrid drugs discussed throughout this review have their drawbacks. NCX4016 has limited NO-mediated antiplatelet effects due to the organic nature of its NO moiety. Its antiplatelet effects *in vitro* are almost exclusively aspirin mediated and in plasma are non-existent, most likely due to instability of the acetyl group [40]. The fact that antiplatelet effects have been demonstrated *ex vivo* [58,86,88,89] suggests that NO can be released from the compounds *in vivo*, probably through remote activation of the organic nitrate group – an inefficient method of delivering NO if platelets constitute the primary target in this therapeutic setting.

A further drawback to NCX4016, and perhaps one more likely to limit its use, is the probability of tolerance with long-term or high-dose use; there is no evidence to suggest that NCX4016 will not be susceptible to the tolerance problems associated with long-term or high dosage use of traditional organic nitrates (for review on tolerance see [155]).

The stability of the acetyl group is a problem that appears to affect the aspirin-like action of both NCX4016 and the furoxan NO-aspirins in plasma. Further chemical modifications are necessary in order that an aspirin function is retained in plasma, although it is perhaps the instability of the aspirin moiety in plasma which has been turned to advantage in the use of NCX4016 in diabetes.

For a drug to be classed as a hybrid drug, it is reasonable to presume that both components of the drug contribute to its clinical effect. There are examples of furoxan derivatives (B8) where dominance of the NO moiety suggests that it acts almost exclusively as an NO-donor, rather than a true NO-aspirin hybrid. The furoxan NO-aspirin B7, has a better balance between the NO and aspirin moieties and, therefore, has greater potential as a hybrid drug [40]. It is likely that chemical modification may be necessary to drugs of the furoxan NO-aspirin family in order that both elements remain active, otherwise the co-administration of an NO-donor with aspirin may be a more suitable option. The nitro-oxy ester compound NCX4016 is also affected by 'balance' problems and thus does not necessarily act as a true hybrid. Its effect in platelets is limited to an aspirin-only mediated action due to lack of NO release [40], at least *in vitro*. The effects of the novel furoxan hybrids have not been as extensively examined as NCX4016 and further study is required before fair comparisons can be made. However, the

furoxan class appears to be a competitive alternative to NCX4016. The NO-release mechanism does not rely on enzymatic degradation and their specific release in the presence of cellular antioxidants instils potential for targeted cellular delivery of NO [40]. The furoxan NO moiety is not nitro-oxy ester and does not require the same enzyme-mediated mechanism to release NO, and so their long-term use would not be expected to be limited by tolerance, although this remains to be tested. Data obtained with NO-aspirins so far definitely warrant further research into this class of drugs. From a gastric safety perspective, the results seem promising and drugs of this class may offer an effective alternative to COX-2 inhibitors. However, further work is needed to perfect the art of hybrids in this arena, particularly with respect to balancing NO release with COX activity, stabilising the compounds in plasma to retain the acetyl group and looking for alternatives to nitro-oxy esters that would avoid tolerance issues for long-term and/or high-dose arthritis treatment.

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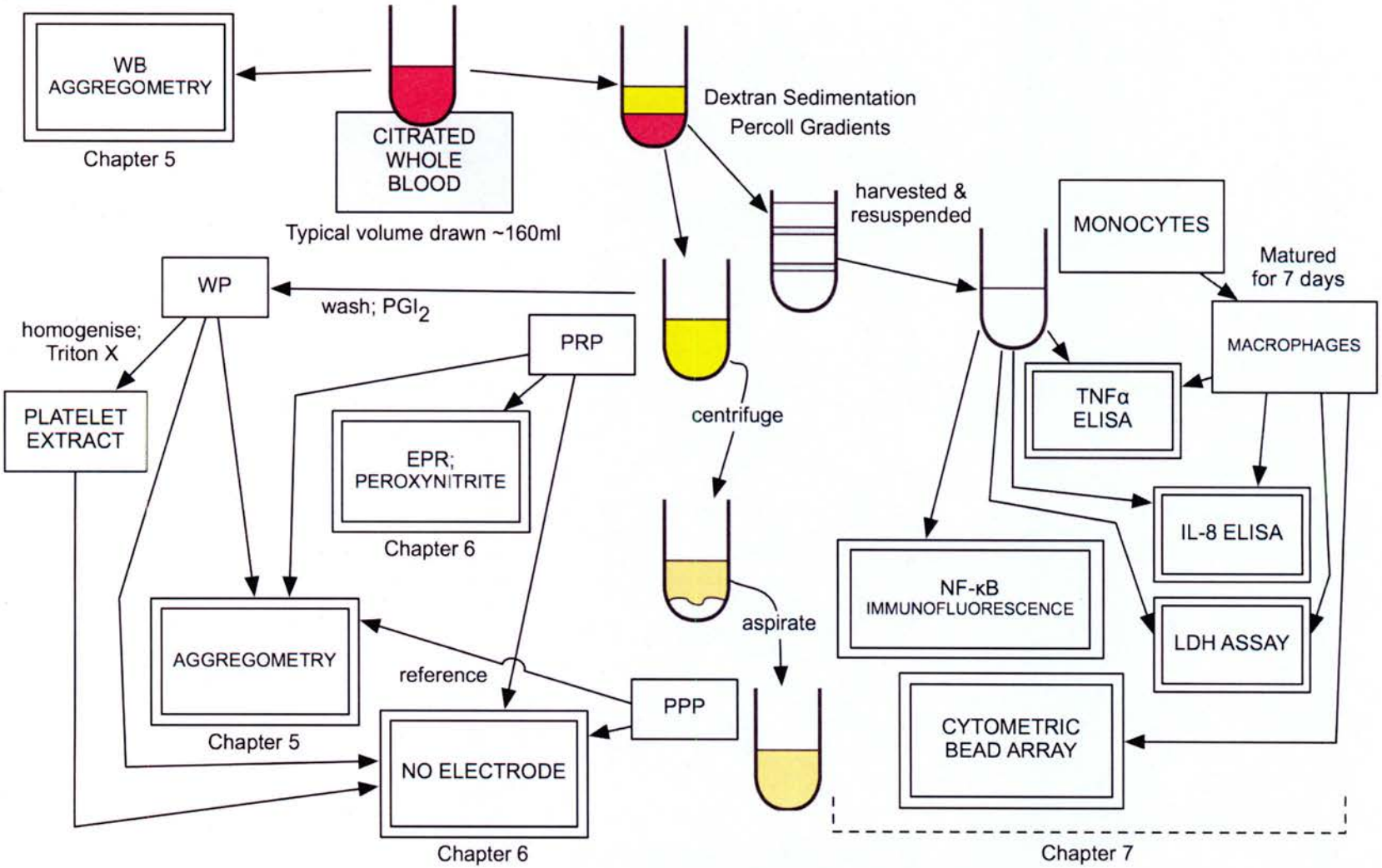
WB
AGGREGOMETRY
Chapter 5
CITRATED
WHOLE
BLOOD
Typical volume drawn ~160ml
Dextran Sedimentation
Percoll Gradients
harvested &
resuspended
MONOCYTES
Matured
for 7 days
MACROPHAGES
WP
wash; PGI_2
homogenise;
Triton X
PRP
PLATELET
EXTRACT
EPR;
PEROXYNITRITE
Chapter 6
centrifuge
TNFα
ELISA
IL-8 ELISA
NF-κB
IMMUNOFLUORESCENCE
LDH ASSAY
aspirate
AGGREGOMETRY
Chapter 5
reference
PPP
NO ELECTRODE
Chapter 6
CYTOMETRIC
BEAD ARRAY
Chapter 7