Cholangiocarcinoma cell lines: proteomic analysis and enhancing response to chemotherapy.

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Abstract

Cholangiocarcinoma (CCA) is a rare cancer with a poor prognosis. Much of medical research has focused on investigating cancers with a higher incidence and little focus has been devoted to this disease.

The aim of this thesis was to perform a protein analysis of CCA and cholangiocyte cell lines. Differences between immortalised cancer and normal cells were sought in order to identify potential therapeutic targets and/or diagnostic tools.

A variety of CCA cell lines were used, reflecting both intra and extrahepatic disease. The different subtypes of CCA through the developed and developing world are also represented so differences were also sought between them. Proteomic analysis was performed using DIGE with subsequent spot selection. Identified spots were extracted and processed using mass spectrometry.

In addition, available chemotherapy agents were tested in vitro against the same cell lines to check for their action and how this could be enhanced. A benzodiazepine receptor antagonist (PK11195) was used to demonstrate apoptosis promotion in the presence of established cytotoxic agents (gemcitabine, etoposide, 5 fluorouracil and cisplatin). Cytotoxic assays were carried out using the SRB (Sulphorhodamine B) assay. Cell lines were tested for benzodiazepine receptor status using qRTPCR and response was correlated.

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Declaration of Originality

This thesis is my own work, unless referenced and has not been submitted in any previous application for a degree.

Sources of information have been specifically acknowledged.

Stephanos Pericleous

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1 General Introduction

1.1 Cholangiocarcinoma

Cholangiocarcinoma (CCA) is a rare hepatobiliary tumour that arises from biliary epithelium. The word's etymology comes from Greek for bile (*χολή*, chole) duct (*αγγείον*, angion) and cancer (*καρκίνωμα*, carcinoma). The disease was first described in 1911 where it was distinguished from hepatomas (hepatocellular carcinomas) on the basis of its cellular derivation (Goldzieher and von Bokay, 1911). Although infrequent, it represents the second most common primary hepatobiliary malignancy.

1.1.1 Incidence and epidemiology

In the western world the annual incidence of cholangiocarcinoma is typically 1 to 2 cases per 100,000 population (Renard et al., 1987, Landis et al., 1998). This translates to roughly 1,000 cases per year in the United Kingdom and 9,000 cases per year in the United States. However, the incidence varies considerably around the world and can be much higher in developing countries, especially in the Far East, often reflecting the disease's different aetiologies. In Thailand for example, annual incidence has been calculated to be in excess of 100 cases per 100,000 population translating to about 60,000 cases per year (Green et al., 1991).

Data reveal that over the past few decades the incidence of CCA has been on the increase both in the west and in the Far East (West et al., 2006, Khan et al., 2002b). In the United States, intrahepatic CCA saw a rise in incidence of 165% between the

periods 1975-1979 and 1995-1999 (Shaib et al., 2004). The cause of this rise is unknown and does not appear to be explained simply by changes in coding practice or by improvements in diagnosis (Khan et al., 2002a).

CCA accounts for less than 2% of all cancer diagnoses worldwide (Chamberlain and Blumgart, 2000), however, it accounts for approximately 10 to 15% of all primary hepatobiliary malignancies making it the second most common in this group (Chapman, 1999, Ries LAG, 2005).

1.1.2 Classification and histology

Cholangiocarcinomas can develop anywhere along the biliary tree from the ampulla of Vater to the intrahepatic biliary radicals. The term "cholangiocarcinoma" was originally used to refer exclusively to primary tumours of the intrahepatic bile ducts. It was not used in reference to extrahepatic bile duct tumours, however, the term is nowadays regarded as inclusive of intrahepatic, perihilar, and distal extrahepatic tumours of the bile ducts (Albores-Saavedra et al., 1991).

CCAs are usually categorised according to their anatomic location:

- intrahepatic bile ducts (intrahepatic CCA is also sometimes referred to as 'peripheral'), which represent 20-25% of the total
- extrahepatic bile ducts (extrahepatic CCA), which also represent 20-25%
- hilar CCAs, representing 50-60% of all cases of cholangiocarcinoma. Tumours that involve the bifurcation of the ducts are often referred to as Klatskin tumours and

are sub categorised using the Bismuth classification (Bismuth and Castaing, 1994).

- Type I - tumours found below the confluence of the right and left hepatic ducts;
- Type II - tumours reaching the confluence but not involving the right or left hepatic ducts;
- Type III - tumours occluding the common hepatic duct and either the right (IIIa) or left (IIIb) hepatic duct;
- Type IV - tumours that are multicentric or they involve the confluence and both the right and left hepatic ducts.

Hilar CCAs have often been grouped with either intra or extrahepatic CCAs. Examples include death certification, where Klatskin tumours have been recorded as intrahepatic (Khan et al., 2002a) whilst in other instances they have been classified as extrahepatic (Chamberlain and Blumgart, 2000).

A method for the **macroscopic** classification of CCAs has yet to be universally agreed on, on an international base at least. Several, mostly regional, classifications have been used in the past and present, which often creates difficulties and confusion particularly when comparing data. A commonly used classification system is the one proposed by The Liver Cancer Study Group of Japan which characterises them as mass forming, periductal infiltrating or intraductal (Lim and Park, 2004). Of these morphological types, intraductal cholangiocarcinomas are the least common, but

have a more favourable prognosis than either the mass-forming or periductular infiltrating types.

Histologically cholangiocarcinomas are most commonly found to be well-differentiated adenocarcinomas (95%) (Patel, 2006). The other 5% can be squamous cell carcinomas, mucoepidermal carcinomas, rhabdomyosarcomas, leiomyosarcomas, cystadenocarcinoma, granular cell carcinoma, lymphomas or carcinoid tumours (Chapman, 1999).

1.1.3 Staging

There are currently three staging systems for CCA: the AJCC/UICC (American Joint Cancer Committee/Union for International Cancer Control) TNM staging system, the LCSGJ (Liver Cancer Study Group of Japan) (Yamasaki, 2003) and the NCCJ (National Cancer Center of Japan) (Okabayashi et al., 2001). Intra, perihilar and extra hepatic CCAs are staged according to different criteria. The 7th and most recent edition of the TNM classification of malignant tumours was published in 2009 and Table 1 illustrates the staging of intrahepatic CCA (Sobin et al., 2010). A subsequent validation study demonstrated that it was accurate in correlating increasing stage with poor patient survival (Ribero et al., 2011).

Table 2 illustrates the TNM staging for perihilar CCA and Table 3 that of extra hepatic CCA.

T1	Solitary tumour without vascular invasion
T2a	Solitary tumour with vascular invasion
T2b	Multiple tumours, with or without vascular invasion
T3	Tumour(s) perforating the visceral peritoneum or involving the local extra hepatic structures by direct invasion
T4	Tumour with periductal invasion
N0	no regional lymph node metastases
N1	regional lymph node metastases
M0	No distant metastases
M1	Distant metastases present
Stage	
I	T1 N0 M0
II	T2 N0 M0
III	T3 N0 M0
IVa	T4 N0 M0, Any T N1 Mo
IVb	Any T, Any N, M1

Table 1 TNM classification and staging of intra hepatic CCA

Tx	Primary tumour cannot be assessed		
Tis	Carcinoma in situ		
T1	Tumour confined to the bile duct, with extension up to the muscle layer or fibrous tissue		
T2A	Tumour invades beyond the wall of the bile duct to surrounding adipose tissue		
T2B	Tumour invades adjacent hepatic parenchyma		
T3	Tumour invades unilateral branches of the portal vein or hepatic artery		
T4	Tumour invades main portal vein or its branches bilaterally; or the common hepatic artery; or the second-order biliary radical bilaterally; or unilateral second order biliary radicals with contralateral portal vein or hepatic artery involvement		
N0	No regional lymph node metastases		
N1	Regional lymph node metastases present (including nodes along the cystic duct, common bile duct, hepatic artery, and portal vein)		
N2	Metastases to periaortic, pericaval, superior mesenteric artery, and/or celiac artery lymph nodes		
M0	No distant metastases		
M1	Distant metastases present		
Stage			
0	Tis	N0	M0
I	T1	N0	M0
II	T2A-B	N0	M0
IIIa	T3	N0	M0
IIIb	T1-3	N1	M0
IVa	T4	N0-1	M0
IVb	Any T	N2	M0
	Any T	Any N	M1

Table 2 TNM classification and staging of perihilar CCA

TX	Primary tumour cannot be assessed		
T0	No evidence of primary tumour		
Tis	Carcinoma in situ		
T1	Tumour confined to the bile duct histologically		
T2	Tumour invades beyond the wall of the bile duct		
T3	Tumour invades the gallbladder, pancreas, duodenum, or other adjacent organs without involvement of the celiac axis, or the superior mesenteric artery		
T4	Tumour involves the celiac axis, or the superior mesenteric artery		
NX	Regional lymph nodes cannot be assessed		
N0	No regional lymph node metastases		
N1	Regional lymph node metastases present		
M0	No distant metastases		
M1	Distant metastases present		
Stage			
0	Tis	N0	M0
IA	T1	N0	M0
IB	T2	N0	M0
IIA	T3	N0	M0
IIB	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
III	T4	Any N	M0
IV	Any T	Any N	M1

Table 3 TNM classification and staging of extra hepatic CCA

1.1.4 Clinical presentation

Patients with extrahepatic or hilar cholangiocarcinoma almost invariably present with symptoms of obstructive jaundice (90-98%). Weight loss is present in 29% of patients, whilst 20% have abdominal pain and 9% have fever (Chamberlain and Blumgart, 2000).

Patients with intrahepatic lesions often present with malaise, abdominal pain or just weight loss. Some are identified incidentally as mass lesions on imaging studies performed during investigation of other symptoms or pathologies. On clinical presentation alone, it is often difficult to distinguish intrahepatic cholangiocarcinoma from hepatic metastases from other malignancies (Patel, 2006).

1.1.5 Risk factors and aetiology

At presentation, most patients are at an advanced age with 65% of patients being over 65 years old. The overall average age of presentation is 50 years. Males have a higher incidence with a 3:1 ratio to females (Chapman, 1999).

The strongest association of cholangiocarcinoma development is with diseases that cause chronic inflammation of the biliary tract. Not surprisingly, primary sclerosing cholangitis (PSC) is the commonest known predisposing factor for cholangiocarcinoma in the UK with a calculated lifetime risk of 5–15% for the PSC patient. This association persists irrespective of the presence of ulcerative colitis, a disease closely linked to PSC.

Other risk factors include:

- Chronic intraductal gall stones.
- Bile duct adenoma and biliary papillomatosis.
- Caroli's disease (an inherited condition characterised by cystic dilatation of intrahepatic bile ducts giving a lifetime risk for CCA of 7%).
- Choledochal cysts (about 5% will transform, risk increases with age).
- Cirrhosis of any aetiology (Welzel et al., 2007).
- Smoking (increased risk in association with PSC).

- Thorotrast, a radiological contrast medium with a half life of approximately 400 years. It is no longer licensed for clinical use and was withdrawn from the market in the 1950s. After exposure, the relative risk for all hepatobiliary malignancies has been reported between 39.2 and 47 and lasts for several decades as Thorotrast is retained in the reticuloendothelial system and continues to produce ionising radiation (Zhu et al., 2004).

Some risk factors such as Hepatitis C (especially with intrahepatic cholangiocarcinoma) and heavy alcohol consumption have been associated with cholangiocarcinoma but their role could not be independently evaluated from cirrhosis (Shaib et al., 2007).

In South East Asia, where the tumour has a higher incidence, the additional associated risk factors are:

- Liver flukes (*Opisthorchis viverrini* and *Clonorchis sinensis*), especially in Thailand, Laos and northern Malaysia (Sripa and Pairojkul, 2008). This association was first recognised in the literature approximately 60 years ago (Viranuvatti et al., 1955). It has been suggested that the association of *O. viverrini* and CCA is the strongest between any parasite and a cancer (Sripa et al., 2012).

- Chronic typhoid carrier status (which causes a six fold increased risk of all hepatobiliary malignancies) (Welton et al., 1979).

1.1.6 Molecular pathogenesis

The malignant transformation of cholangiocytes, a process often referred to as 'cholangiocarcinogenesis' is still not fully understood. Current evidence implicates conditions leading to an environment of chronic inflammation of the liver. Other factors such as injury to the bile duct epithelium and alteration to bile flow, especially obstruction, are usually linked to this chronic inflammation (Wise et al., 2008). DNA damage as a result of the above conditions promotes tissue proliferation, in a local environment that becomes rich in growth factors and cytokines. Cells are therefore in a position to develop autonomous proliferation through activation of pro-proliferative intracellular signalling pathways and enhanced production of mitogenic factors.

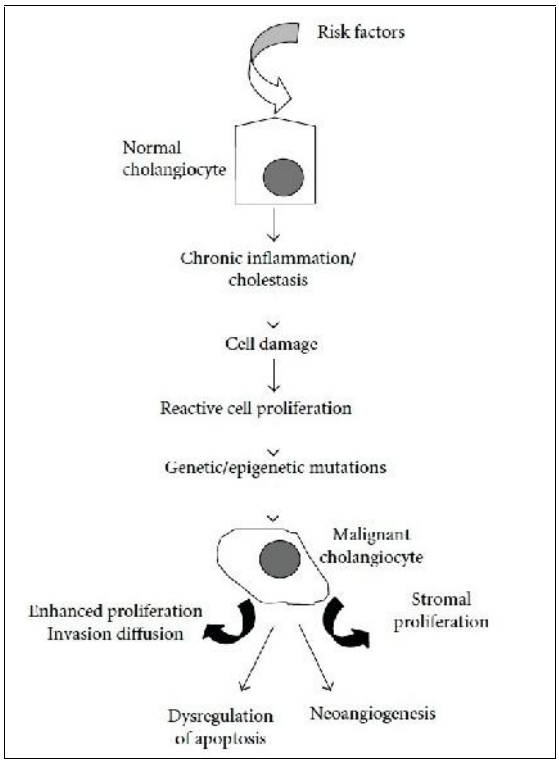

Figure 1-1 Proposed mechanisms leading to transformation of normal biliary cells into malignant cholangiocytes.

Cholangiocarcinoma cells express altered molecular mechanisms, which enhance cell proliferation, decrease apoptosis, and increase the capacity of tissue invasion, stromal proliferation, and angiogenesis (Fava and Lorenzini, 2012).

Cytokines released by cholangiocytes and that have been implicated in cholangiocarcinogenesis include: interleukin 6 (IL-6), transforming growth factor-β (TGF-β), IL-8, tumour necrosis factor-α (TNF-α) and platelet–derived growth factor (PDGF). These can act in both an autocrine and paracrine fashion (Fava and Lorenzini, 2012). They stimulate several intracellular pathways involved in growth and survival of malignant cholangiocytes.

IL-6 for instance activates the pro-survival p38 mitogen activated protein kinase and up-regulates Mcl-1, an anti-apoptotic protein in the Bcl-2 family of apoptotic

proteins (Kobayashi et al., 2005). This tips the balance in the pro and anti-apoptotic equilibrium thereby shifting affected cells towards cell survival.

Mcl-1 up regulation in cholangiocytes also increases cancer cell resistance to TRAIL (tumour necrosis factor related apoptosis inducing ligand) promoting cell survival.

The cytokine TGF-β along with its signalling pathway are associated with several cell functions such as growth, survival, apoptosis, differentiation and immunity. In the presence of cholestasis, cholangiocytes express TGF-β, however CCA cell mutations to the receptors of TGF-β induce a resistance to the cytokine's effect. The impediment in TGF-β signalling also accounts for the enhanced deposition of fibrotic (stromal) tissue, a characteristic feature of CCA (Yazumi et al., 2000).

Nitric oxide is an agent with recognised mutagenic properties. Cytokines released in response to inflammation activate iNOS (inducible nitric oxide synthase) which in turn generates nitric oxide. Nitric oxide in turn can directly or through the formation of peroxynitrite species lead to the deamination of guanine and DNA adduct formation thereby promoting DNA mutations (Jaiswal et al., 2000, Jaiswal et al., 2001). iNOS has also been shown to promote the up-regulation of COX-2 in in-vitro studies of immortalized mouse cholangiocytes suggesting that COX-2 and COX-2 derived prostanoids could have a key role in cholangiocarcinogenesis. COX-2 also up regulated the expression of Notch-1, a trans membrane receptor involved in cell proliferation, which has been implicated in other cancers (Ishimura et al., 2005).

1.1.7 Current treatment of cholangiocarcinoma

1.1.7.1 Surgery

1.1.7.1.1 Resection

Patients are usually assessed for surgery before being considered for other treatments. Surgical resection for CCA gained favour in the early 1970s (Okaro et al., 2002). It remains a major undertaking with relatively high morbidity and mortality rates, however, it is the only treatment modality that can offer potential for cure. Morbidity figures range for 31 to 85% and perioperative mortality from 5 to 10% (Meza-Junco et al., 2010).

Less than 25% of patients with CCA are amenable to surgical resection at the time of presentation. When R0 resection is achieved, reported five-year survival rates range from 30% to 41% for hilar CCA, 31% to 63% for intrahepatic tumours and 27% to 37% for extrahepatic tumours. This rate has not appreciably improved over recent years (Meza-Junco et al., 2010, Wade et al., 1997).

1.1.7.1.2 Liver transplantation

More recently, liver transplantation has been championed as an alternative surgical option for peripheral CCA with potential curative outcomes. Early attempts of its use were fraught with high recurrence rates (Robles et al., 2004). However, the latest outcomes presented by the Mayo Clinic Rochester, where an aggressive neo-adjuvant regime is employed, claim a 5 year survival rate (in 90 transplanted patients) in excess of 70% (Rosen et al., 2008). These figures compare favourably to

surgical resection. Very importantly however, is that they also compare well to survival figures in patients that receive liver transplants for other diagnoses. This justifies the argument of using donor livers for this purpose at least on a clinical trial stage. To date however, the evidence as illustrated above comes from non-randomised case series and has not been widely replicated in other parts of the world.

1.1.7.2 Palliative treatment

The remaining 75% of patients, who are not candidates for surgical resection, can be considered for palliative treatment. There are various supportive treatment regimes and the algorithm of treatment can be complex (see Figure 1-2). Factors that can affect treatment decisions include:

- Anatomical location of primary and/or metastatic disease
- Symptoms (eg jaundice)
- Patient's current state of fitness
- Other co-morbidities
- Patient wishes
- Local availability of technology and expertise
- Loco regional involvement and recruitment in clinical trials

Palliative surgical options can be tailored to clinical needs. Various bypass procedures can be employed to circumvent or drain the biliary system, an effective

treatment for the jaundiced patient. Less invasive options such as endoscopic or percutaneous biliary stenting can also be used with either plastic or metal devices. Stenting with drug eluting covered metal stents is still at an experimental stage.

Chemotherapy has had limited success on this disease. It was not until 2010 that the first phase III chemotherapy based treatment showed any survival benefit. The ABC-02 (Advanced Biliary Cancer) was a multi centre UK study that demonstrated an overall survival benefit using gemcitabine and cisplatin when compared to gemcitabine alone (Valle et al., 2010b). It currently represents the chemotherapy treatment standard for unresectable CCA.

The ABC-02 trial became an extension of the ABC-01, a phase II trial that used the same treatment regime (Valle et al., 2009). Prior to this study, gemcitabine monotherapy had been one of the most commonly used agents in CCA (Gebbia et al., 2001). The ABC-02 trial recruited 410 adult patients with unresectable and histopathology or cytology proven CCA from 37 centres. Patients were randomised to either of the above two regimes. The study demonstrated that the combination regime (gemcitabine with cisplatin) improved median overall survival (OS) by 3.6 months (11.7 vs. 8.1 months respectively) when compared to monotherapy with gemcitabine. The group receiving combination therapy also had improved progression free survival (PFS) by 3 months (8.0 vs. 5.0 months respectively). The same investigators have launched two further phase II trials (ABC-03 and ABC-04) that are investigating the effect of tyrosine kinase inhibitors on patients with advanced CCA.

The following table (Table 4) summarises all the phase II and phase III trials published in the literature to date involving patients with CCA. Table 5 that follows, summarises the current ongoing phase III trials on patients with CCA.

Study	Year of Publication no of patients	Treatment	Type Phase	Outcome
(Harvey et al., 1984)	1984 17	5-FU mitomycin doxorubicin	Palliative Phase II	PR 31% SD 41%
(Falkson et al., 1984)	1984 34	5-FU streptozotocin Methyl-CCNU	Palliative Phase II	OR 8%
(Ellis et al., 1995)	1995 25	5-FU epirubicin cisplatin	Palliative Phase II	RR 40%
(Polyzos et al., 1996)	1996 13	5-FU mitomycin-C folinic acid	Palliative Phase II	RR 23% MS 22 w
(Jones et al., 1996)	1996 15	paclitaxel	Palliative Phase II	CR 0% PR 0%
(Patt et al., 1996)	1996 35	5-FU rIFN alpha-2b	Palliative Phase II	MS 12m PR 34%
(Eckel et al., 2000)	2000 30	5-FU Leucovorin cyclophosphamide	Palliative Phase II	MS 7.3 m
(Park et al., 2005)	2005 40	epirubicin, cisplatin, uracil/tegafur, and leucovorin	Palliative Phase II	PR 22.5% MS 34w MTTP: 16w
(Ducreux et al., 2005)	2005 58	5-FU folinic acid cisplatin	Palliative Phase II	CR 0-4% PR 7-15% OR 7-9% DS 44-46%

Study	Year of Publication no of patients	Treatment	Type Phase	Outcome
(Park et al., 2006)	2006 43	epirubicin, cisplatin capecitabine	Palliative Phase II	PR 40% MS 8m SD 23%
(Kim et al., 2006)	2006 29	gemcitabine cisplatin	Palliative Phase II	PR 34.5% OR 34.5% SD 13.8% PD 44.8% MS 11m
(Hong et al., 2007)	2007 32	capecitabine cisplatin	Palliative Phase II	PR 41% CR 0% SD 9.4% MTTP 3.5m MS12.4m PD 34.4%
(Ciuleanu et al., 2007)	2007 22	OSI-7904L 5-FU leucovorin	Palliative Phase II (Randomised)	SD 0-36% MTTP 7.4-18w MS 23.7-29.4w
(Riechelmann et al., 2007)	2007 75	gemcitabine capecitabine	Palliative Phase II	OR 29% CR 4% PR 25% MS 12.7m SD 49% MTTP 6.2m

Study	Year of Publication no of patients	Treatment	Type Phase	Outcome
(Kim et al., 2008)	2008 51	Cisplatin S-1 (=tegafur gimeracil oteracil K)	Palliative Phase II	OR 30% CR 4% PR 26% SD 42% PD 18% MTTP 4.8m MS 8.7%
(Lee et al., 2008)	2008 39	gemcitabine cisplatin	Palliative Phase II	PR 17.1% SD 28.6% PD 45.7% MS 8.6m MTTP 3.2m
(Meyerhardt et al., 2008)	2008 33	gemcitabine cisplatin	Palliative Phase II	PR 21% SD 36% PFS 6.3m MS 9.7m OYS 39%
(Yu et al., 2008)	2008 41	gemcitabine capecitabine	Palliative Phase II	PR 30.1% SD 30.1% MS 10m MTTP 6m OYS 40%
(Takezako et al., 2008)	2008 39	Cisplatin epirubicin 5-fluorouracil	Palliative Phase II	PR 10% MS 9.1m PFS 5.1m OYS 21%

Study	Year of Publication no of patients	Treatment	Type Phase	Outcome
(Oh et al., 2008)	2008 15	S-1 oxaliplatin	Palliative Phase II	PR 6.7% SD 26.7% MTTP 1.4m MOS 3.1m
(Jang et al., 2010)	2010 53	Gemcitabine oxaliplatin	Palliative Phase II	OR 18.9% CR 2% SD 50.9% DC 69.8% PFS 4.8m MOS 8.3m
(Sasaki et al., 2010)	2010 35	Gemcitabine S-1	Palliative Phase II	OR 34.3% DC 82.9% MOS 11.6m MTTP 5.9m
(Bengala et al., 2010)	2010 46	Sorafenib	Palliative Phase II	OR 2% DC 32.6% PFS 2.3m MOS 4.4m
(Lubner et al., 2010)	2010 53	Bevacizumab erlotinib	Palliative Phase II	PR 12% SD 51% MOS 9.9m TTP 4.4m

Study	Year of Publication no of patients	Treatment	Type Phase	Outcome
(Williams et al., 2010)	2010 48	Gemcitabine with carboplatin	Palliative Phase II	OR 31.1%. MPFS7.8 OS10.6 6MS 85.4%
(Gruenberger et al., 2010)	2010 30	Cetuximab, gemcitabine oxaliplatin	Palliative Phase II	OR 63% CR 10% PR 53% DC 80% SD 17% PD 20% PFS 8.8m MOS 15.2m
(Karachaliou et al., 2010)	2010 28	irinotecan oxaliplatin	Palliative Phase II	OR 17.9% MOS 9.2m MPFS 2.7m
(Lassen et al., 2011)	2011 41	Emcitabine oxaliplatin capecitabine	Palliative Phase II	OR 34% SD 51% DC 85% MOS 12.5 m MPFS 6.9 m
(Glimelius et al., 1996)	1996 90, (37 biliary)	5FU, etoposide leucovorin	Palliative **Phase III**	36% ↑ QALY MS 6m

Study	Year of Publication no of patients	Treatment	Type Phase	Outcome
(Rao et al., 2005)	2005 54	5FU, etoposide leucovorin cisplatin	Palliative **Phase III**	MS 9.02-12.03m RR 15-19.2%
(Takada et al., 2002)	2002 139	5FU mitomycin-C	Adjuvant **Phase III**	No benefit in treatment arm of CCAs
(Valle et al., 2010a)	2010 410	Gemcitabine +/- cisplatin	Palliative **Phase III**	Combination arm ↑MOS by 3.6m and ↑MPFS by 3m
Abbreviations: DS: Disease Stabilisation, CR: Complete Response, OR: Overall Response, PR: Partial Response, MS: Median Survival, RR: Response Rate, SD: Stable Disease, m: months, w: weeks, QALY: Quality of life index, MTTP: Median Time to Progression, PD: Progressive Disease; OYS: One Year Survival; DC: Disease Control; 6MS: 6 month survival; CR Complete Response Notes: Some trials do not separate CCA from GB cancers				

Table 4 Published clinical trials on chemotherapy for biliary tract tumours

Study	Started/No of Patients	Treatment	Type
NCT00262769*	May 2005 400	Gemcitabine +/- Cisplatin	Palliative
NCT00363584	March 2006 360	Capecitabine or Observation	Adjuvant
NCT00658593	March 2008 320	Gemcitabine +/- Capecitabine	Palliative
NCT00939848	April 2011 136	Cisplatin/Gemcitabine +/- Cediranib	Palliative
NCT01149122	January 2009 180	Gemcitabine/Oxaliplatin +/- Erlotinib	Palliative
NCT01313377	July 2009 190	Gemcitabine/Oxaliplatin or Observation	Adjuvant

Table 5 Ongoing Phase III studies for patients for biliary tract tumours

* NCT denotes a unique clinical trial registry number (identifier) at clinicaltrials.gov

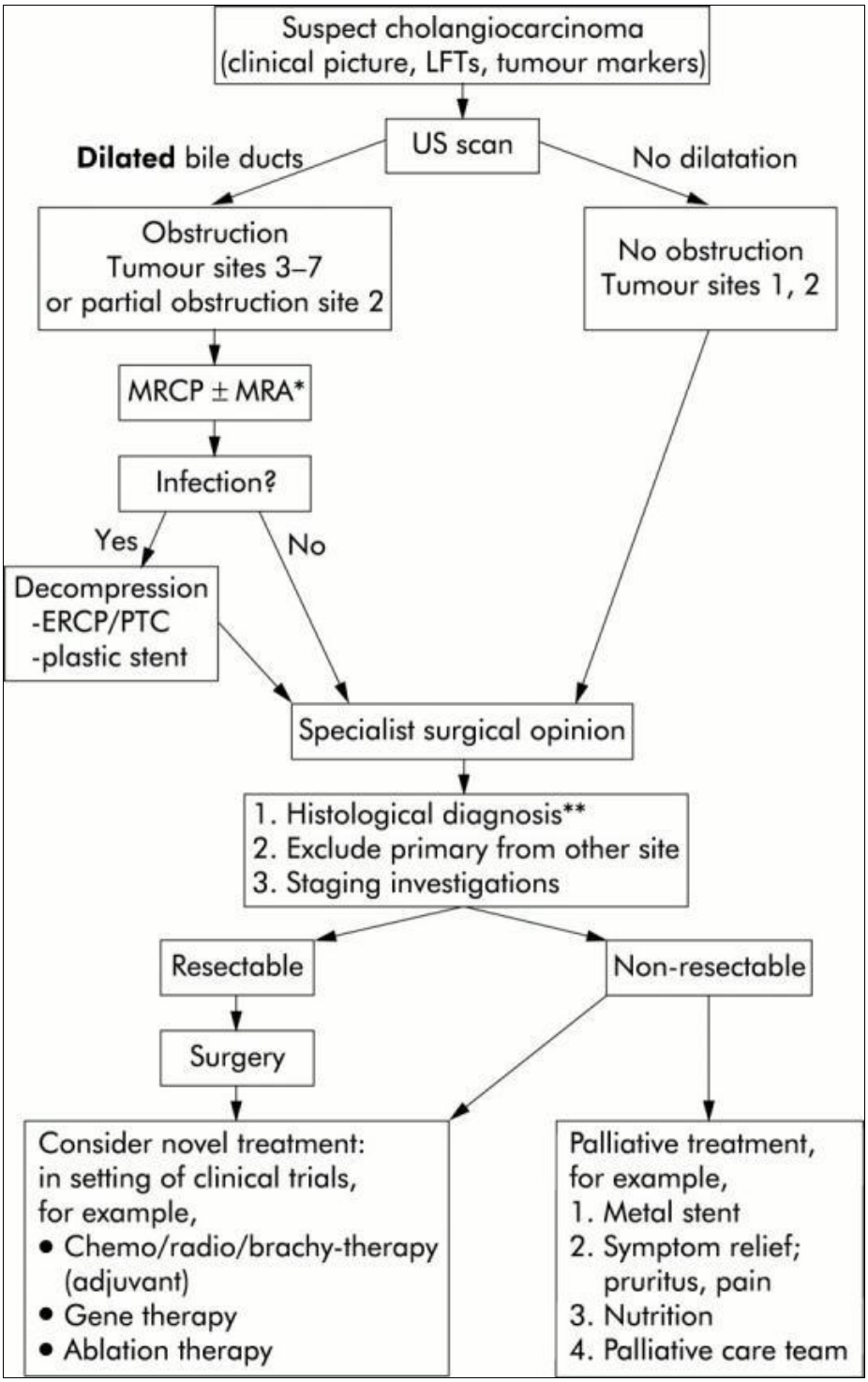

Figure 1-2 Management algorithm for cholangiocarcinoma.

***Where magnetic resonance imaging/MRCP is not possible, patients should have contrast enhanced spiral/helical computed tomography. **Fine needle biopsy or biopsy is ideally avoided until resectability has been assessed by a specialist surgeon (Khan et al., 2002a).**

1.1.7.3 Experimental therapies

- Photodynamic therapy is gaining favour as a candidate treatment for patients with non resectable CCA. Photodynamic therapy relies on the favourable accumulation of photosensitizers, such as porphyrins, in neoplastic or dysplastic cells. Following local or systemic administration the photosensitizer is activated by application of nonthermal laser light of an appropriate wavelength. The photochemical process generates oxygen radicals resulting destruction of the tissue. Two recent phase II randomised controlled trials (RCTs) showed that photodynamic therapy improved survival, jaundice and quality of life, and was well tolerated (Zoepf et al., 2005, Ortner et al., 2003). Currently there are two phase III trials looking to validate this [NCT01439685, NCT00869635][†]. Although photodynamic therapy can be considered an alternative to chemotherapy, it remains a treatment confined to large specialised centres with a particular interest and expertise (Ortner, 2011).

- Another recent and novel approach to treating obstructing biliary tumours has been the use of drug eluting stents. Paclitaxel has been used with some reports of success but at an experimental level (Lee, 2009). At least two phase III trials are investigating the clinical outcomes of this technology [NCT01413386, NCT00453076].

- Trans-arterial chemoembolization (TACE) with or without drug eluting beads are another approach championed for their loco regional targeting

[†] NCT denotes a unique clinical trial registry number (identifier) at clinicaltrials.gov

properties. A few small pilot studies have recently reported their results but no phase III trials are currently underway (Kuhlmann et al., 2012).

1.1.8 Cholangiocytes

1.1.8.1 Anatomy

Cholangiocytes are epithelial cells that make up 3-5% of total liver mass. In the adult human liver they have an estimated total length of approximately 2 km (ANDREWS, 1955).

The biliary tree is formed by a network of ducts that channel bile in one direction of flow. One way flow is achieved synergistically through simple mechanical pressure and a constant concentration gradient of bile along the biliary tree.

The most peripheral and smallest ducts are the bile ductules (which have a diameter of less than 15 μm) that drain into the canals of Herring. Bile ducts then build up in size ending in the extrahepatic ducts and finally the common bile duct (which is roughly 5mm in diameter) that drains into the duodenum. Ducts below the order of the canals of Herring are lined partly by cholangiocytes and partly by hepatocytes. Ducts that are larger than these are lined only by cholangiocytes. In the human biliary tree, the smallest orders of bile ducts have a circumference of 4 to 5 cells while the larger ducts may be lined by up to 40 cholangiocytes.

The microscopic appearance of cholangiocytes differs depending on their anatomical position. The lower order (more peripheral cholangiocytes) lining the narrower ducts

are smaller in size and flatter in shape. The cholangiocytes lining the bigger ducts become larger in size and more columnar in appearance.

Cholangiocytes possess a luminal (apical) and a basolateral surface. They are also inter-connected by gap junctions that allow the exchange of ions and small molecules between neighbouring cells (Bode et al., 2002). The apical surface area is increased fivefold through the presence of microvilli within the bile duct lumen. As such, cholangiocyte morphology is considered typical of an epithelial cell with secretory and absorptive properties.

1.1.8.2 Physiology

Apart from morphological heterogeneity cholangiocytes also display physiological differences according to their anatomical location. This is commonly seen in other organs too such as the kidney and the small intestine. Transporters, receptors, enzymes, exchangers, and channels are expressed in different amounts (both on the apical and basolateral membranes) in fitting with how far down the biliary tree a cell is located. For instance, animal studies show that the ABAT transporter (the apical Na^+-dependent bile acid transporter which allows internalization of bile acids in cholangiocytes and which responds to bile acids with changes in cholangiocyte secretory processes) is expressed in large bile ducts but not in small ones (Alpini et al., 1997).

The different protein expression of proteins in cholangiocytes serves in regulating the transepithelial movement of ions, solute, and water. The involvement of transporters, exchangers and channels in this process results in the formation of bile,

suitable for secretion into the gut lumen. These are regulated by heterogeneity in receptor expression which is acted upon by hormones, regulatory peptides, neurotransmitters, and by bile-borne regulatory factors (Johnson, 2006).

1.1.9 Selective (targeted) drug delivery

In vivo targeted drug therapy confers two potential benefits:

1. It results in the delivery of higher concentrations of drug to an intended target maximising its effect and in doing so reducing the total amount needed to administer.

2. It minimises the impact of these drugs on areas other than the intended destination (non-target sites). Many of the challenges facing current anticancer therapies are related to the toxic side effects of the drugs employed. Gastrointestinal disturbances, nausea, myelosuppression and neuropathy for instance arecommon and serious problematic side effect of many cytotoxic drugs used in the treatment of solid tumours. If the physical presence of these drugs in non cancerous tissues could be reduced then the potential side effects could be minimised.

Attempts to achieve selective drug delivery have been pursued through the use of various means.

If the target is a solid tumour, agents can be delivered to a particular anatomical location. One way this can be achieved is by the injection of a drug directly into an identifiable tumour, if necessary under image guidance.

Another anatomical strategy is capitalising on tumour blood supply. An example that is already in practice is the use of TACE (trans arterial chemo embolisation) to treat hepatomas. Hepatomas derive the majority of their blood supply from branches of the hepatic artery. The feeding vessels can be identified and accessed radiologically where agents can be delivered.

1.2 Experimental models to study the effect of chemotherapy in CCA

1.2.1 In vitro preclinical models

Experimental model systems have been central to providing basic and preclinical insights into many cancers. The usual starting point in assessing efficacy of chemotherapy agents is by investigating the effects of the agents under investigation on cultured cell lines in an in vitro setting. In vitro models are the first step in rational drug discovery and development but serve only the purpose of selecting compounds for secondary, more comprehensive, in vivo testing. This approach is employed by the largest preclinical screening scheme to date, the NCI-60 project (the US National Cancer Institute (NCI) 60 human tumour cell line anticancer drug screen) (Shoemaker, 2006). This project which started in the 1980s and is still running uses a panel of 60 cell lines derived from the most common tumours (leukaemia, melanoma, non small cell lung, central nervous system, colorectal, ovarian, renal prostate and breast cancer). To date, these cell lines have been exposed to over 100,000 different agents using a standardised automated high throughput protocol.

Cell culture studies have several advantages that have established them as a starting point in cytotoxicity testing. They are relatively inexpensive; they are straightforward

and can be performed relatively quickly. They are carried out in a controlled environment and can easily be standardised and reproduced.

Critics argue that they fail to take into account the microenvironment of malignant cells, as they cannot mimic the complexities of drug delivery, metabolism and excretion found in a complete organism. Furthermore they can have a substantially different genome to the disease they are used to represent.

The low incidence of CCA is reflected in the number of cell lines developed for this disease. There are currently only a handful of CCA and immortalised biliary epithelium cell lines available, most of which were developed at academic institutions. Some major cell line collections such as the European Collection of Cell Cultures (ECACC) and the American Type Culture Collection (ATCC) do not have CCA or cholangiocyte cell lines available (ECACC, 2012, ATCC, 2012).

1.2.2 In vivo preclinical animal models

A reliable orthotopic animal model for CCA has not been developed yet. Several approaches have been employed. Early attempts used animal exposure to a combination of parasitic infection and systemic carcinogenic agents. More recent approaches have been to use local or systemic administration of toxins to induce CCA development (Yeh et al., 2004). Another approach has been the direct injection of CCA cell lines into the liver (Sirica et al., 2008).

Farazi et al, described a murine model where p53 mutant mice were subjected to chronic exposure to intermittent intra-peritoneally instilled toxin (carbon tetrachloride (CCl_4)) (Farazi et al., 2006). The exposure induced a state of chronic

inflammation in the biliary tree and the mice eventually developed intrahepatic CCA. However, their model had a long latency time (4 months of treatment with some mice developing CCA a few weeks after the end of exposure). In addition many mice developed cirrhosis and a variety of other tumours including sarcomas, lymphomas, and hepatocellular cancers. Most mice succumbed to the other tumours.

Sirica et al, presented an orthotopic animal model claimed to be more "patient like". Rats were inoculated with a rat CCA cell line (BDEneu or BDEsp) by direct injection of cultured cells into the biliary tree or liver (Sirica et al., 2008). They reported a 100% success rate within 20 days post inoculation with BDEneu in the liver injected rats. Bile duct injected rats were less successful in growing CCA with a success rate of 56% when the BDEsp cell line was used. This model also involved suture ligation of the bile duct, to emulate the cholestasis experienced by human patients and some rats developed extrahepatic metastatic disease, whilst establishing the model takes 3 to 4 weeks. However, despite this model's apparent advantages, the only publications come from the research group that developed it (last date of PubMed search: February 2012).

1.3 Research aims

This study set out to:

1. To investigate the effect of various chemotherapeutic agents either alone or in combination in the treatment of cultured cholangiocarcinoma cells.

2. To investigate the effect of adding PK11195 as a sensitising agent to enhance the action of various chemotherapeutic agents in cultured cholangiocarcinoma cells.

3. To analyse the proteome of a panel of cholangiocarcinoma cell lines and to assess the presence of potential biomarkers and therapeutic targets.

2 Verification of cell lines by immunohistochemistry

2.1 Introduction

In vitro cell line testing is a convenient first step for cytotoxic drug evaluation; however, this experimental method comes with several limitations (see chapter 1.2.1). A well-recognised problem in cell line use is their misidentification or contamination with other cell types. Numerous studies have estimated that up to 36% of cell lines used in research are of a different origin or species to that claimed (Nature, 2009, Masters et al., 2001).

Immunostaining is a technique often employed in clinical practice to determine the origin of tissue specimens. The histopathological diagnosis of cancers can often pose a clinical challenge especially for metastatic disease of unknown origin. In addition, diagnostic dilemmas can arise where adjacent structures can be similar. A common diagnostic problem particular to HPB malignancies is the differentiation of cholangiocarcinoma from hepatocellular cancer. Immunohistochemistry is a valuable tool in such settings as it can help identify the tissue of origin which in turn can assist in guiding what form of treatment a patient should receive, especially when optimising chemotherapy (Bateman and Hubscher, 2010).

Immunostaining was therefore performed to the cell lines used in this study, in an attempt to prove their epithelial origin but also to ensure that they expressed a profile consistent with tissue of biliary origin. Cells were stained for cytokeratin 7, cytokeratin 19 and epithelial membrane antigen.

Cytokeratins are proteins of keratin-containing intermediate filaments found in the cytoskeleton of cells with epithelial tissue origin and form the bedrock of the immunohistochemical evaluation of tumours (Jain et al., 2010).

Cytokeratin 7 (CK7) is a basic type II cytokeratin found on many glandular and transitional epithelia. It is encoded by the KRT7 gene and is usually present in adenocarcinomas of the lung, breast, ovary, serous and endometrial tumours, uterine cervical tumours, transitional cell carcinoma of the bladder, biliary epithelium and cholangiocarcinoma. It is not found on hepatocytes and hepatocellular carcinoma and it is hence used to differentiate HCC from CCA (Dennis et al., 2005).

Cytokeratin 19 (CK19) is a type I keratin protein that in humans is encoded by the KRT19 gene. It is expressed in most epithelial cells and many tumours such as prostate (Pu et al., 2007) and breast cancer (Sakaguchi et al., 2003). Cholangiocytes and CCA cells usually express CK19, unlike hepatocytes and HCC so it is used in conjunction with CK7 to differentiate between the two (Bateman and Hubscher, 2010).

Epithelial membrane antigen (EMA) also referred to as Mucin 1, cell surface associated (MUC1) and polymorphic epithelial mucin (PEM) is expressed by most epithelial cancers (Davidson et al., 1988). In contrast to hepatocellular cancer, it is often expressed by cholangiocarcinoma cells (Bonetti et al., 1983, Haratake and Hashimoto, 1995).

2.2 Methods

2.2.1 Immunostaining with CK7, CK19 and EMA

Cells were grown to sufficient quantities in tissue culture flasks as described elsewhere (see chapter 3.2.1). Cells were then lifted with trypsin and placed on an 8 well glass chamber slide (LabTek, Cat no 177402). Four replicates of the same cell passage were used for each cell line. Cells were allowed to culture in medium overnight in order to adhere to the chamber slide surface (for relevant medium and recipe, see section 3.2.1).

On removal from the incubator, one slide was set aside for haematoxylin and eosin (H&E) staining. Three slides were exposed to cold (4^{o}C) paraformaldehyde (4% concentration) for 10 minutes to ensure fixation. The paraformaldehyde was removed and cells were exposed to a few drops of blocking agent for 10 minutes (Power Block™ Universal Blocking, cat no HK085-5K, Biogenex). Slides were then washed with PBS twice. Two drops of antibody (CK7, CK19 and EMA) were added to the slides which were then allowed to stand overnight at 4^{o}C. The silicon chamber separators were dissected using a sharp surgical scalpel on the following day. Slides were then exposed to pre prepared secondary anti mouse antibody (1:300 dilution) (Cy3 conjugated Affinipure F(ab')2 Fragment donkey Antimouse IgG, cat no. 715-166-150, Jackson immunoresearch labs, West Grove, PA) and left for 45 minutes in a horizontal position in a dark cupboard. Slides were then placed in a slide rack and washed twice by submersion in PBS with DAPI. Slide covers were attached using water based glue.

2.2.2 Staining with haematoxylin and eosin (H&E)

One slide for each cell line was reserved for H&E staining. Following fixation, slides were washed with water and covered with haematoxylin for 5 minutes. They were then washed again with water and covered with eosin for 3 minutes. After washing in water again, a cover-slip was attached using aqueous glue.

2.3 Results

Slides were viewed and images were captured at X 20 and X 40 magnification using an inverted fluorescence microscope (Olympus IX-71).

2.3.1 H69

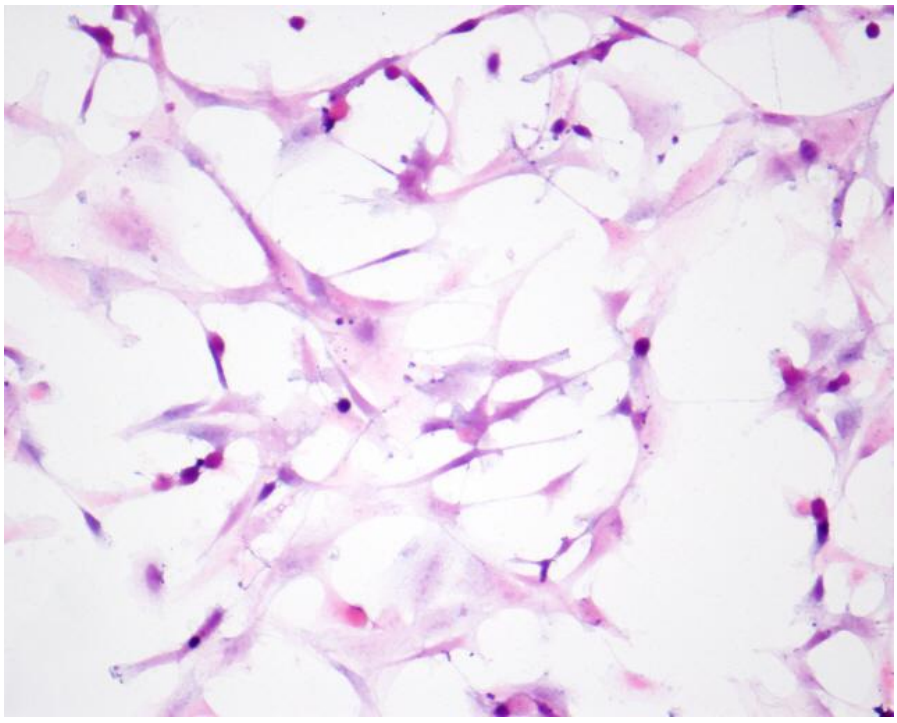

Figure 2-1 H69 cells stained with H&E (x40 magnification)

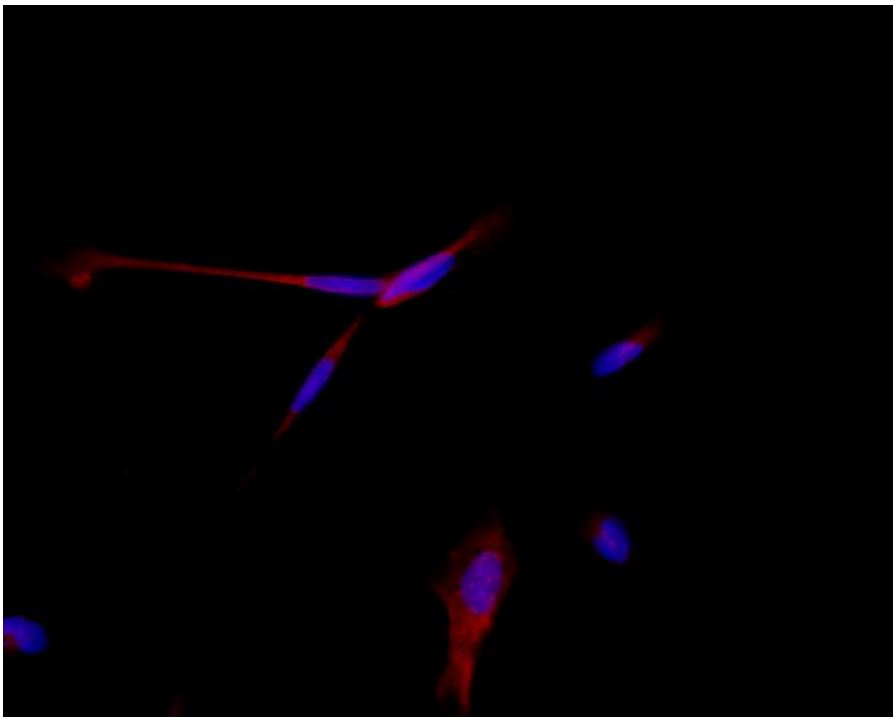

Figure 2-2 H69 cells stained with EMA (x40 magnification)

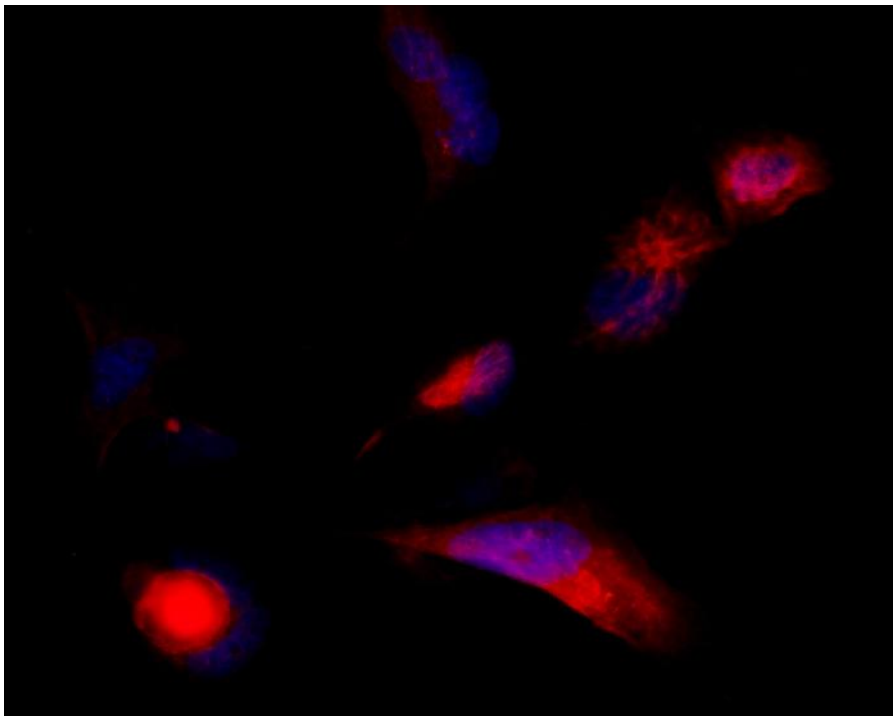

Figure 2-3 H69 cells stained with CK7 (x40 magnification)

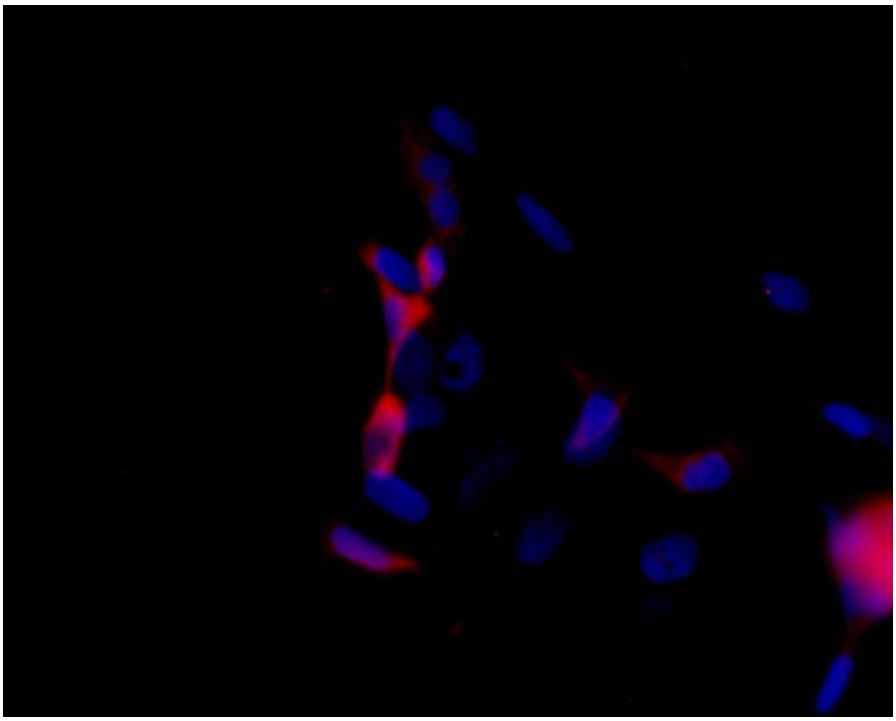

Figure 2-4 H69 cells stained with CK19 (x40 magnification)

2.3.2 HUCC

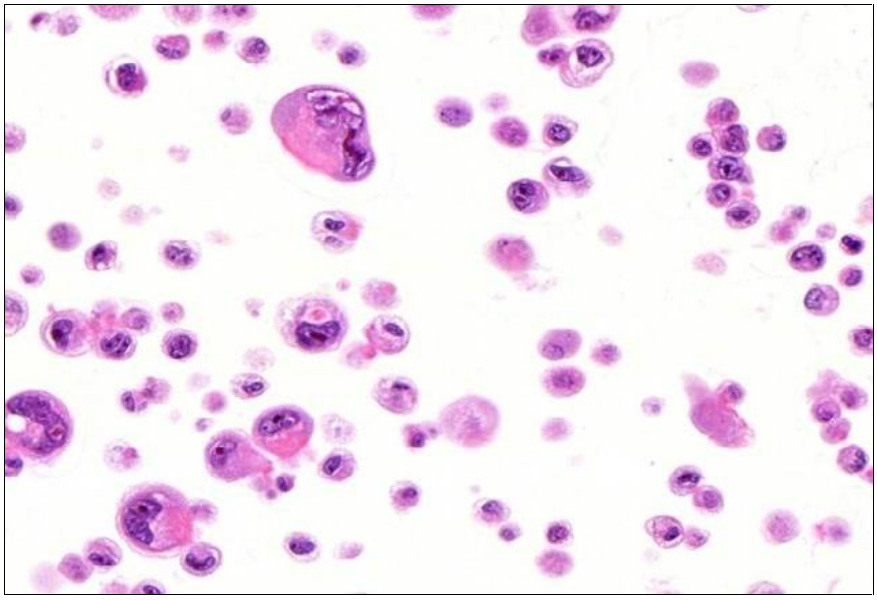

Figure 2-5 HUCC-T1 cells stained with Haematoxylin and Eosin (x20 magnification)

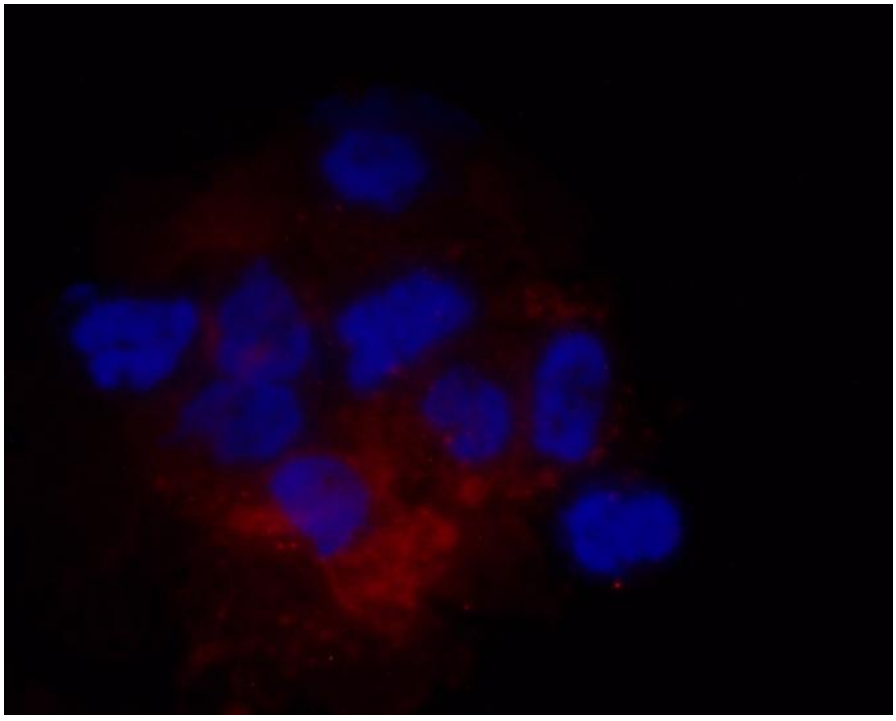

Figure 2-6 HUCC cells stained with EMA (x40 magnification)

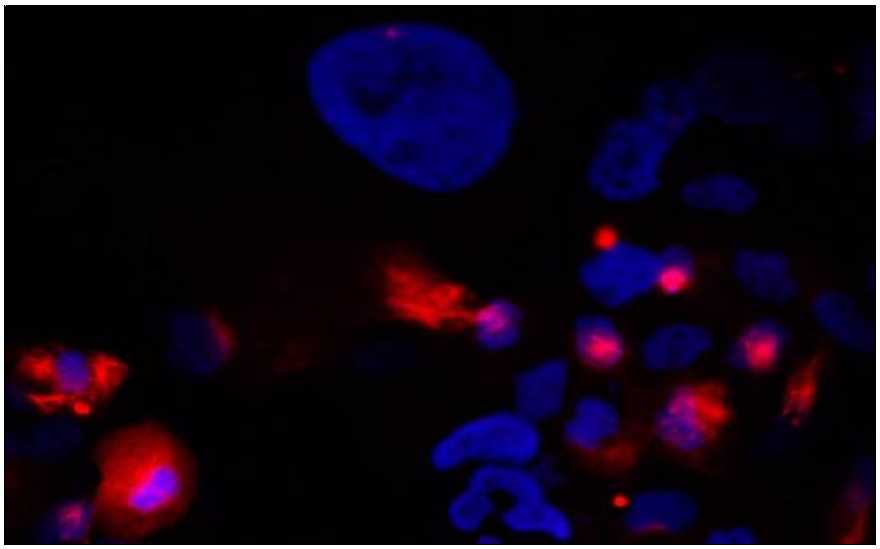

Figure 2-7 HUCC-T1 cells stained with CK7 (x40 magnification)

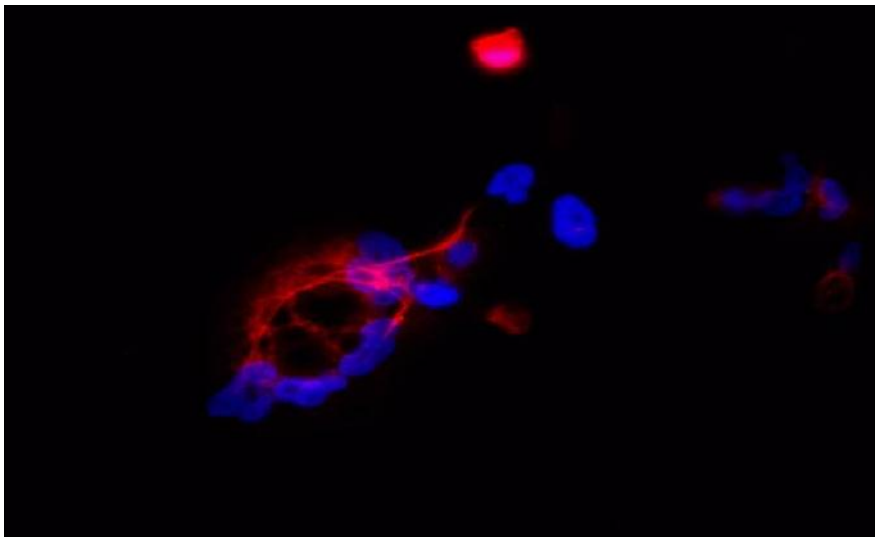

Figure 2-8 HUCC cells stained with Ck19 (x40 magnification)

2.3.3 SkChA1

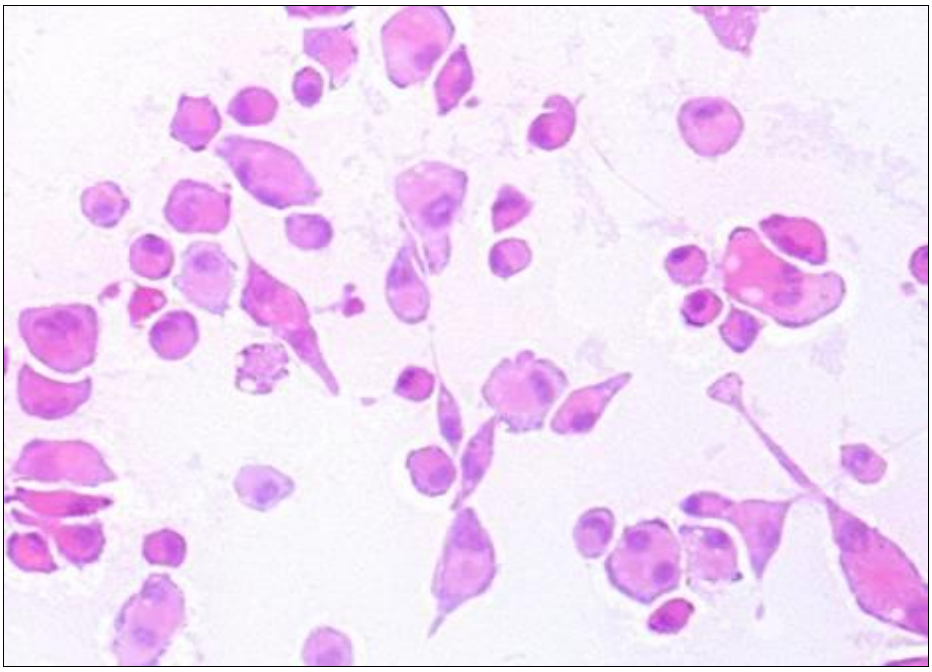

Figure 2-9 SkChA-1 cells stained with Haematoxylin and Eosin (x40 magnification)

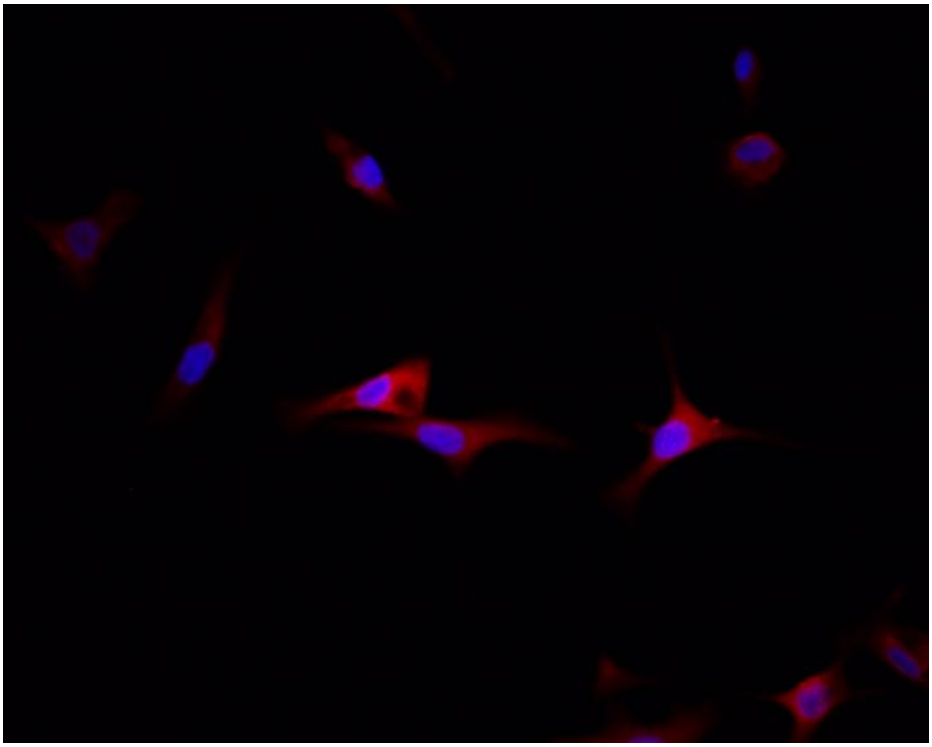

Figure 2-10 SkChA-1 cells stained with EMA (x40)

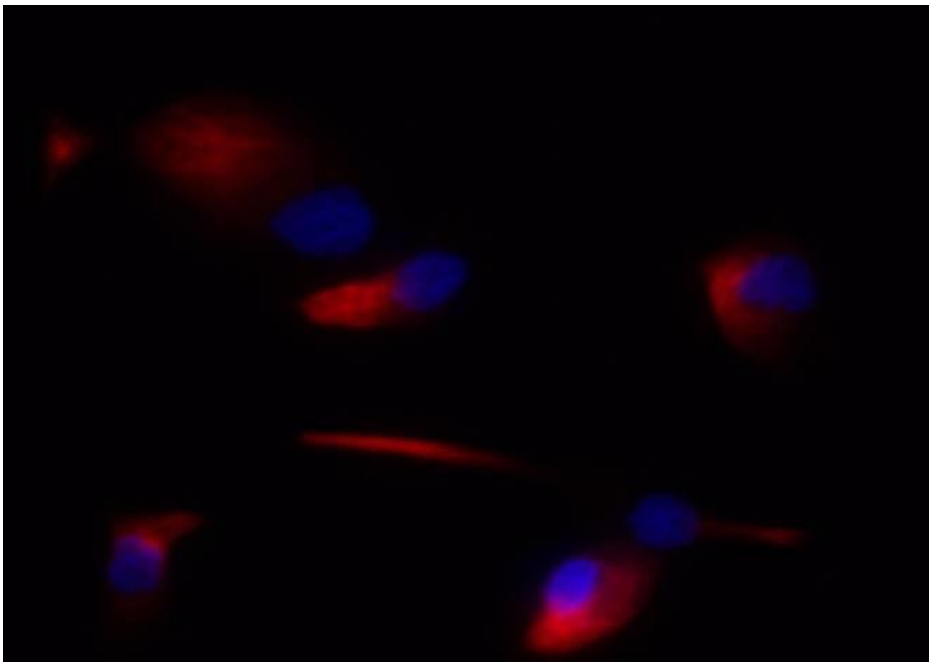

Figure 2-11 SkChA1 cells stained with CK7 (x40 magnification)

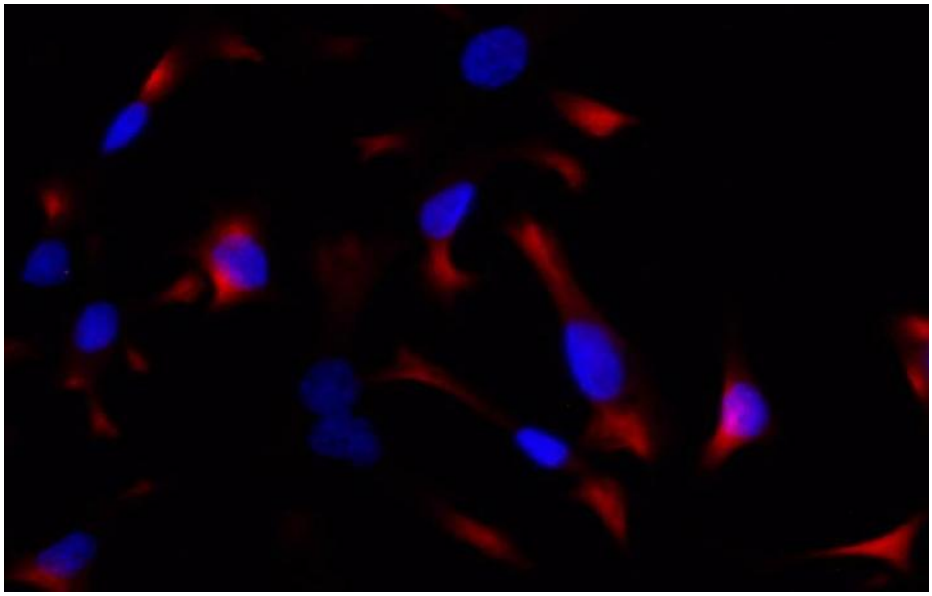

Figure 2-12 SkChA1 cells stained with CK19 (x40 magnification)

2.3.4 M213

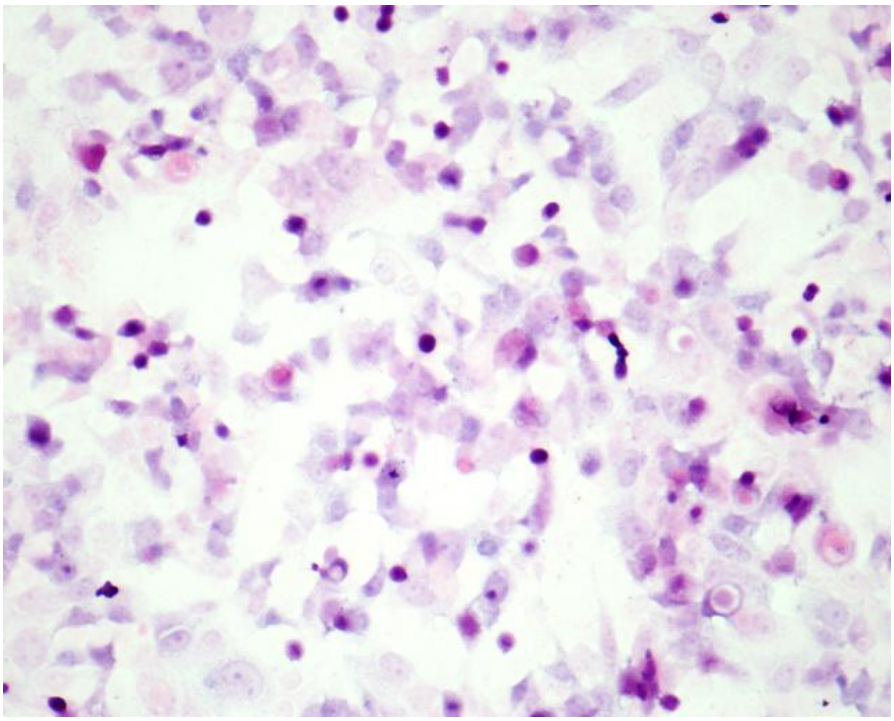

Figure 2-13 M213 cells stained with H&E (x20 magnification)

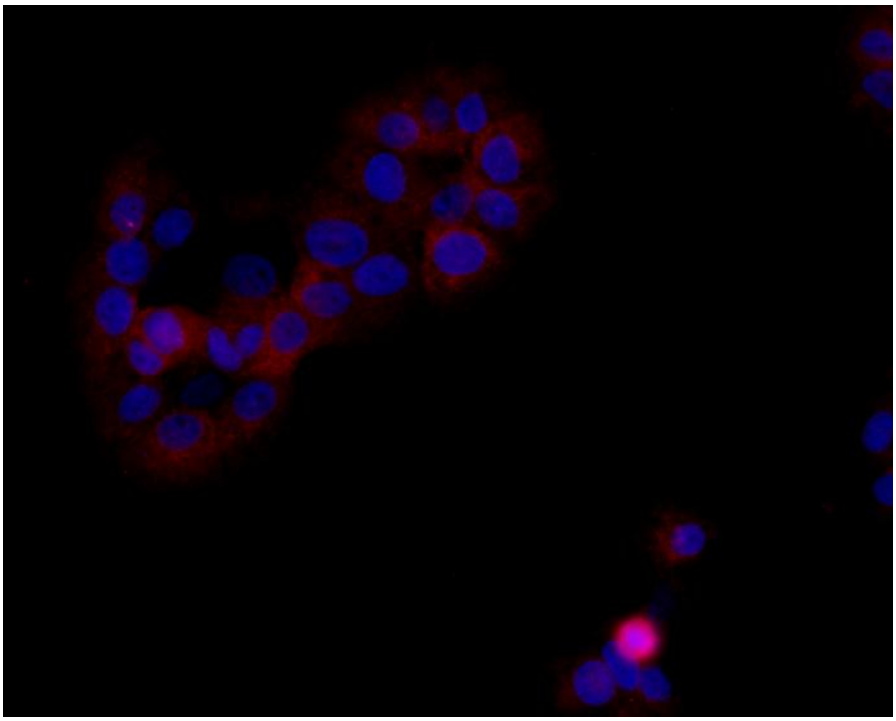

Figure 2-14 M213 cells stained with EMA

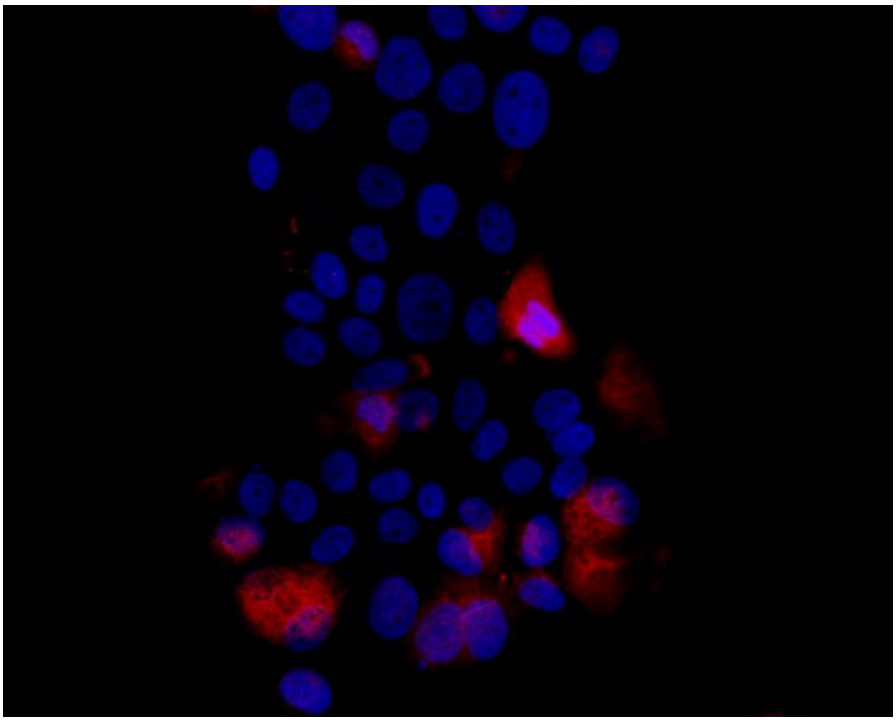

Figure 2-15 M213 cells stained with CK7 (x40 magnification)

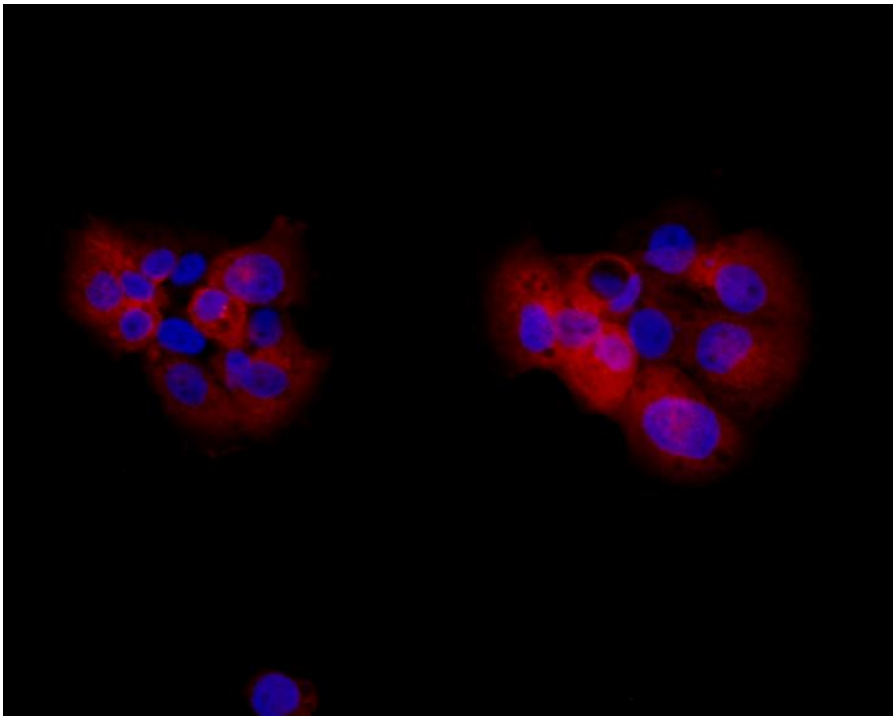

Figure 2-16 M213 cells stained with CK19 (x40 magnification)

2.4 Discussion

The objective of this section of the study was to establish whether or not the panel of cell lines to be used was consistent with having originated from the biliary tree. All cell lines tested positive to CK7, CK19 and EMA which suggests that this is true. They were therefore deemed relevant to the subject of study. These results are consistent with previously published data on these cell lines (Harnois et al., 1997, Yoshikawa et al., 2009).

3 Cell line cytotoxicity assays

3.1 Introduction

3.1.1 Cytotoxicity testing

Cytotoxicity testing is based on one or more mammalian cell lines cultured under conditions where they are actively growing and dividing. Cells are grown in a microtitre plate (such as a 96 well plate) and the rate of multiplication and growth is quantified indirectly by formation of a colour, the intensity of which is directly proportional to the cell volume present. A variety of experiments can be employed but a commonly used method is to compare the rate of proliferation of a cancer cell line in the presence and absence of the test substance, usually after a specified time. Ideally several different cancer cell lines should be used to assess selectivity (Houghton et al., 2007). The two most established cytotoxicity assays are the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the Sulforhodamine B (SRB) assay. The MTT assay was developed in 1986 and relies on mitochondrial reduction of the MTT (which has a yellow colour) to a formazan (deep purple). The formazan can be then quantified through spectrophotometry measurement in an automated plate reader.

The MTT assay's main limitation arises from its dependence on mitochondrial function. Factors affecting their activity can influence the assay outcome. Examples such as variations in cellular levels of NADH and glucose can mimic the effect of cytotoxics.

The SRB assay on the other hand does not rely on mitochondrial function. It is more sensitive, simple, reproducible and more rapid than the MTT assay and gives better linearity, a good signal-to-noise ratio and has a stable end-point that does not require a time-sensitive measurement (Keepers et al., 1991). A detailed explanation of the SRB assay is described further down (see section 3.2.4).

Assessment of cytotoxicity to CCA cell lines was performed *in vitro* using the SRB assay. Three separate cell lines, each representing a different form of CCA, were exposed to agents either alone or in combination. The same cytotoxic agents were also tested in the presence and absence of PK11195.

3.1.2 Cytotoxic agents

3.1.2.1 Gemcitabine

Gemcitabine (20-20 difluorodeoxycytidine) is a water soluble intravenously administered chemotherapeutic agent developed in the late 1980s. It is a nucleoside analog that functions as a pyrimidine antimetabolite by imitating the structure of the natural nucleoside deoxycytidine. It is incorporated into the end of the elongating DNA strand instead of deoxycytidine and works as a masked chain terminator in DNA synthesis. Damaged DNA then triggers an apoptotic pathway (Plunkett et al., 1995).

In the UK, it is currently licensed for the treatment of several solid cancers and is marketed under the brand name of *Gemzar*. Gemcitabine is licensed to be used alone or in combination with other agents in the treatment of metastatic pancreatic cancer, locally advanced or metastatic non-small cell lung cancer, advanced bladder cancer and metastatic breast cancer (BNF, 2012). In the US, gemcitabine has further

approval in the treatment of advanced ovarian cancer (FDA, 2012). In the European Union, Elli Lilly held the patent on gemcitabine until March 2009 after which it became available in its generic form by non-proprietary manufacturers.

Gemcitabine, in combination with cisplatin has recently been shown to be effective in the treatment of patients with cholangiocarcinoma and represents the current treatment of choice (see section 1.1.7.2) (Valle et al., 2010b).

Gemcitabine is generally well tolerated. The most common side effects are mild gastro-intestinal disturbances, musculoskeletal pain, influenza-like symptoms and rashes. Less frequently patients can suffer from renal impairment and pulmonary toxicity.

3.1.2.2 Cisplatin

Cisplatin (also referred to as cisplatinum, or cis-diamminedichloroplatinum(II) (CDDP) chemical formula: $Cl_2H_6N_2Pt$) is a widely used, intravenously administered, platinum-based chemotherapy agent. It has a molar mass of 300g/mol.

Although as a compound it was first described in 1845, its biological activity was not recognised until 1965 (Rosenberg et al., 1965). It received its first FDA licence for human use in 1978. Today, it is used either alone or in combination with other agents to treat patients with a variety of cancers, including sarcomas, lymphomas, germ cell tumours and certain carcinomas (such as small cell lung and bladder cancer). It was the first member of the platinum based class of anti-cancer drugs which now also includes carboplatin and oxaliplatin.

Cisplatin works by crosslinking DNA in several different ways, interfering with mitotic cell division. DNA that has been damaged then triggers DNA repair mechanisms, which in turn force the cell to an apoptotic pathway.

Cisplatin is toxic, and can cause nephrotoxicity, ototoxicity, peripheral neuropathy, hypomagnesaemia and myelosuppression.

Cisplatin, in combination with gemcitabine has recently been shown to be effective in the treatment of patients with cholangiocarcinoma (see section 1.1.7.2) (Valle et al., 2010b).

3.1.2.3 Etoposide

Etoposide was first synthesised in 1966, and was first granted FDA approval in 1983 (Hande, 1998). It is a chemotherapeutic agent that can be administered orally or by slow intravenous infusion. It is used either alone or in combination with other agents and is licensed for use in small cell carcinoma of the bronchus, the lymphomas and testicular cancer (BNF, 2012).

Etoposide's mechanism of action is by inhibiting type II topoisomerase, an enzyme responsible for unwinding DNA. This inhibition results in breakage of DNA strands. Damaged DNA then triggers apoptosis.

Toxic effects of etoposide include alopecia, myelosuppression, nausea, and vomiting.

3.1.2.4 Fluorouracil

Like gemcitabine, fluorouracil (commonly referred to as 5-FU), belongs to a class of chemotherapeutic agents called antimetabolites. Antimetabolites are incorporated into newly manufactured DNA activating apoptotic pathways.

5-FU was first synthesised in 1957 and it is an intravenously administered drug. However, it is also available as capecitabine, an oral prodrug that is converted to 5-FU in the tissues (Shirasaka, 2009). It is licenced for use in the treatment of a number of solid tumours, including gastro-intestinal tract cancers and breast cancer (BNF, 2012).

Toxicity is unusual, but may include myelosuppression, mucositis, and rarely a cerebellar syndrome. On prolonged infusion, a desquamative hand–foot syndrome may occur.

3.1.2.5 PK11195

PK11195 (or 52028 RP; 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline carboxamide) is a peripheral benzodiazepine receptor ligand. It is a synthetic molecule that binds to the mitochondrial benzodiazepine receptor. It can antagonize both Bcl-2 proper and Bcl-XL function thereby stopping the inhibition of apoptosis (Hirsch et al., 1997). Bcl-2 proper and Bcl-XL belong to the Bcl-2 family of proteins.

Addition of PK11195 to CCA cell lines (Egi-1 and Tfk-1) reduced the threshold for ultraviolet, radiation and chemotherapy (etoposide and 5FU) induced apoptosis

(both in vivo and in vitro) (Okaro et al., 2002). Furthermore, in some cell types, PK11195 alone is able to induce apoptosis (Santidrián et al., 2007). PK11195 has not been tested before with the CCA cell lines used in this experiment. Furthermore, its potentiating effects have not been tested in combination therapies.

Oral and intravenous administration of incremental doses of PK11195 to healthy volunteers was well tolerated without any significant side effects (Ferry et al., 1989).

3.2 Materials and methods

3.2.1 Cell lines

Several human derived CCA cell lines were used, each representing a subtype of the disease. All cell lines were of the adherent type. Acquisition of cell lines proved to be a challenge due to the scarce availability of CCA cell lines. Most can only be acquired through non commercial sources. Indicative of this is that the European Collection of Cell Cultures (ECACC), the largest of its kind in Europe and possessing more than 1,100 cell lines, does not have offer a CCA cell line. The American Type Culture Collection (ATCC), an equivalent organisation in the United States does not have any CCA cell lines in its collection either.

3.2.1.1 HUCC T1

HUCC-T1 is derived from the ascitic fluid of a 56 year old male patient from Japan (Miyagiwa et al., 1989). The patient died 2 months after the cell line was established from an intra hepatic CCA. HuCC-T1 cells, kindly provided by Dr. Giles Smith (University College London) were routinely cultured in RPMI 1640 (Sigma-Aldrich,

Dorset, UK), supplemented with 10% (volume to volume) heat inactivated foetal calf serum (FCS), 2 mM L-Glutamine (Gibco-Invitrogen, Paisley, UK) streptomycin and penicillin.

3.2.1.2 SK-ChA-1

SkChA1 is derived from the ascitic fluid of a 47 year old female patient in Germany (Knuth et al., 1985). The patient died one month after the cell line was established of an extra hepatic CCA. Sk-ChA-1 cells were also provided by Dr. Giles Smith and were cultured in the same fashion and medium as HUCC-T1 cells.

3.2.1.3 SG231

SG231 is a cholangiocarcinoma cell line, however, its original anatomical location has not been made clear by the authors (Storto et al., 1990). SG231 were also provided by Giles Smith and were cultured in Dulbecco's Modified Eagle Medium (DMEM - Gibco 21969) supplemented with 10% heat inactivated FCS. To a 500 ml bottle of DMEM the following were added: 5 ml of L-Glutamine, 5 ml of NEAA (x100, Gibco 11140) and 0.5 ml of gentamicin (Sigma G1272).

SG231 cells were sub-cultured using 0.5% trypsin/EDTA (Sigma-Aldrich).

3.2.1.4 KKU-M213

KKU-M213 is an intrahepatic adenosquamous cholangiocarcinoma cell line derived from a 58 year old male patient from Thailand. The cells were kindly donated by Dr Temduang Limpaiboon and Ruethairat Sriraksa from the Liver Fluke and

Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Thailand.

KKU-M213 cells were cultured in the same medium as HUCC-T1 cells.

3.2.1.5 H69

H69 cells are a cell line derived from normal human intrahepatic cholangiocytes and were produced using retroviral transduction of SV40 and were also provided by Dr. Giles Smith (Grubman et al., 1994).

H69 cells were cultured in medium, the recipe for which was kindly provided by Dr. Sharon DeMorrow from the Texas A&M Health Science Center. The medium was made up with the following: DMEM with high glucose (Invitrogen, Cat No. 11095098), DMEM/F-12, (Invitrogen, Cat No. 11330057), 50ml Foetal bovine serum (Invitrogen, Cat No. 16000044), Penicillin/Streptomycin (Invitrogen, Cat No. 15140155), Adenine (Sigma, Cat No. A2786), Insulin (Sigma, Cat No. I2643), Epinephrine (Sigma, Cat No. E4250), 3,3 Triiodo-l-thyronine (Sigma, Cat No. T6397), Epidermal Growth Factor (Millipore, Cat No. 01-102), Hydrocortisone (Sigma, Cat No. H0888).

Since the H69 cell line represents an immortalised normal cholangiocyte, it was not used in chemotherapy experiments. It was used in experiments described later (see section 4.2.2) as a reference for comparison.

3.2.2 Cell storage and culture

All cell lines were stored in liquid nitrogen tanks (-196^{o}C), at a volume of roughly 1 million cells aliquot per cryovial. Cells were suspended in 1mL of the appropriate medium containing 20% heat inactivated FCS and 10% DMSO prior to freezing.

Revival of cells from liquid nitrogen storage was performed by quick thawing in a 37^{o}C water bath. Thawed cells were washed in 10 ml of medium, harvested by centrifugation for 5 minutes at 1000 g and were then transferred to 25 cm^2 culture flasks containing fresh culture medium.

Cell culture was carried out in Class 2 laminar flow cabinets (Scanlaf Mars) and all materials were sterile and disposable. All cell lines were adherent and were grown as monolayers in plastic tissue culture flasks incubated in a humidified atmosphere of 5% CO_2 at 37^{o}C. Cells were regularly checked and once they had covered the surface available for growth (to approximately 80% confluence) were sub-cultured in a 1:3 split. The smallest culture flasks used were 25cm^2 whilst large volumes were cultured in triple layer flasks providing 500cm^2 of surface area for growth. Exhausted media, due to cell metabolism and natural degradation of ingredients was changed periodically as required, to ensure optimal growing conditions.

3.2.3 Cell counting

Cell numbers and concentrations were calculated using an improved Neubauer haemocytometer visualised through an inverted microscope. The chamber was loaded with a 10µl volume of medium containing suspended cells mixed with equal volume of trypan blue and covered with a disposable glass cover slip. Trypan blue is

a stain that selectively transverses cell membranes of dead cells. It hence allows identification of viable cells via dye exclusion.

3.2.4 The sulforhodamine B assay

Growth characteristics and sensitivity of CCA cell lines to chemotherapy agents were measured by using the SRB assay as described in 1990 by Skehan et al (Skehan et al., 1990). The SRB assay remains one of the most commonly used methods for *in vitro* cytotoxicity screening.

Sulforhodamine B is a bright pink protein dye. The assay relies on the ability of SRB to bind to the basic amino acid residues of cells fixed with TCA, in an electrostatic and pH dependent manner. In mildly acidic conditions, SRB binds to basic amino acids and in mild basic conditions it dissociates. This property can be exploited by extracting SRB and then solubilising it for colorimetric measurement. Quantification of the dye directly relates to the amount of protein present, which is linearly proportional to the number of cells present. As such SRB acts as a surrogate endpoint for cell mass at the end of the cell growth experiment.

The assay has several advantages: it is practical, it is not destructive, treated culture plates remain indefinitely stable at several steps and it is relatively low cost. As such it is considered an appropriate and sensitive assay to measure drug-induced cytotoxicity even at large-scale application. It is worth mentioning that the SRB assay is the method of choice for the NCI-60 project - a high-flux anticancer drug screening program that has tested the activity of several hundred thousand compounds on a

panel of 60 cancer cell lines (Monks et al., 1991). None of these cell lines are CCAs (Sharma et al., 2010).

Cells suspended in 100µl of growth medium per well, were added at the relevant inoculation densities (see section 3.2.5) to 96 well microtitre plates and left to adhere for 24 hours. At this stage the medium was aspirated and replaced with the agent(s) under investigation at the relevant concentration, diluted in 200µl of fresh growth medium and left to incubate for two cell cycles. Each agent was routinely prepared and tested at five to six concentrations ranging from $1x10^{-4}$ to $1x10^{-9}$M with 10-fold dilutions in between. Each concentration was replicated three to four times on the same plate. Experiments were replicated identically on three separate 96 well plates, carried out on different days thus givng a total of at least 9 replicates for each experiment.

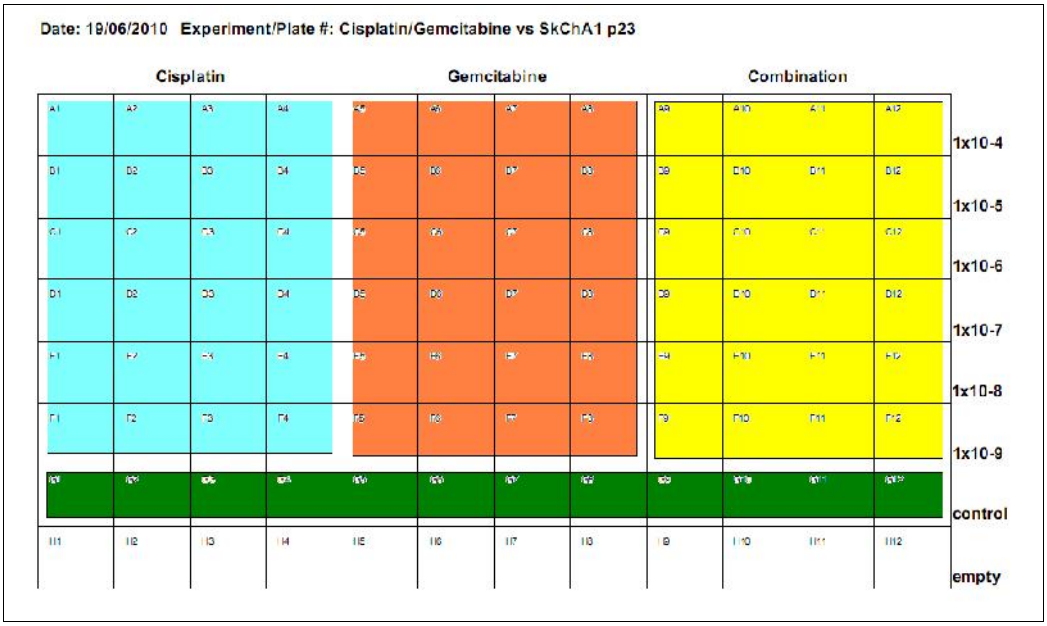

Figure 3-1 Typical 96 well plate layout for cytotoxicity testing

The plates were read on a Tecan SPECTRAFLUOR plus plate reader which gave the optical densitometry (OD) of each well. The automatic plate reader had the following parameters set: a 10 second orbital shake, followed by four random location absorbance readings per well, at a wavelength of 492nm.

Cell survival was calculated using the mean optical density (OD) of treated cells as a percentage of the mean OD of controls, using the following equation:

$$\text{Cell survival (\%)} = \frac{OD\ of\ treated\ cells}{OD\ of\ control\ cells} \times 100$$

Dose response curves were generated using commercially available statistical software (Graphpad Prism 5.0). Two-way analysis of variance (ANOVA) with Bonferoni post hoc tests were used to compare different concentrations and regimes. The methodology for statistical analysis was discussed with a medical statistician (Dr Nicos Middleton, PhD, Harvard School of Public Health).

3.2.5 Growth characteristics

The SRB assay should ideally be performed after cells have been exposed to a cytotoxic for about 2 cell cycles (Skehan et al., 1990). For practical reasons, inoculation densities that would produce a cell cycle of about 48 hours were sought. To establish the ideal inoculation densities which would achieve these parameters, a separate experiment was carried out. Incremental numbers of cells, ranging from 1,000 cells per well to 80,000 cells per well, were added to 96 well plates and incubated for up to 5 days. Every 24 hours one plate was sacrificed, and the SRB assay was performed. As optical density is directly proportional to the number of

cells present, the results were plotted on a graph and the cell doubling time was determined for each inoculation density.

3.2.6 Cytotoxicity assays

Cytotoxicity assays were carried out using the SRB protocol as described in paragraph 3.2.4.

3.2.6.1 Preparation of cytotoxic agents

Although the risks of occupational low level exposure to cytotoxic agents has not been determined, all agents were treated as hazardous materials. All cytotoxics were prepared in a safety laminar flow cell culture cabinet to ensure maximum sterility and surfaces were wiped with 70% alcohol afterwards.

3.2.6.1.1 Gemcitabine preparation

Gemcitabine has a molar mass of 263.198 g/mol. It was acquired as a stock liquid solution with a concentration of 38mg/ml from the Hammersmith Hospital pharmacy and stored at -80^{o}C until use (Humbert et al., 2010). It was diluted to concentration of 1x10^{-4} to 1x10^{-9} mM with cell culture medium prior to in vitro use.

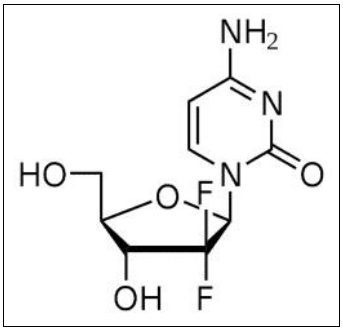

Figure 3-2 Structure of gemcitabine

3.2.6.1.2 Cisplatin preparation

Cisplatin comes in an off-white to orange powder form and must be solubilised prior to experimental use in cell culture (Enzo Life Sciences (UK) LTD, Exeter, Product code: ALX-400-040-M050). It is sparingly soluble in water and insoluble in ethanol. Although readily soluble in DMSO, a commonly used solvent, DMSO substitutes the chloride ligands resulting in a variety of different compounds (Kerrison and Sadler, 1977). This reduces the effect of cisplatin (Gebel and Koenig, 1999).

Hence, cisplatin was solubilised in NNDMF (dimethyl formamide) as suggested by the product distributor. Ten milligrams of cisplatin were added to 1.333ml of NNDMF, resulting in a 25mM concentration. Solutions were then made up for $1x10^{-4}$ to $1x10^{-9}$ mM with cell culture medium.

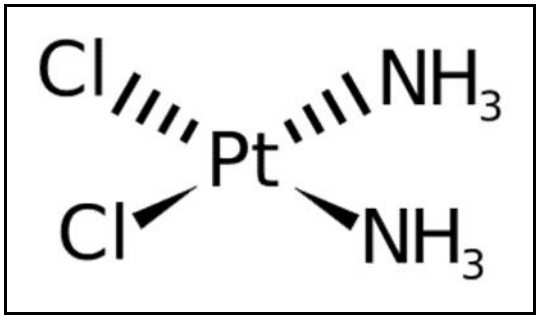

Figure 3-3 Cisplatin Structure

3.2.6.1.3 Etoposide preparation

Etoposide has a molar mass of 588.557 g/mol and comes as a white powder (Sigma Aldrich, UK, Prod code: E1383). Ten milligrams of etoposide were solubilised in 0.34 ml of DMSO to create a stock solution. DMSO was used as a solvent in accordance with the product literature and previous publications (Olmos et al., 2004, Joel et al., 1995). No evidence was found to suggest that DMSO affects the potency of

etoposide in a similar manner to cisplatin. Solutions were then made up for $1x10^{-4}$ to $1x10^{-9}$ mM with cell culture medium.

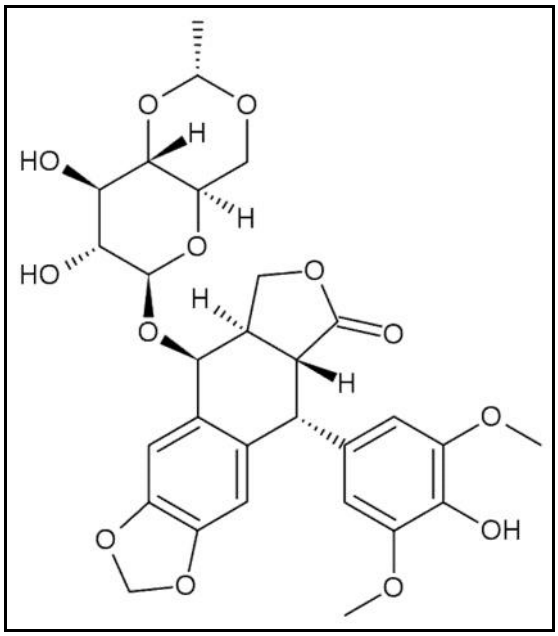

Figure 3-4 Structure of etoposide

3.2.6.1.4 5 FU preparation

5-Flurouracil has a molar mass of 130.077 g/mol (Sigma Aldrich, Prod Code:F6627). It is water soluble so it was made up to concentrations of $1x10^{-4}$ to $1x10^{-9}$ mM with cell culture medium (Krishnaiah et al., 2002).

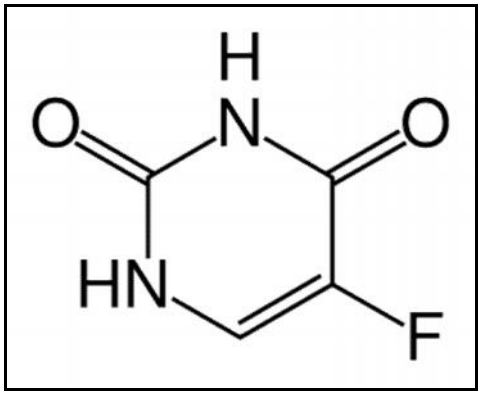

Figure 3-5 Structure of 5-FU

3.2.6.1.5 PK11195 preparation

PK11195 has a molar mass of 352.856 g/mol (Sigma Aldrich UK, Prod Code: C0424). It was solubilised in DMSO and then diluted to $1x10^{-4}$ to $1x10^{-9}$ mM with cell culture medium (Ryu et al., 2005). No published evidence was found to suggest that DMSO affects PK11195 in a similar manner to cisplatin. DMSO, a colourless liquid is generally considered safe and non toxic (Brown et al., 1963). DMSO is used as a topical analgesic and anti inflammatory and is championed as a 'natural healer', to the extent that several 'self-help' books in popular culture have been published (Walker, 1993, Walters, 1993). However, at high concentrations above 10%, DMSO can be cytotoxic in cell culture (Da Violante et al., 2002).

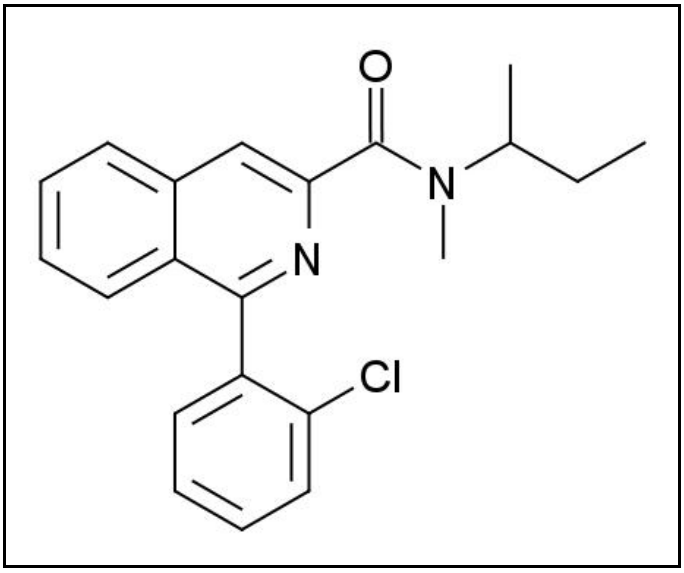

Figure 3-6 Structure of PK11195

3.2.6.2 Combination chemotherapy

Cells were exposed to monotherapy and all different permutations of combination chemotherapy. The agents used in combination chemotherapy were used simultaneously reflecting usual clinical practice:

Gemcitabine and cisplatin

Gemcitabine and etoposide

Gemcitabine and fluorouracil

Cisplatin and etoposide

Cisplatin and fluorouracil

Etoposide and fluorouracil

3.2.6.3 Chemotherapy in combination with PK11195

To determine whether or not PK11195 affected the sensitivity of cell lines to chemotherapy agents, each single chemotherapeutic agent was assessed in combination with PK11195. Cell lines were exposed to a chemotherapy regime alone, PK11195 alone or a combination of PK11195 plus agent at the same concentrations. Furthermore combination chemotherapy regimes were also tested in the presence and absence of PK11195.

The concentrations ranged from $1x10^{-4}$ to $1x10^{-9}$ M. Four wells of each concentration were used and experiments were replicated 3 times, giving a total of at least 12 replicates per experiment. More details on the protocol are given in chapter 3.2.4.

3.2.7 BCL-2 expression

3.2.7.1 RNA extraction

Cells (H69p35, M213p36, SkChA1p58 and HUCCp99) were grown to sufficient quantities as described previously (section 3.2.1). Cells were lifted and washed in PBS and pellets were stored at -80^{o}C. RNA extraction and purification was performed using the RNeasy Mini Kit (Qiagen, Cat No. 74104). The protocol used was the HCSG modification to the published protocol by Qiagen, as outlined below:

A cell pellet was added to 600 µl of RLT buffer and passed through a QIA shredder column (Qiagen, Cat No. 79654) by centrifuging for 2 minutes at full speed. The flow-through was kept and 600µl of 100% EtOH was added and mixed by pipetting. The mixture was transferred to an RNeasy spin column in a 2 ml tube at 700µl at a time and centrifuged at maximum speed for 15 seconds (>8,000g). The flow through was discarded, 500µl of RPE buffer was added and centrifuged again at maximum speed for 15 seconds. The flow through was discarded and a 700µl of RWI buffer was applied to the column and centrifuged for 15 seconds at maximum speed. The flow through was again discarded and 500µl of RPE buffer was applied and centrifuged again for 15 seconds at maximum speed. The flow through was discarded and 500µl of RPE buffer was applied and centrifuged again for 2 minutes at 8,000g. The flow through was discarded and the columns were spun for 1 minute at full speed to collect any remaining buffer, which was then discarded. The samples were collected by applying two successive aliquots of 50µl of water and spun for 1 minute. The flow through was collected and the columns discarded.

3.2.7.2 RNA quantification

RNA was quantified using a Nanodrop ND 1000 spectrophotometer and samples were stored in 50µl aliquots at -80^{o}C.

3.2.7.3 cDNA conversion

cDNA conversion was carried out using the 'High Capacity cDNA Reverse Transcription Kit' (Invitrogen, Applied Biosystems, Cat No. 4368814). A mastermix was made up in advance using the following recipe: 10X Reverse Transcription Buffer 2µl per reaction, 25X dNTPs 0.8µl per reaction, 10X random primers 2µl per reaction and MultiScribe™ Reverse Transcriptase 1 µl per reaction making a total of 5.8µl of mastermix per reaction.

Each mastermix aliquot of 5.8µl was added to 14.2µl of RNA and added to a well on the reaction plate. RNA was diluted if necessary in advance using H_2O, to achieve the recommended range by the manufacturer between 0.002 and 0.2 µg/µL. The plate was covered, briefly centrifuged and placed on the thermal cycler. The settings used for the reaction were as follows: 10 minutes at 25^{o}C, 120 minutes at 37^{o}C, 5 minutes at 85^{o}C and then at 4^{o}C until discontinuation. The plate was stored at -4^{o}C until the qrt-PCR reaction.

3.2.7.4 TaqMan Assay

BCL-2 expression was assessed using a TaqMan Gene Expression Assay (Applied Biosystems, Assay ID: Hs00236329_m1). β- actin (Applied Biosystems) was selected to act as an endogenous control. Each reaction was performed in duplicate.

The reaction mixture was made up according to the following recipe: 20× TaqMan Gene Expression Assay 1µl, 2× TaqMan Gene Expression Master Mix 10µl, cDNA template 4.0 µl and RNase-free water 5.0 µl per reaction. A MicroAmp 96 well reaction plate was used (Applied Biosystems, Cat No. 4306737) and each reaction was loaded into a separate well. The plate was covered, briefly centrifuged and loaded into the thermal cycler (Applied Biosystems 7900HT Fast Real-Time PCR System). The conditions were set as follows: 10 minutes at 95^{o}C followed by 40 cycles at 95^{o}C for 15 seconds and 60^{o}C for 1 minute.

3.2.7.5 Analysis

Data analysis was performed using SDS 2.4, supplied by Applied Biosystems on CD-ROM as part of the TaqMan Assay kit.

3.3 Results

3.3.1 Growth characteristics

The mean values of the absorbance for each cell line at incremental inoculation densities are depicted in the figures below.

3.3.1.1 SkChA1

The target of a 48 hour doubling time was best served by an inoculation density of 20,000 cells per well (calculated by non-linear regression as 50.60 hours).

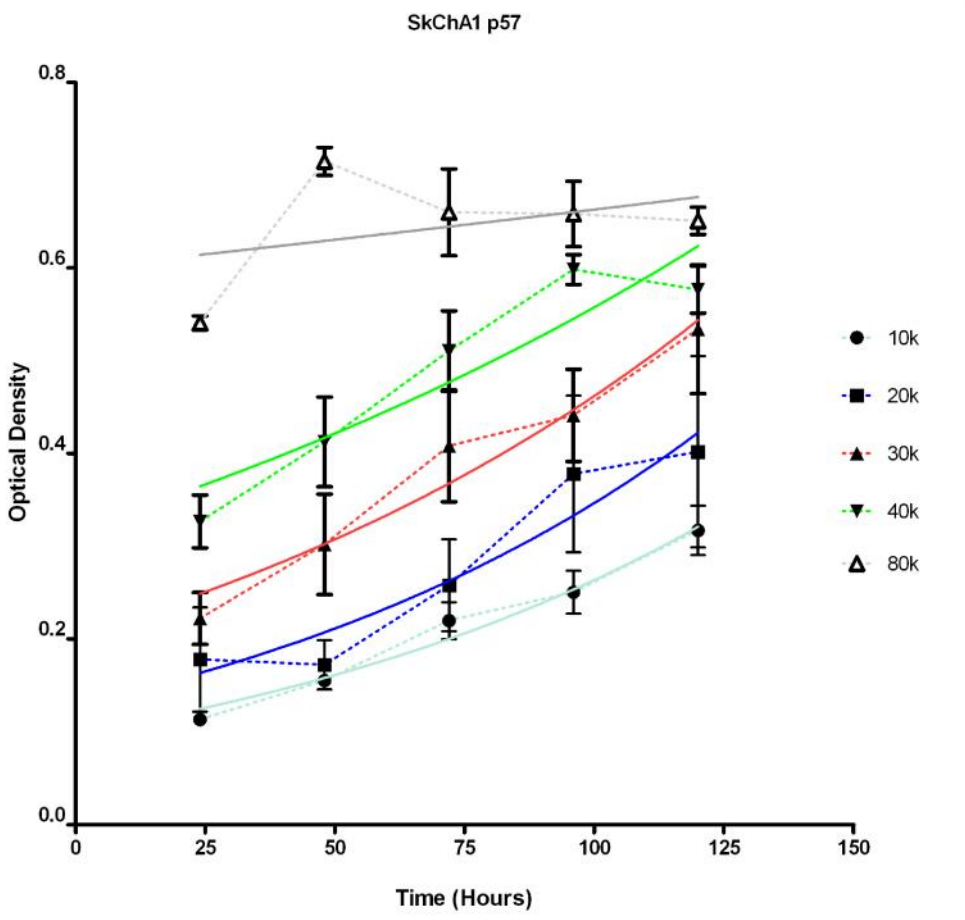

Figure 3-7 SkChA1 growth characteristics

3.3.1.2 HUCC T1

The target of a 48 hour doubling time was best served by an inoculation density of 20,000 cells per well (calculated by non-linear regression as 45.71 to 53.97 hours).

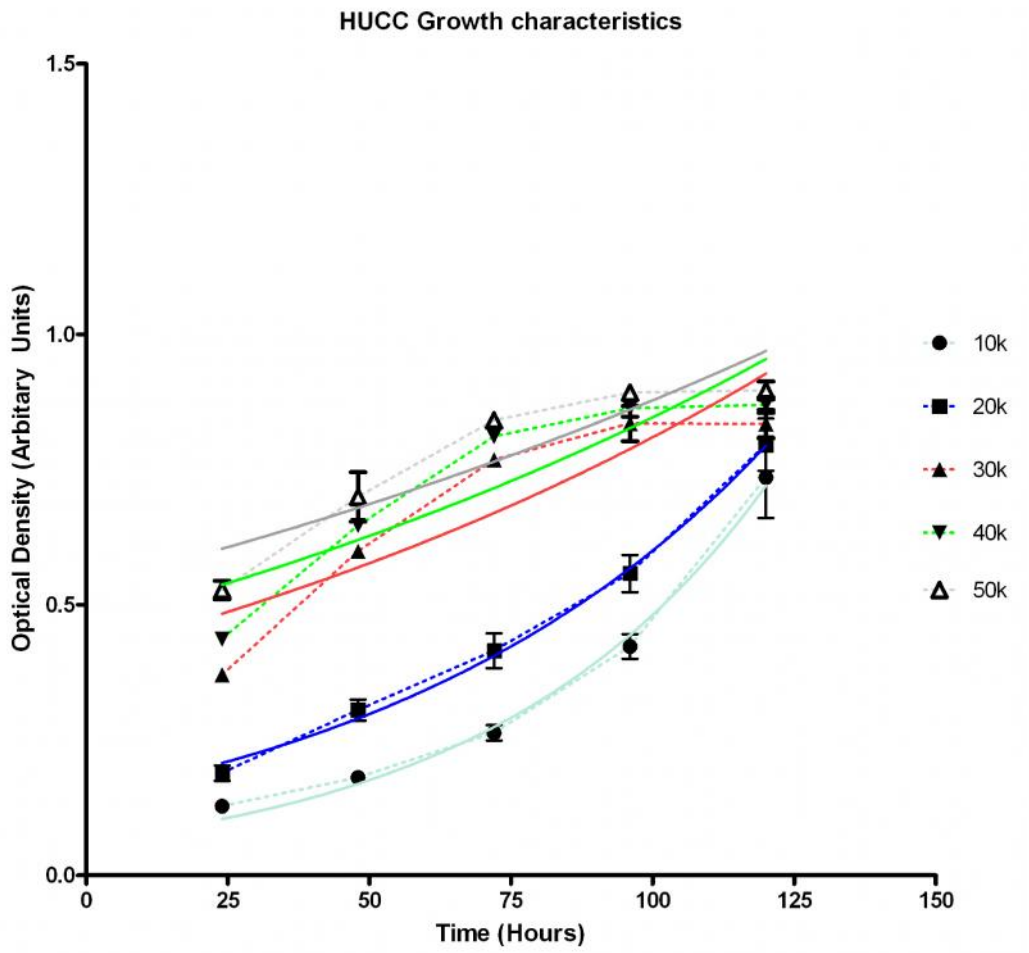

Figure 3-8 HUCC growth characteristics

3.3.1.3 KKU M213

The target of a 48 hour doubling time was best served by an inoculation density of 10,000 cells per well (Calculated by non-linear regression as 41.80 to 57.22 hours).

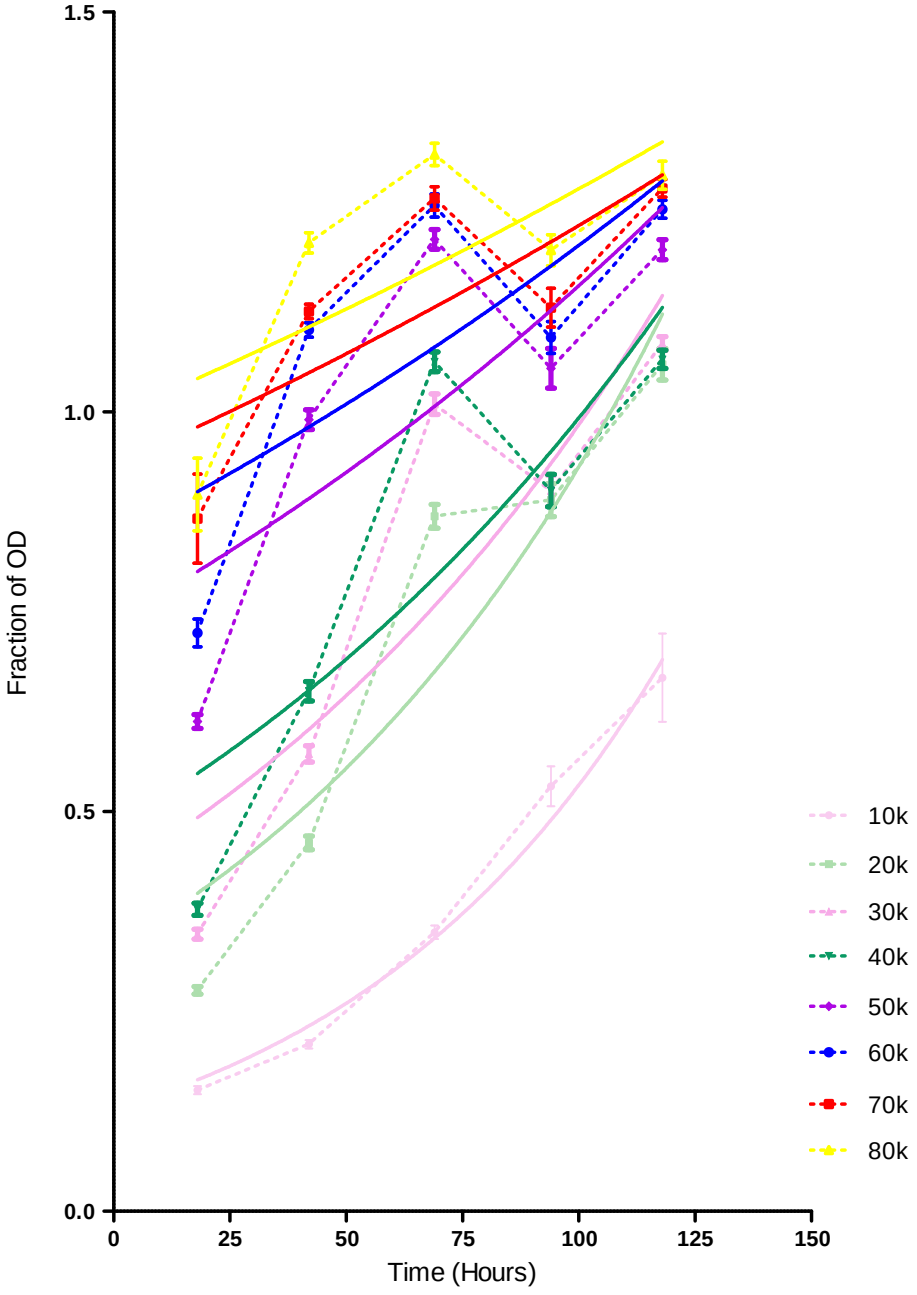

Figure 3-9 Growth Characteristics KKU M213 cells

A summary of the inoculation densities used in CCA cell lines throughout cytotoxicity testing in 96 well plates are shown in Table 6.

Cell Line	Inoculation density (per well)
HUCC-T1	20,000
SkChA-1	20,000
M213	10,000

Table 6 Inoculation densities used in CCA cell lines

3.3.2 Cytotoxicity assays

3.3.2.1 Preliminary experiments

To ensure experimental validity, a series of quality control experiments were carried out to optimise the methodology.

Cell lines were exposed to DMSO in the absence of cytotoxic agents to ensure that this solvent was not contributing to cell death at the concentrations used. Although previously shown not to affect cell studies at the concentrations used in these experiments, DMSO has not been tested with the particular cell lines used in these experiments (see section 3.2.6.1.5).

The method of adding chemotherapy agents to growing cells varied from reviewing published protocols (Houghton et al., 2007, Vichai and Kirtikara, 2006). Once cells were added to a 96 well plate suspended in 100µL of culture medium, they were allowed 24 hours to adhere before addition of the agent under scrutiny. One option

was to remove the original medium and replacing it with 200µL of fresh medium containing the agent at the appropriate concentration. The other option was to leave the 100µL of medium in the 96WP and just add another 100µL of agent suspended in medium at double the intended final concentration. Preliminary experiments were performed to check whether there would be a difference in these two methods.

3.3.2.1.1 Exposure to DMSO

The graphs below illustrate the effect of DMSO on CCA cell lines. Increasing concentration of DMSO has not altered cell culture growth pattern.

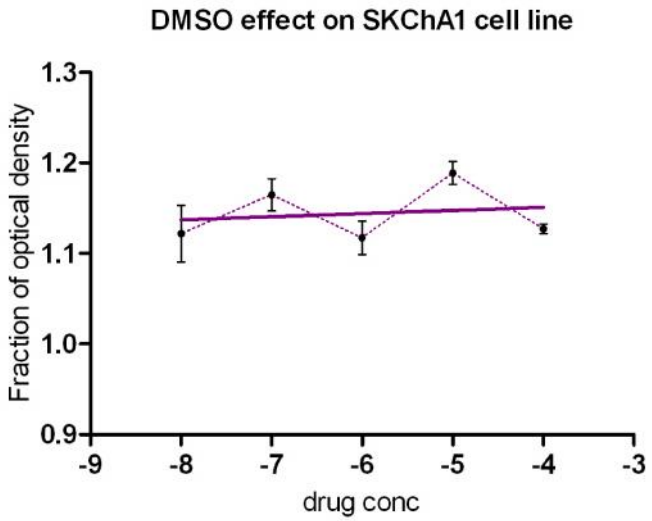

Figure 3-10 DMSO effect on SkChA1

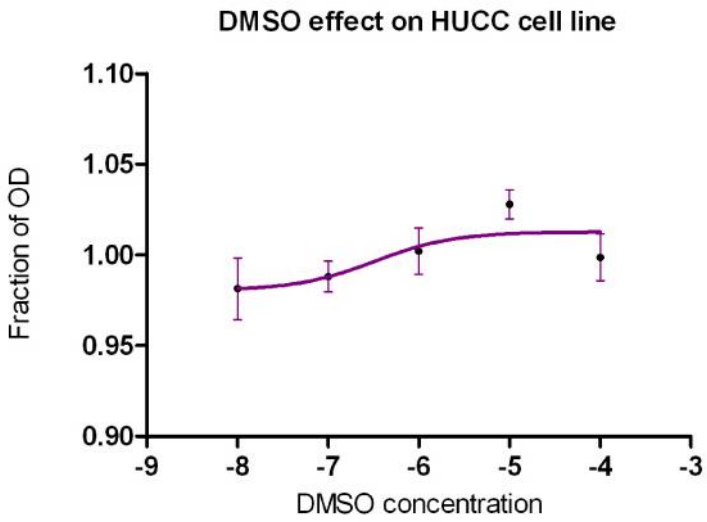

Figure 3-11 DMSO effect on HUCC

3.3.2.1.2 Removal vs non removal of medium prior to addition of cytotoxic agent

The graphs below represent a series of experiments to check the effect of removal of medium prior to the addition of cytotoxic agent as compared to addition of further medium with double concentration of the cytotoxic agent.

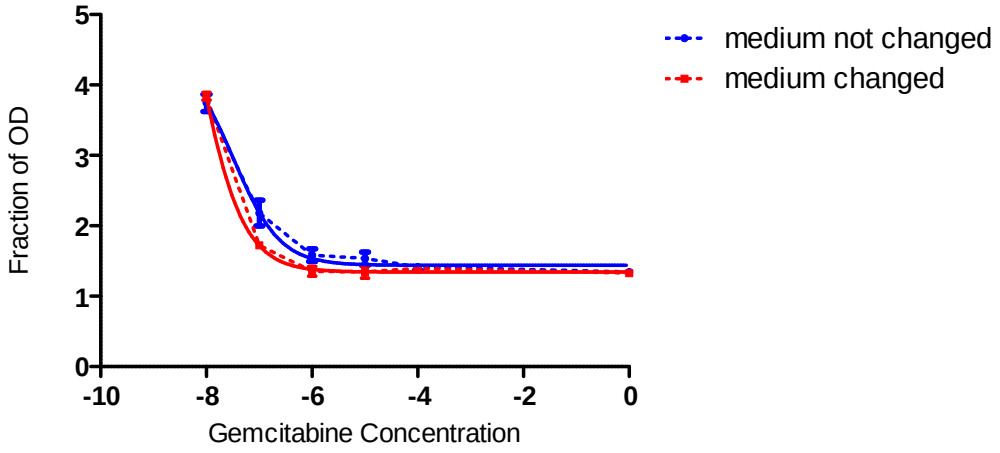

Figure 3-12 SkChA1 cell line exposed to Gemcitabine (comparison between two methodologies)

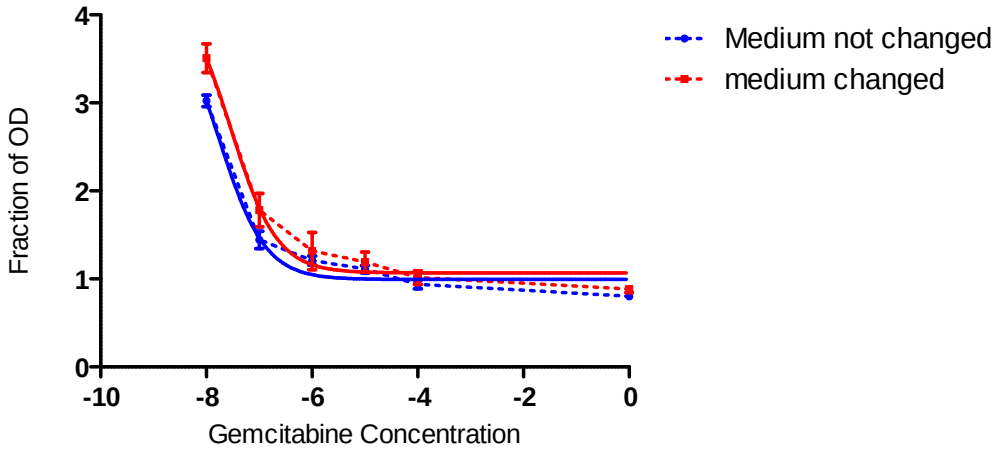

Figure 3-13 HUCC cell line exposed to Gemcitabine (comparison between two methodologies)

Complete renewal increases the amount of culture medium available and removes waste products of metabolism. On the other hand by removing the medium from the 96WP, there is a risk of aspirating adherent cells, and this could be done disproportionately between wells. The extra step also adds extra risk of contamination, either by introducing an infection or by cross contaminating wells with the wrong cells. Finally there is a risk of dilution discrepancies, as aspirating 100% of the 100µL is technically challenging. There was no significant difference observed between the two methodologies above. As such the method of 'no medium change' was used thereafter in all cytotoxic experiments.

3.3.2.2 Single agent testing

3.3.2.2.1 Cisplatin

The dose response curves of the selected cell lines to cisplatin monotherapy are shown below.

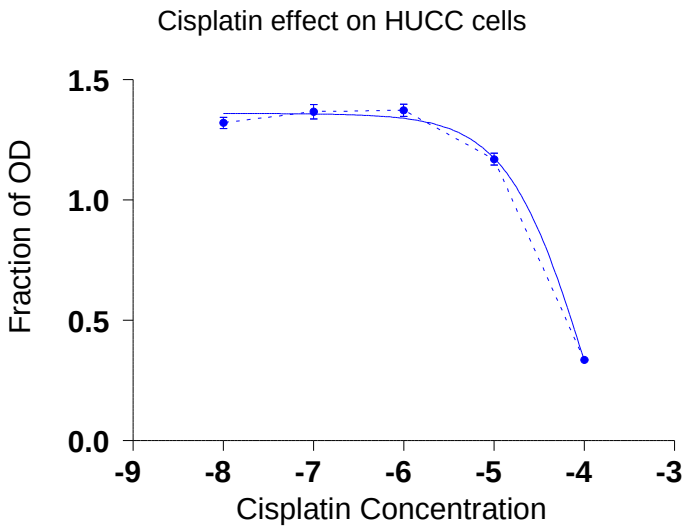

Figure 3-14 Cisplatin effect on HUCC Cells

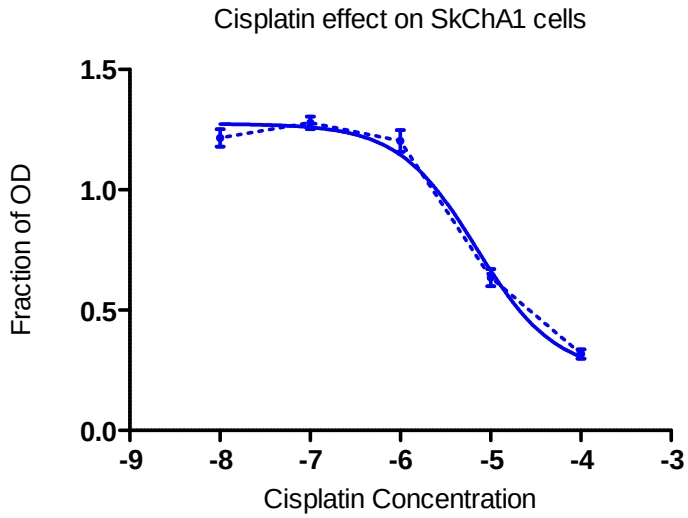

Figure 3-15 Cisplatin effect on SkChA1 cells

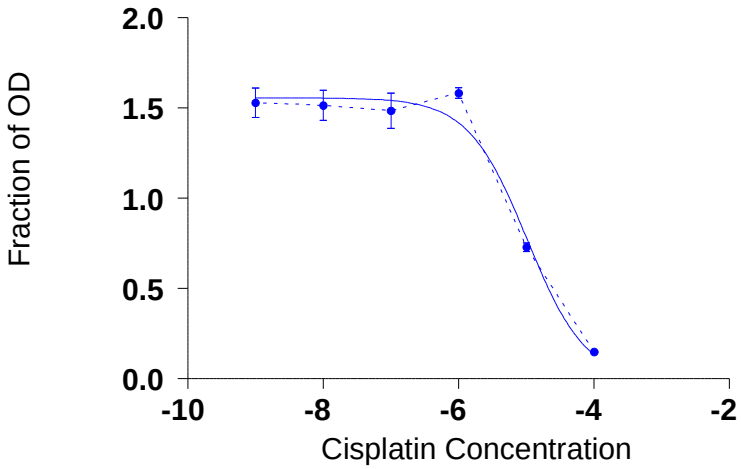

Figure 3-16 Cisplatin effect on KKU M213 cells

3.3.2.2.2 Cisplatin in combination with PK11195

The dose response curves of cell lines exposed to cisplatin monotherapy in the presence or absence of PK11195 are shown below. Visual inspection of the graphs shows that PK11195 enhanced the effect of cisplatin in all cell lines at varying degrees. However, when examined using 2 way ANOVA, this enhancement was not always statistically significant. If statistically significant, the greatest percentage enhancement is given in the text following each graph along with the concentration of cytotoxic agent at which this occurred.

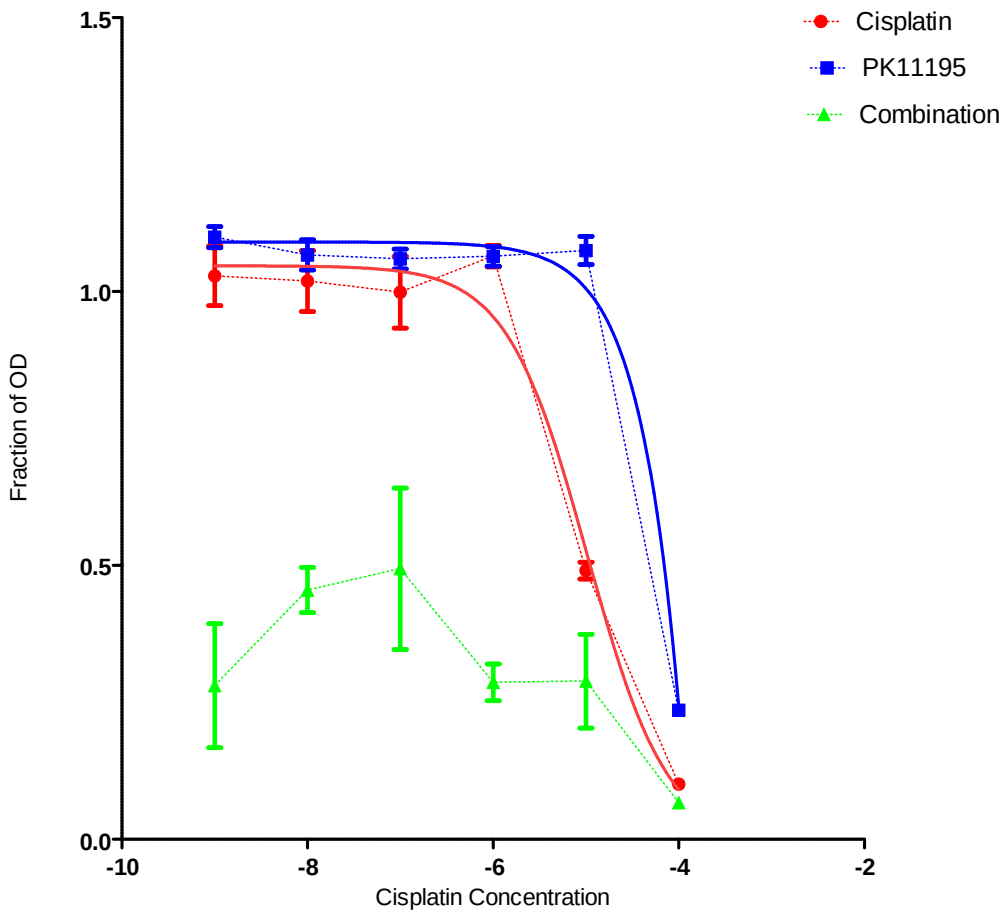

Figure 3-17 Effect of PK11195 addition to cisplatin on KKU M213 cells

The addition of PK11195 to cisplatin therapy in KKU M213 cells resulted in a significant enhancement of cytotoxic effect. The largest effect was seen at a concentration of $1x10^{-6}$ mM as indicated by the graph above (Figure 3-17). At this concentration PK11195 enhanced the action of cisplatin by 73.10% (95% CI: 49.44-96.83%, $P<0.001$).

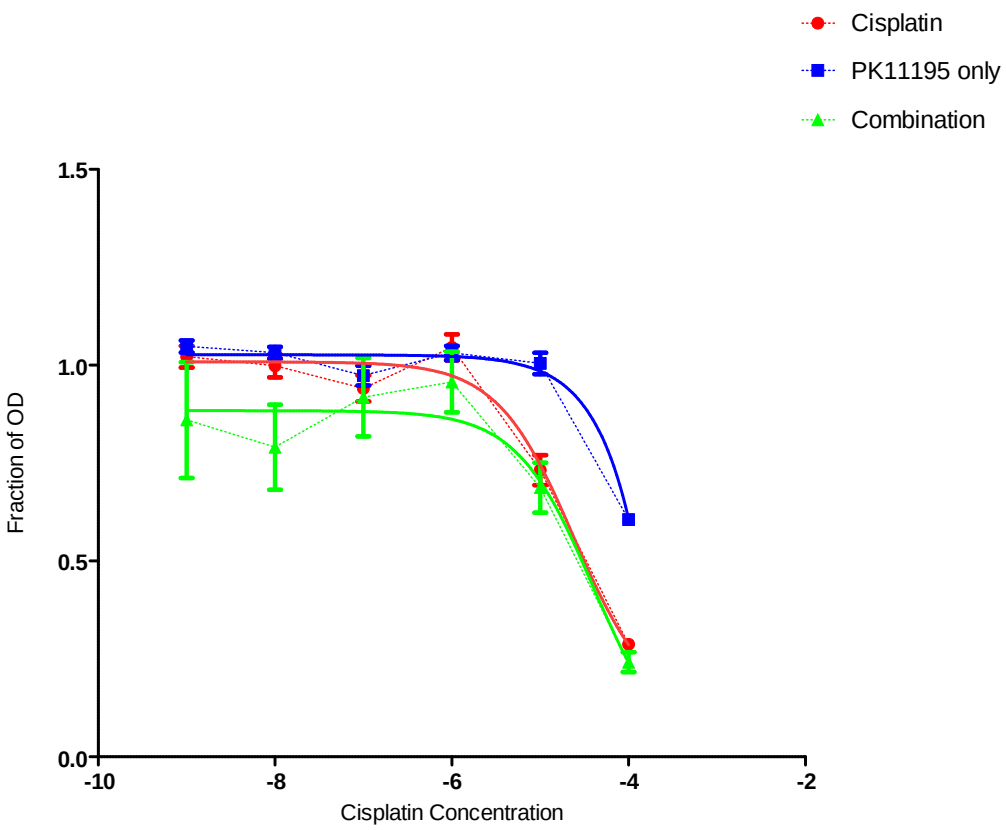

Figure 3-18 Effect of PK11195 addition to cisplatin on SkChA1 cells

The addition of PK11195 to cisplatin therapy in SkChA1 cells did not have a significant effect in any concentration. The only concentration that it had a minimal effect was at $1x10^{-8}$ M, an enhancement of 20%, but this was not statistically significant (P>0.05).

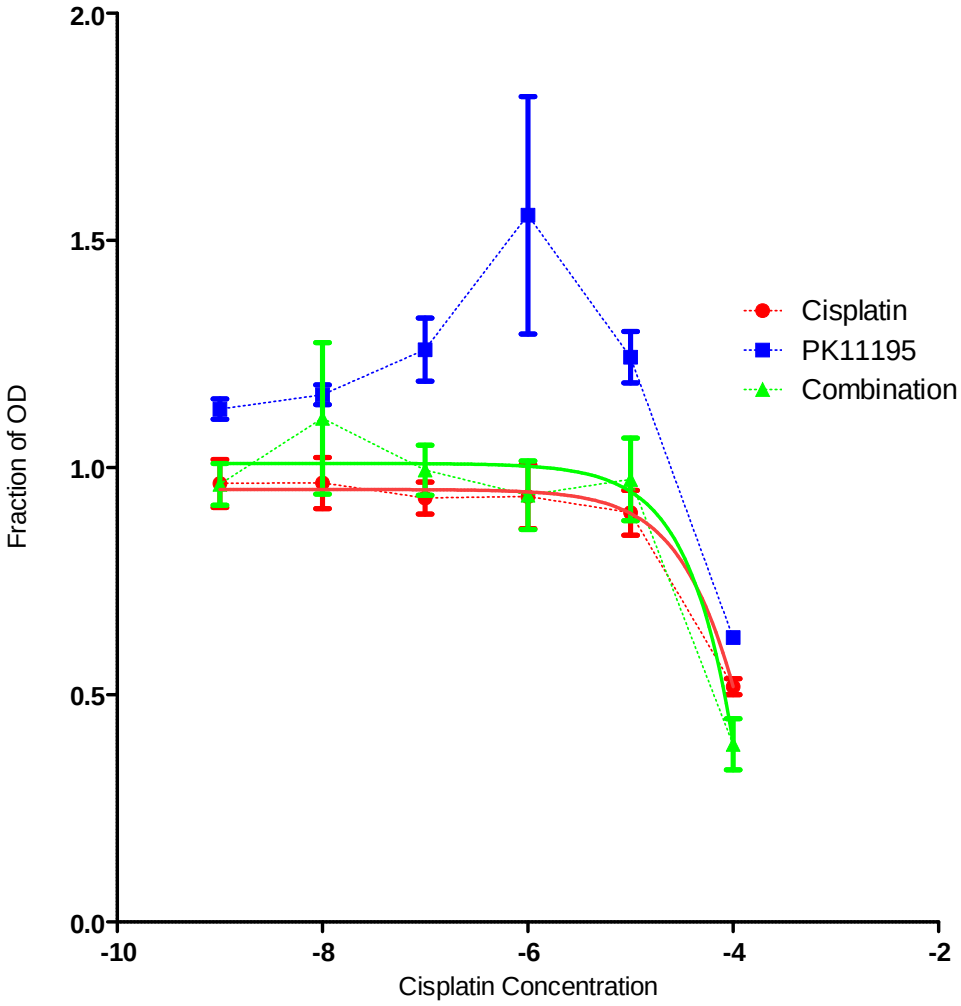

Figure 3-19 Effect of PK11195 addition to cisplatin on HUCC cells

The addition of PK11195 to cisplatin therapy in HUCC cells did not have a significant effect in any concentration. The only concentration that it had a minimal effect was at $1x10^{-9}$ M, an enhancement of 0.2%, which was not statistically significant (P>0.05).

3.3.2.2.3 ***Gemcitabine***

The dose response curves of the cell lines to gemcitabine monotherapy are displayed below.

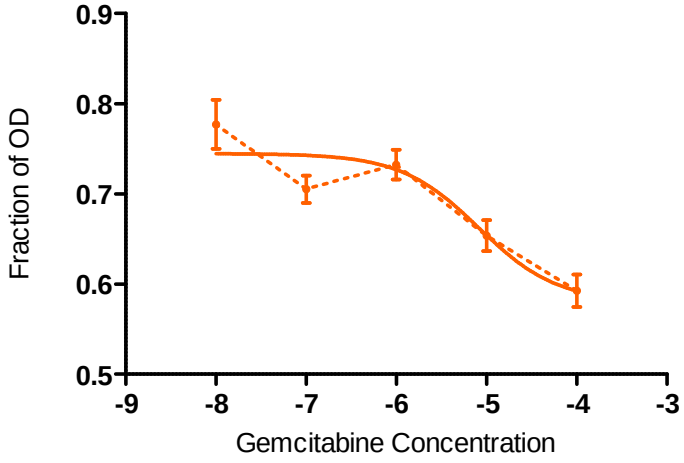

Figure 3-20 Gemcitabine effect on SkChA1 cells

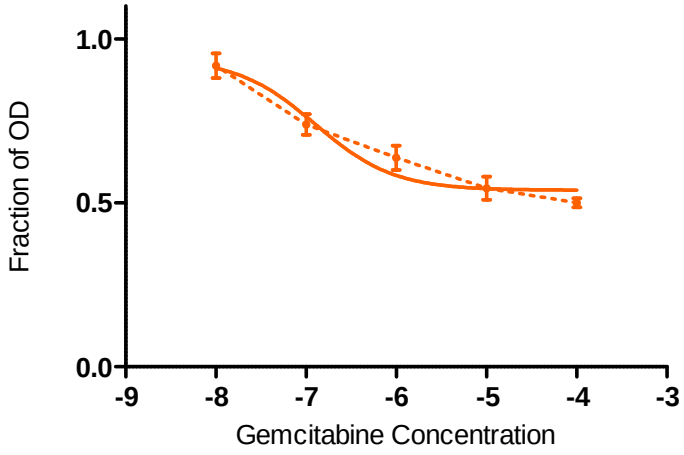

Figure 3-21 Gemcitabine effect on HUCC cells

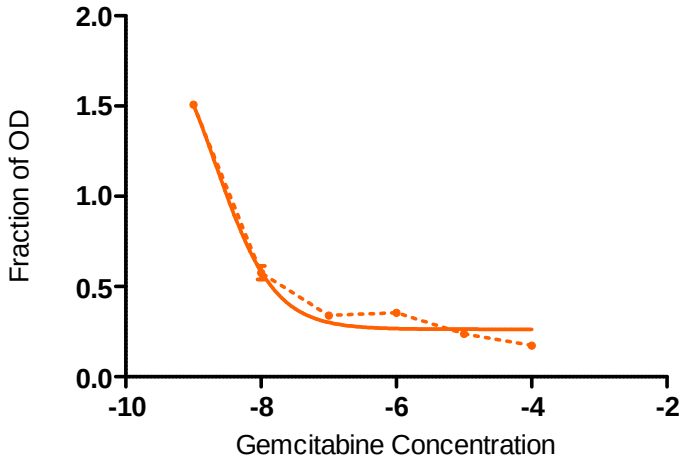

Figure 3-22 Gemcitabine effect on KKU M213 cells

3.3.2.2.4 Gemcitabine in combination with PK11195

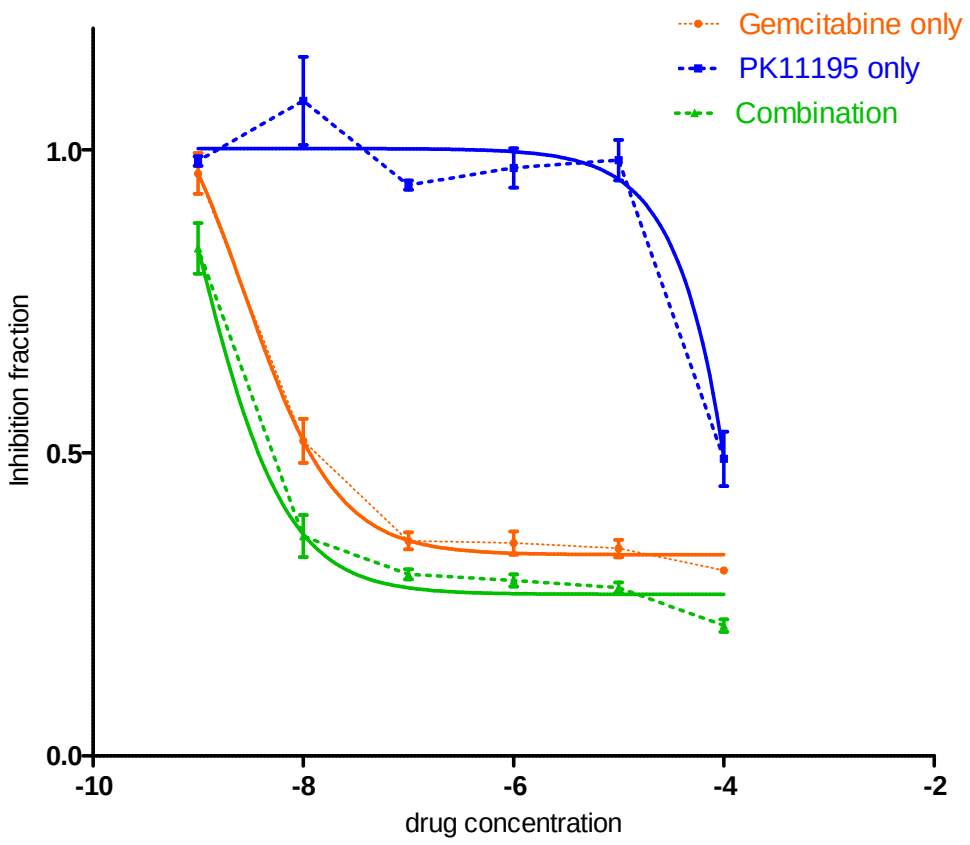

Figure 3-23 Effect of PK11195 addition to gemcitabine on SkChA1 cells

The addition of PK11195 to gemcitabine in SkChA1 cells resulted in a significant enhancement of cytotoxic effect. The largest effect was seen at a concentration of $1x10^{-8}$ mM as indicated by the graph above (Figure 3-23). At this concentration PK11195 enhanced the action of gemcitabine by a mean of 30.24% (95% CI: 4.67% to 55.78%, P<0.001).

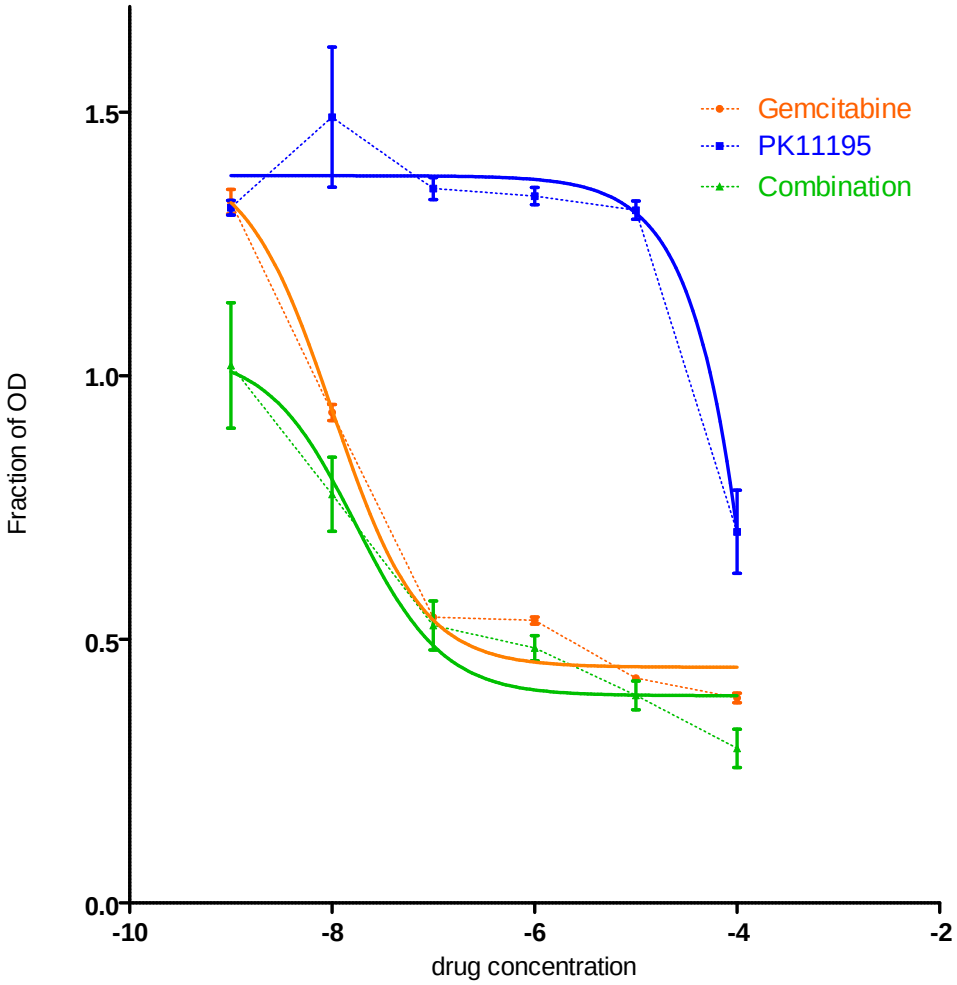

Figure 3-24 Effect of PK11195 addition to gemcitabine on HUCC cells

The addition of PK11195 to gemcitabine in HUCC cells resulted in a significant enhancement of cytotoxic effect. The largest effect was seen at a concentration of $1x10^{-9}$ mM as indicated by the graph above (Figure 3-24). At this concentration PK11195 enhanced the action of gemcitabine by a mean of 23.31% (95% CI: 40.91% to 5.80%, P<0.001).

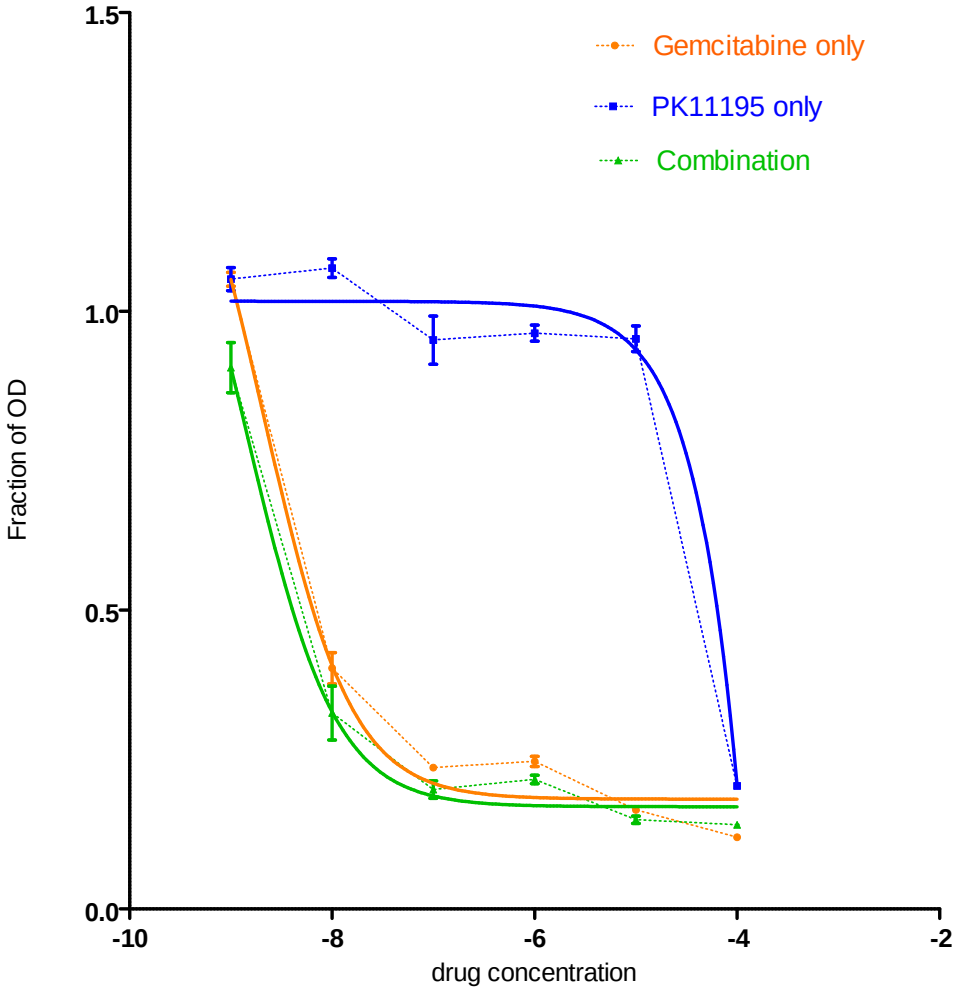

Figure 3-25 Effect of PK11195 addition to gemcitabine on KKU M213 cells

The addition of PK11195 to gemcitabine in KKU M213 cells resulted in a significant enhancement of cytotoxic effect. The largest effect was seen at a concentration of $1x10^{-9}$ mM as indicated by the graph above (Figure 3-25Figure 3-24). At this concentration PK11195 enhanced the action of gemcitabine by a mean of 14.07% (95% CI: 5.83% to 22.87%, P<0.001).

3.3.2.2.5 Fluorouracil

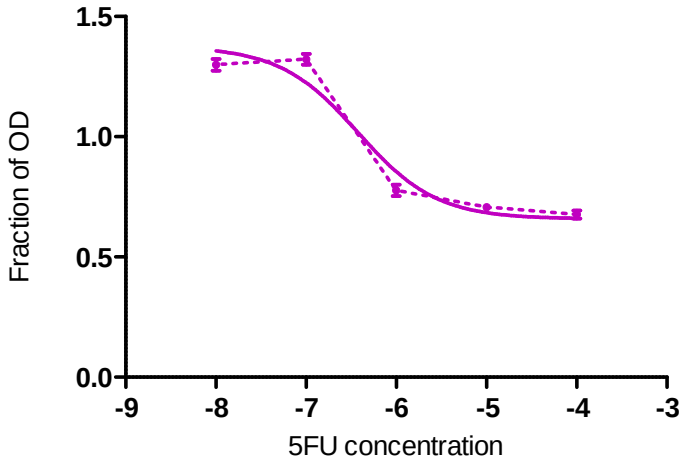

Figure 3-26 Fluorouracil effect on SkChA1 cells

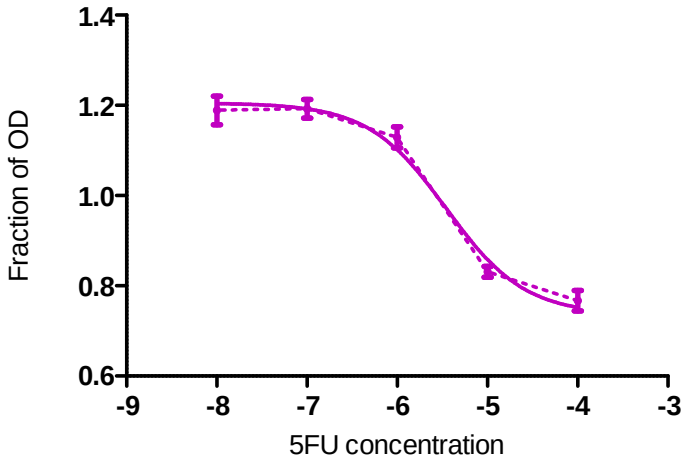

Figure 3-27 Fluorouracil effect on HUCC cells

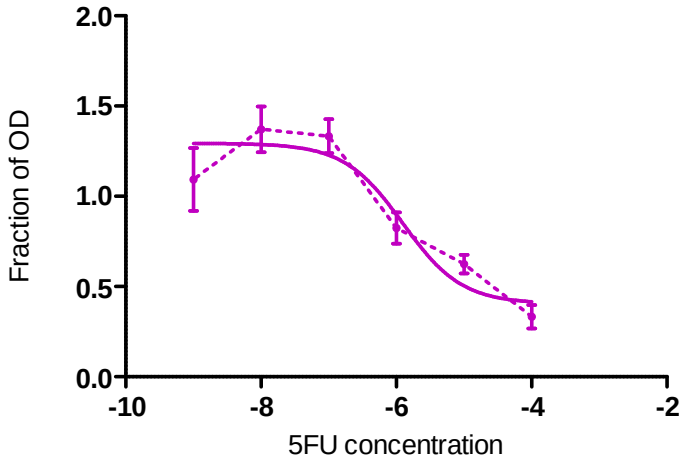

Figure 3-28 Fluorouracil effect on KKU M213 cells

3.3.2.2.6 Fluorouracil in combination with PK11195

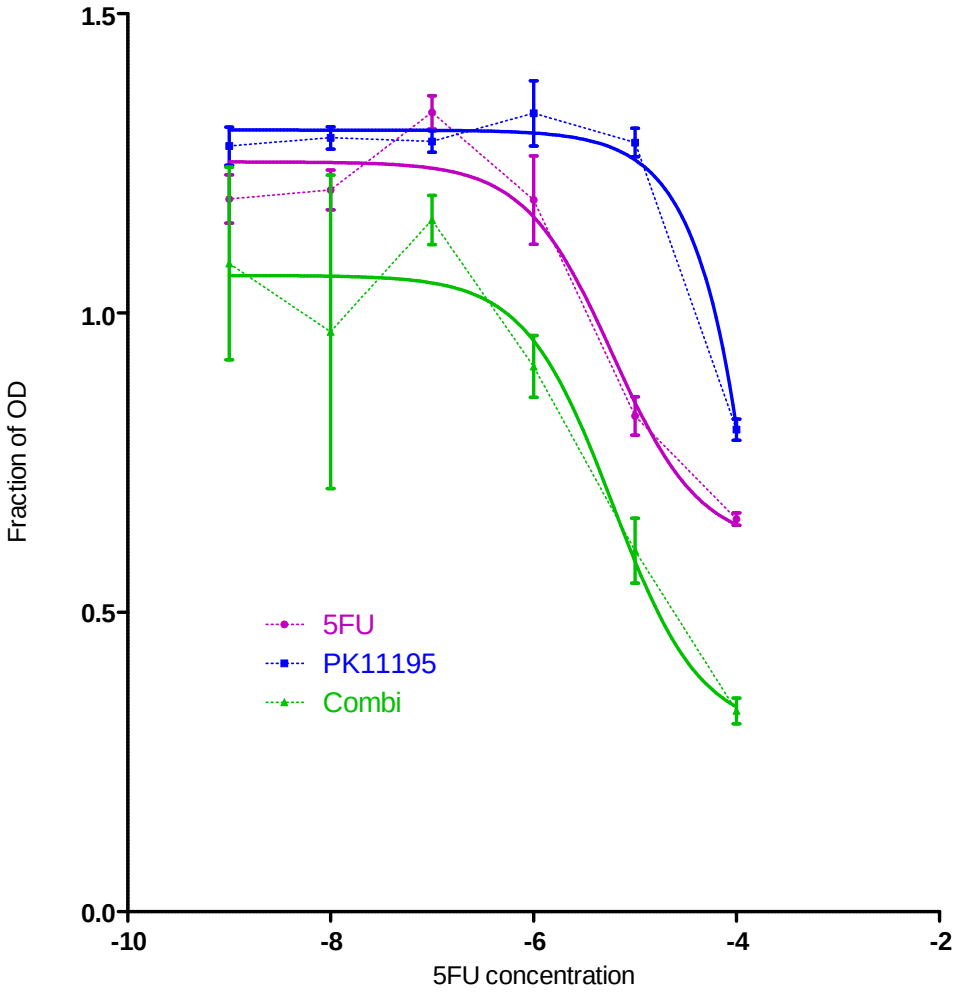

Figure 3-29 Effect of PK11195 addition to 5FU on HUCC cell line

The addition of PK11195 to 5FU therapy in HUCC cells did not have a significant effect in any concentration. An effect was observed at $1x10^{-6}$ M, an enhancement of 23.38% (95% CI: 6.76 to 53.54%) which was, however, not statistically significant (P>0.05).

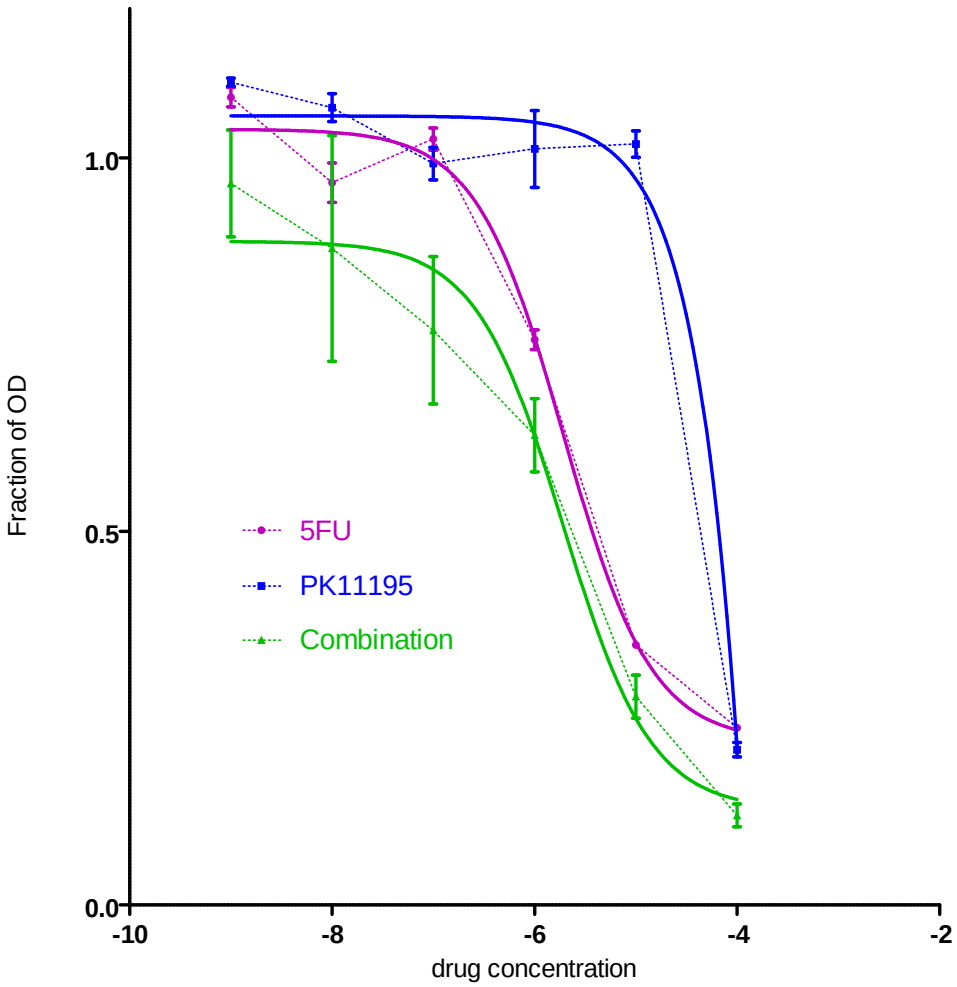

Figure 3-30 Effect of PK11195 addition to 5FU on KKU M213 cell line

The addition of PK11195 to 5FU in KKUM213 cells resulted in a significant enhancement of cytotoxic effect. The largest effect was seen at a concentration of $1x10^{-7}$ mM as indicated by the graph above (Figure 3-30). At this concentration PK11195 enhanced the action of 5FU by a mean of 24.99% (95% CI: 3.02% to 46.89%, $P<0.01$).

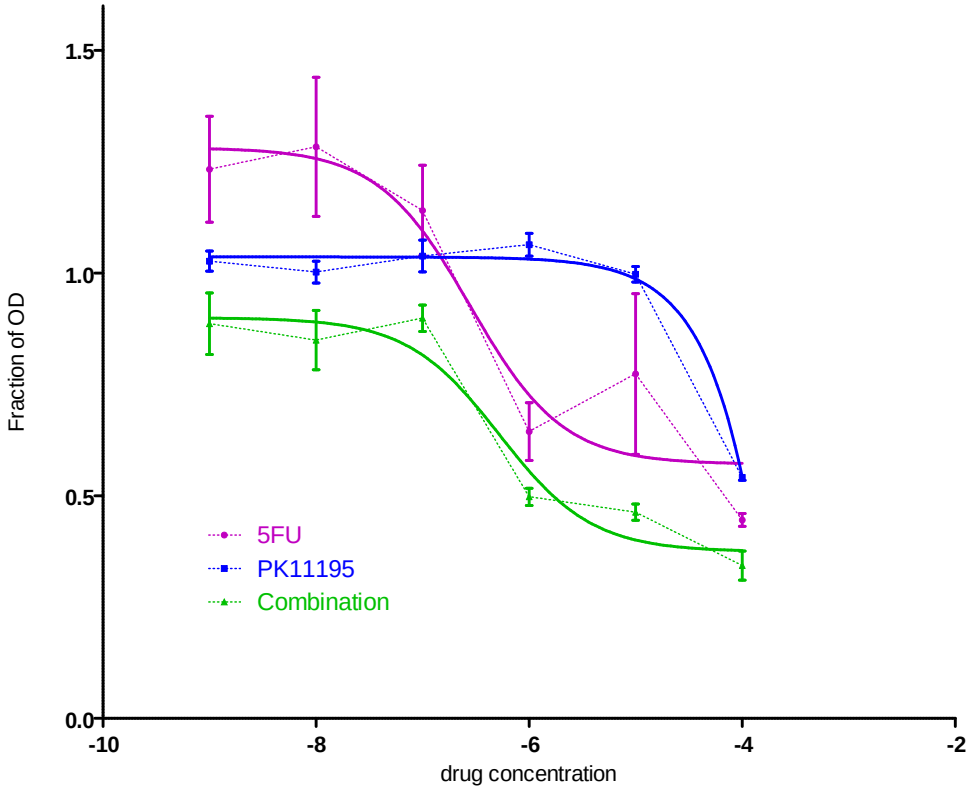

Figure 3-31 Effect of PK11195 addition to 5FU on SkChA1 cell line

The addition of PK11195 to 5FU in SkChA1 cells resulted in a significant enhancement of cytotoxic effect. The largest effect was seen at a concentration of $1x10^{-8}$ mM as indicated by the graph above (Figure 3-31). At this concentration PK11195 enhanced the action of 5FU by a mean of 33.81% (95% CI: 7.93% to 59.71%, $P<0.001$).

3.3.2.2.7 Etoposide

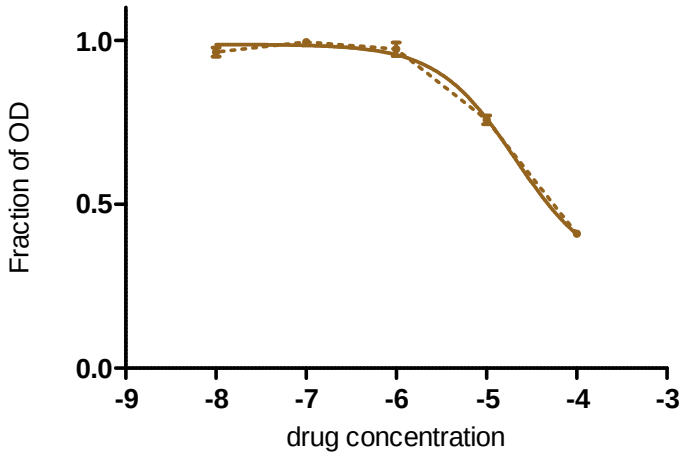

Figure 3-32 Etoposide effect on SkChA1 cells

The IC_{50} was $2.42x10^{-5}$ M.

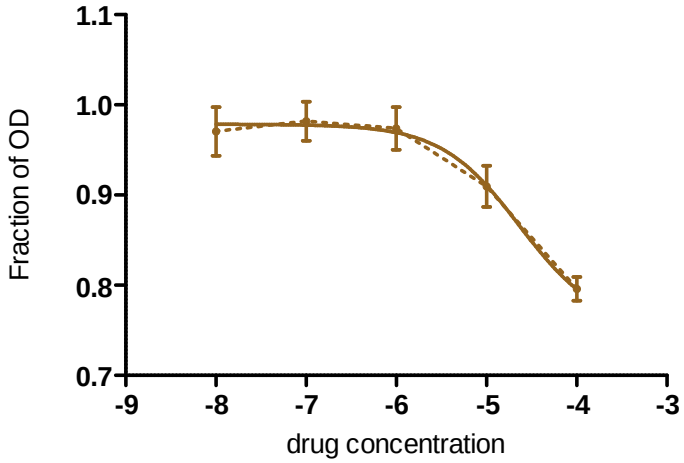

Figure 3-33 Etoposide effect on HUCC cells

The IC_{50} was $2.317x10^{-5}$ M.

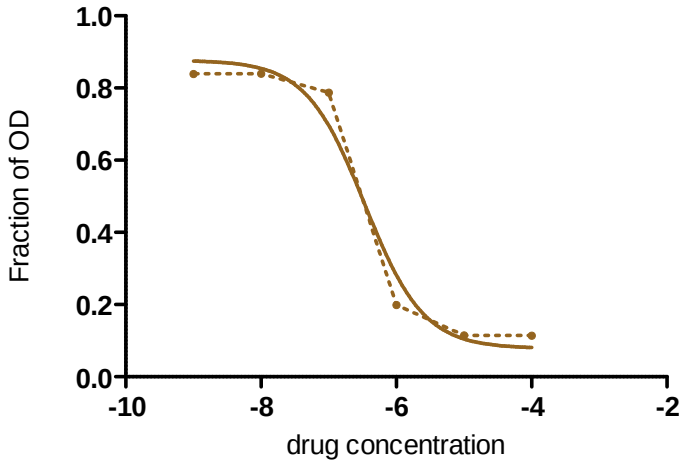

Figure 3-34 Etoposide effect on KKU M213 cells

The IC_{50} was $3.422x10^{-7}$M.

3.3.2.2.8 Etoposide in combination with PK11195.

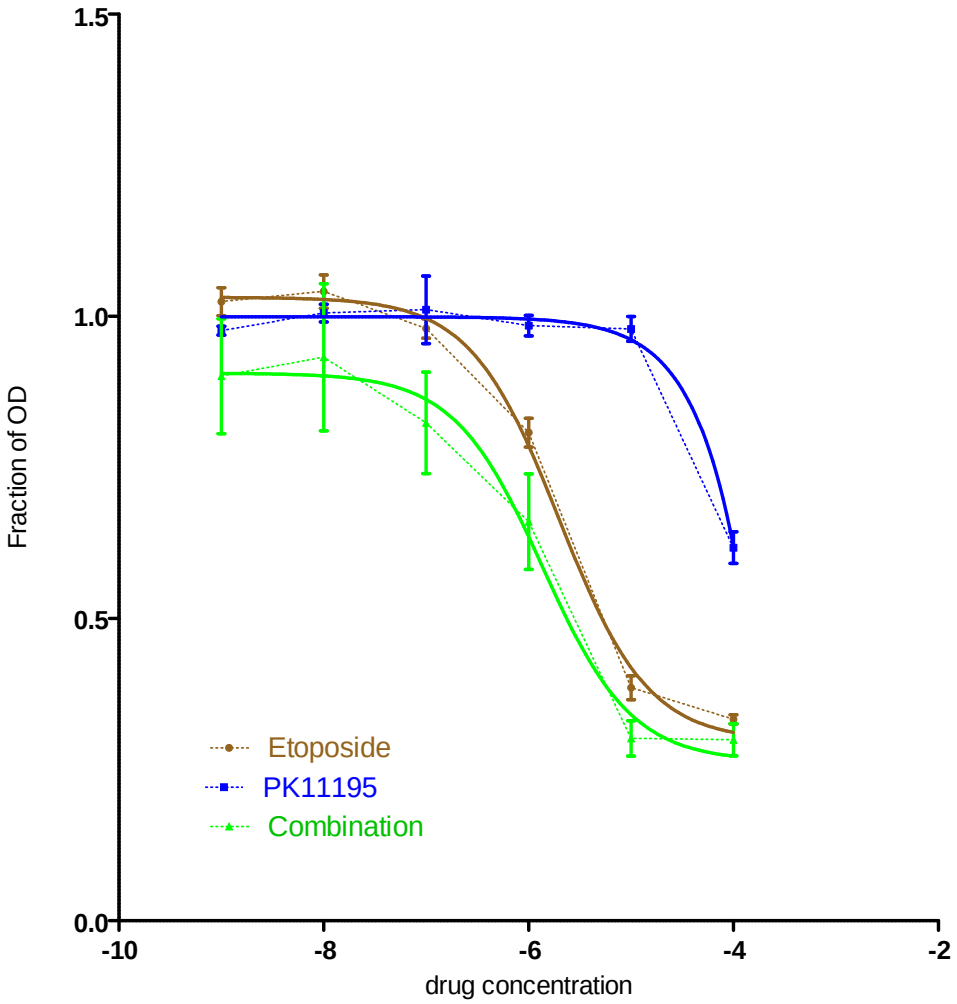

Figure 3-35 Effect of PK11195 addition to Etoposide on SkChA1 cell line

The addition of PK11195 to etoposide therapy in SkChA1 cells did not have a significant effect at any concentration. The largest effect was observed at $1x10^{-7}$mM, an average enhancement of 15.9%, which was not statistically significant (P>0.05).

Comparing the dose response curve as a whole, the IC_{50} was reduced from $1.986x10^{-6}$ mM (etoposide monotherapy) to $1.372x10^{-6}$ mM (combination PK11195/etoposide), representing a 31% reduction.

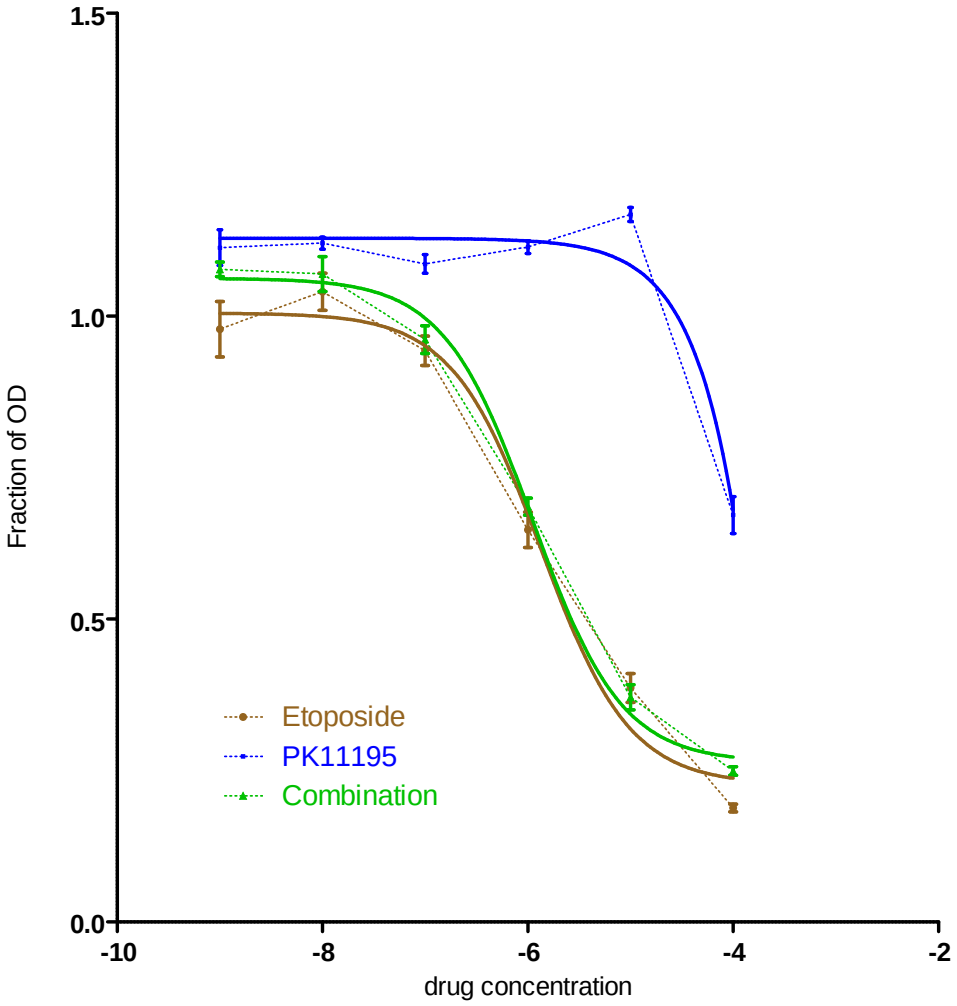

Figure 3-36 Effect of PK11195 addition to Etoposide on HUCC cell line

The addition of PK11195 to etoposide therapy in HUCC cells did not have a significant effect at any concentration. A small effect was observed at $1x10^{-5}$mM, an average enhancement of 3.98%, which was not statistically significant (P>0.05).

Comparing the dose response curve as a whole, the IC_{50} was reduced from $1.34x10^{-6}$ mM (etoposide monotherapy) to $1.11x10^{-6}$ mM (combination PK11195/etoposide), representing a 16.9% reduction.

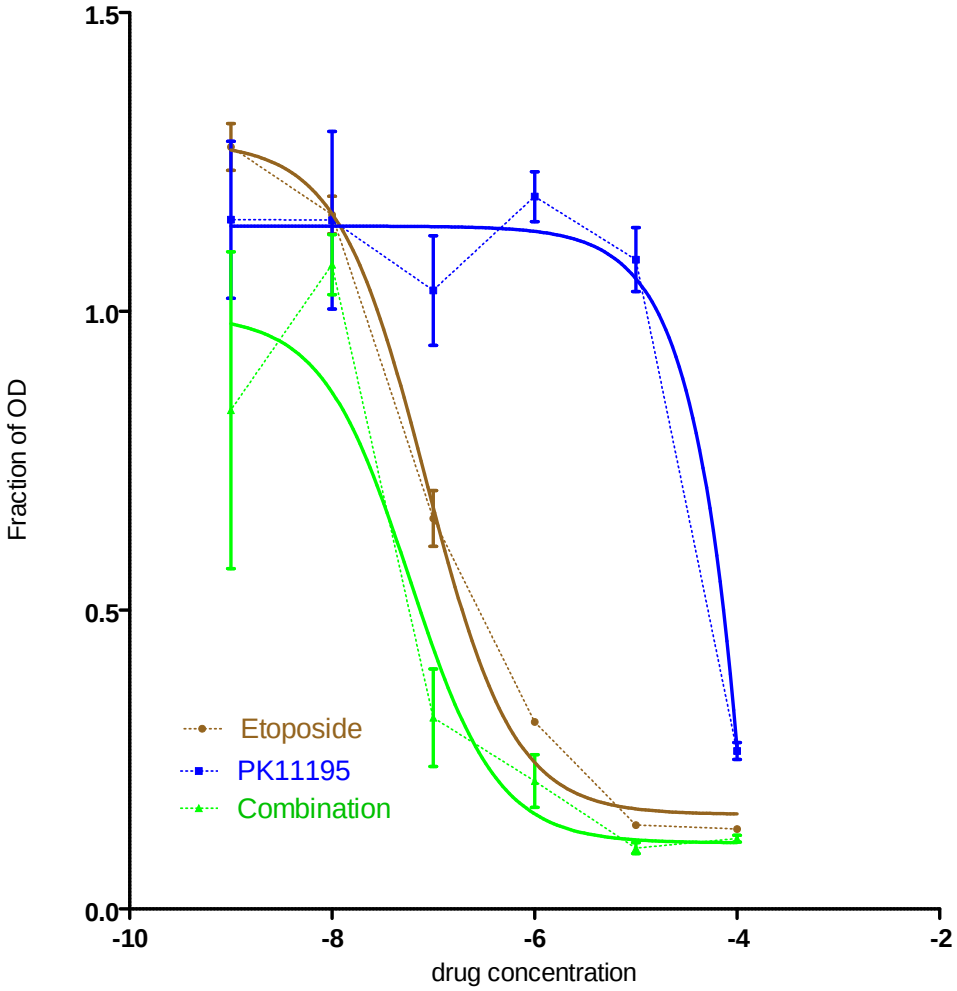

Figure 3-37 Effect of PK11195 addition to Etoposide on KKU M213 cell line

The addition of PK11195 to etoposide in KKU M213 cells resulted in a significant enhancement of cytotoxic effect. The largest effect was seen at a concentration of $1x10^{-9}$ as indicated by the graph above (Figure 3-37). At this concentration PK11195 enhanced the action of 5FU by a mean of 34.54% (95% CI: 4.03% to 65.05%, P<0.01).

Comparing the dose response curve as a whole, the IC_{50} was reduced from $8.39x10^{-8}$ mM (etoposide monotherapy) to $5.83x10^{-8}$ mM (combination PK11195/etoposide), representing a 30.47% reduction.

Cell line	Most effective agent	Concentration (mM)	% enhancement by PK11195 (95% CI)	P - value
SkChA-1	Fluorouracil	$1x10^{8}$	33.81% (7.93-59.71%)	P<0.001
HuCC T-1	Gemcitabine	$1x10^{-9}$	23.31% (5.80-40.91%)	P<0.001
KKU M213	Cisplatin	$1x10^{-6}$	73.10% (49.44-96.83%)	P<0.001

Table 7 Greatest effect achieved with PK11195 for each cell line

3.3.2.3 Cytotoxic agent combination

In this series of experiments all agents were combined in all possible permutations with each other to determine the most effective combination therapy for each particular cell line. The most effective combination (for each cell line) was then re tested in the presence or absence of PK11195 to determine if there was any additional benefit.

3.3.2.3.1 SkChA1 cell line

The most potent combination regime to treat the SkChA1 cell line (when compared to the single agents independently) was gemcitabine and etoposide. This combination regime was most effective at a drug concentration of $1x10^{-9}$mM, the most dilute of concentrations tested. Compared to etoposide alone, the combination was 46.57% more effective.

Subsequent addition of PK11195 to the above regime, however, had little effect (see Figure 3-38). In fact at a concentration of $1x10^{-8}$ mM, PK11195 had statistically significant inhibitory effect of -24.75% on the drug regime (i.e. the addition of PK11195 resulted in a less potent action) ($P<0.01$).

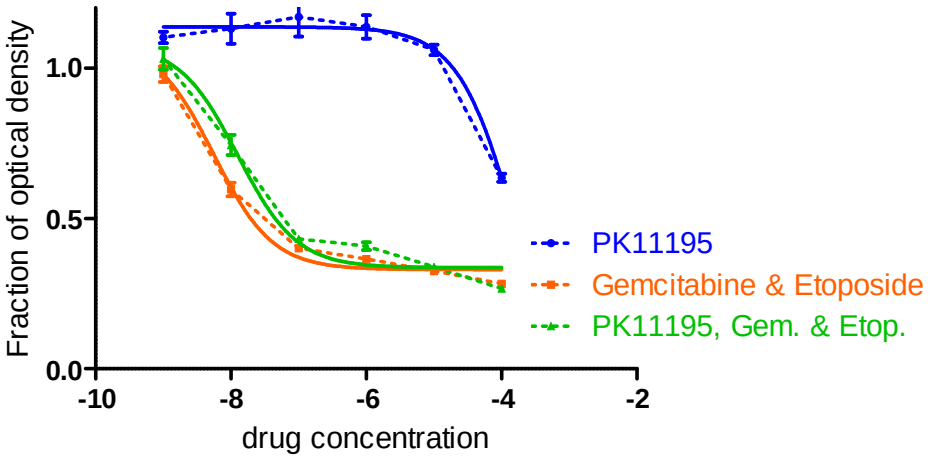

Figure 3-38 Addition of PK11195 to Etoposide and Gemcitabine combination in SkChA1 cells

3.3.2.3.2 HUCC Cell line

In this cell line, the most potent combination was gemcitabine with cisplatin. Addition of PK11195 again did not have the desired effect, reducing the combination's effect at the lowest concentration ($1x10^{-9}$mM), by -18.34% ($P<0.01$)

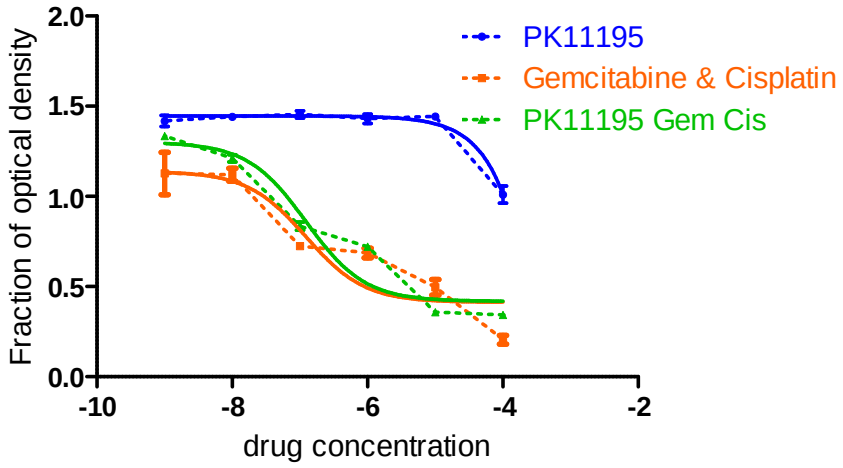

Figure 3-39 Addition of PK11195 to gemcitabine and cisplatin combination in HUCC cells

3.3.2.3.3 KKU M213 cell line

In this cell line, the most potent drug combination was etoposide with 5FU. Addition of PK11195 to the combination therapy had no statistically significant effect at any concentration. In addition, unlike the other two cell lines, there was no reduction in potency by adding PK11195 at any concentration.

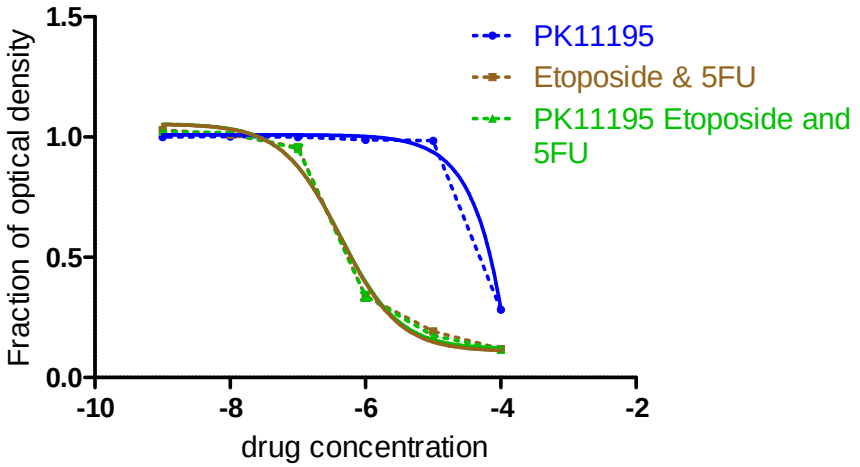

Figure 3-40 Addition of PK11195 to etoposide and 5FU combination in KKU M213 cells

3.3.3 Qrt-PCR results

Each sample was analysed in technical duplicate. All samples were normalised to the endogenous control gene, β-actin. There was no inter analysis variation between the two technical replicates of each sample indicating that there was no evidence of contamination or pipetting error in these experiments.

Figure 3-41 shows the amplification curves, plotting the cycle number against fluorescence (Rn – arbitrary reading) for all the cell lines used. The cell line H69 was also included although not part of the cytotoxic and PK11195 experiments. The ΔCt value represents the point at which the sigmoid curve starts to exponentially

amplify. The sigmoid curves which are lying more to the left indicate that Bcl-2 levels are detected at an earlier PCR cycle, in turn indicating higher levels of expression. From the plot in Figure 3-41 we can see that HUCC has the highest levels of Bcl-2 expression followed by SkChA1, with M213 having the lowest levels.

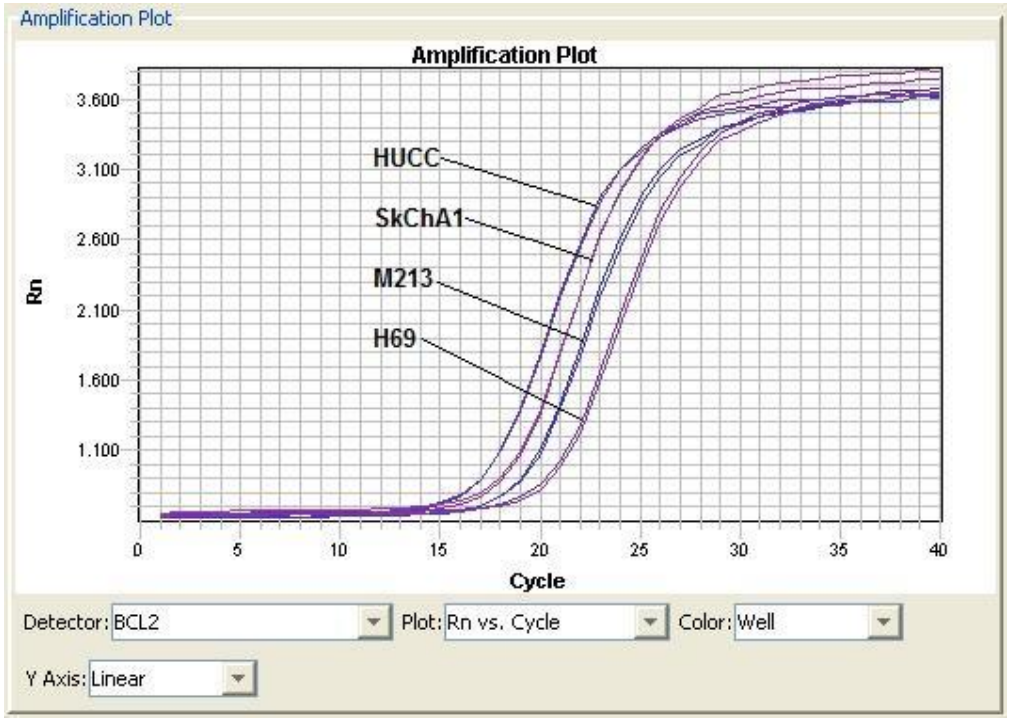

Figure 3-41 Bcl-2 amplification curves

Figure 3-42 represents the relative abundance of Bcl-2 (in arbitrary units) for the three cell lines used in the PK11195 experiments. The lower the ΔCt values in Figure 3-41, the higher the expression of Bcl-2. There was no co-relation between the level of Bcl-2 expression and the response to PK11195 enhancement.

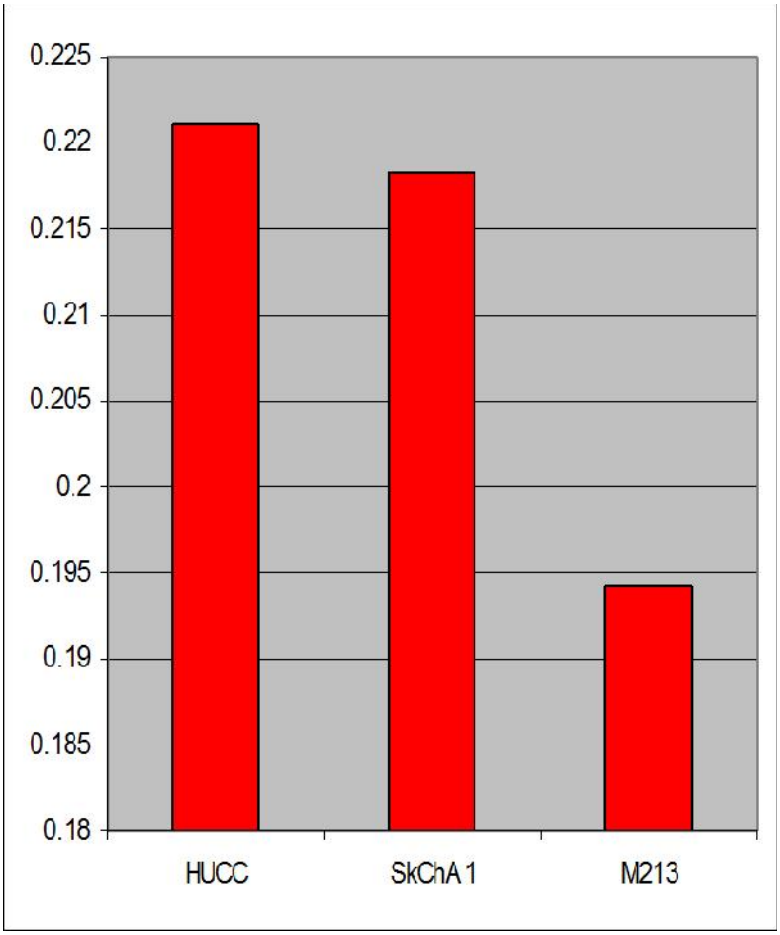

Figure 3-42 Level of Bcl-2 expression (arbitrary values)

3.4 Discussion

Analysis of the action of individual cytotoxics (monotherapy) on all cell lines demonstrated that, to varying degrees, all had an inhibitory effect on cell growth. The growth inhibition curves, however, were quite heterogeneous and consequently the IC_{50} for each cell line also varied.

There could be various explanations for these results, however, two are the most obvious: Firstly, the assumption has been made that the cell lines used represent the original diseases they were harvested from. This is not necessarily true as in cell

culture, daughter cells deviate both in genetic terms and property terms from parent cells, more so as the passage number increases (Wenger et al., 2004). As an indication the cell line SkChA1 was at passage number 50 and HUCC was at passage number 90 at time of use in these experiments. Even if the primary cell cultures of these lines, and by extrapolation the original disease, had been sensitive to the same cytotoxic agents, the actual cells used could have developed resistance to distinct cytotoxic agents.

Secondly, it has been repeatedly suggested that EH (represented by cell line SkChA1) and IH CCA (represented by HUCC) may in fact represent different, distinct diseases. Although traditionally in receipt of a common approach, several groups have suggested a rethink to the above and for tailoring management separately to these subcategories (Braconi and Patel, 2010). Furthermore, the IH CCA originating from South East Asia (represented by cell line KKU M213) may also represent a third, distinct entity (Suzuki et al., 2000). Consequently these potentially 3 different diseases might well respond to three distinct chemotherapy regimens.

Application of two drugs to a system can result in the same response as the sum of the two drugs individually (additive), a greater response (synergistic) or a lesser response (antagonistic, where one drug blocks the effects of the other). Studies in the combined action of biologically active agents, most notably cytotoxic and antimicrobial drugs have generated much debate, particularly in search of potentially potent drug regimens at a preclinical stage. However, there is little consensus over terminology, definitions and models for the evaluation of these interactions (Greco et al., 1996). As such, there has been no standardisation of

methodology despite several (at least 16) published approaches for assessing combined action data (Greco et al., 1995). Some of the described approaches use specialised software and complex data simulation to create 3D graphical representations using response surface methodology (Dressler et al., 1999).

A simple approach for assessing drug interaction was adopted in these experiments, by comparing the IC_{50} of each agent separately and then in combination. The rationale for this was in order to determine the most active combination for each cell line, and then subsequently to see how this combined activity could be enhanced by the addition of PK11195.

Testing of combination therapies revealed similar results to those observed in monotherapy: that different cell lines responded differently to separate regimes. If these results are correlated to clinical trials then one would expect that CCA cell lines would respond best to the regimes currently used in human cancer patients. The standard of care for all CCA patients is currently a combination of gemcitabine and cisplatin (Valle et al., 2010b). Although all cell lines responded (to varying degrees) to this regime, this regime represented the most potent combination only for the HUCC cell line (derived from an IH CCA in a Japanese patient). The patient characteristics of the subjects enrolled in this study included a sizable group of IH CCA, however, this was a multicentre UK-based study, and likely to represent a local population. Arguably the cell line closest to this patient makeup is SkChA1, derived from an EH CCA patient from Germany. However, this cell line responded best to the combination of gemcitabine with etoposide, a regime that is currently at phase II

trials in other cancers, but not tested in CCA to date (Bruzzone et al., 2011, Melnik et al., 2010).

The addition of PK11195 to cytotoxic agents produced some interesting results. Of particular note was that on its own, PK11195 had a consistent effect on all cell lines: unless it was administered at a very high concentration (> $1x10^{-4}$mM), it had little to no effect on cell growth. At very high concentrations, however, it had a marked inhibitory effect on all cell lines.

When PK11195 was added to cytotoxic monotherapy, more often than not it enhanced the effect of that particular cytotoxic agent. The magnitude of this effect again varied according to the cell line and cytotoxic agent used. The explanation for this could be argued along the same lines as above, in the second paragraph of the discussion section.

Time and budget constraints did not allow for testing of PK11195 on all the possible chemotherapy combinations in all the cell lines. Hence, it was only tested against the most potent combination for each particular cell line. Addition of PK11195 to these regimens though did not have the same effect as for monotherapy testing. It failed to enhance the effect of any of the tested regimes, in some cases causing an apparent inhibitory effect, although in most cases this was not statistically significant.

The levels of Bcl-2 expression were investigated in an attempt to explain the differences in response to PK11195, especially as illustrated by its additional effect on monotherapy regimens. Bcl-2 is an anti-apoptotic protein, higher levels of which

result in cell survival advantage. It is well documented that cancer cells can express higher levels of Bcl-2 thus evading apoptosis (Placzek et al., 2010). High levels of Bcl-2 in some cancers have been described as a marker of poor prognosis. For example in prostate cancer, Bcl-2 detection was much more common in androgen independent samples (McDonnell et al., 1992). This observation has led to targeting apoptosis suppressors as a route of developing cancer therapeutics.

PK11195 is a peripheral benzodiazepine receptor inhibitor. It acts by inhibiting Bcl-2 and therefore rebalancing the equilibrium and restoring the sensitivity of cancer cells to pro-apoptotic signals. Therefore, in theory at least, cells expressing higher levels of Bcl-2 (expressed as Bcl-2 mRNA) should have an enhanced response to chemotherapy when exposed simultaneously to PK11195. Simply put, cell damage from cytotoxic agents would channel them into apoptosis, and PK1195 would allow that process to occur.

These experiments did not demonstrate this. Although all cell lines responded better to chemotherapy in the presence of PK11195, the maximum difference was observed in the cell line KKU M213, where PK11195 enhanced the action of cisplatin by 73.1% (at a concentration of $1x10^{-6}$ mM, see Figure 3-17). Cell line KKU M213, however, expressed the lowest level of Bcl-2 of all three cell lines used (see Figure 3-42). On the other hand, cell line HUCC which showed the highest levels of Bcl-2 expression had the smallest enhancement when PK11195 was added to gemcitabine.

Whether Bcl-2 plays a significant role in cholangiocarcinogenesis is not clear from the current literature. One study reported 8 out of 11 human CCA samples demonstrating Bcl-2 on immunohistochemistry with the authors going as far as

suggesting Bcl-2 could represent a distinguishing feature from hepatocellular carcinoma (Charlotte et al., 1994). However, this observation has not been consistent with other studies finding little or no expression of Bcl-2 in CCA (Okaro et al., 2001).

4 The proteomic analysis of cholangiocarcinoma cell lines

4.1 Introduction to Proteomics

Proteomics is the study of the entire protein complement of an organism. As a science it has exponentially expanded over the last two decades, principally driven by improvements in the annotation of the human genome and technical developments in mass spectrometry. The human genome contains 20-25,000 genes which encode over 100,000 different proteins (Orchard et al., 2005, Keren et al., 2010). The transcripts that link the genome to the proteome have variable degradation rates and translation efficiencies and are subjected to different processes that differentially alter translation, such as alternative splicing, micro RNA-induced terminations and translation in different reading frames. The resultant proteins are then subject to post-translational modifications that also impact on their function such as phosphorylation and glycosylation. This increasing level of complexity from the genome through the transcriptome to the proteome renders the assumption that accurate protein expression can be determined by studying genes or transcripts questionable. Indeed, a review of the published literature suggests that the link between gene and protein expression is perhaps better described as a trend than a correlation (Maier et al., 2009). Therefore, as the only discipline which studies the functional end-product of gene expression, proteomics could be viewed as the “gold standard” method by which to identify candidate biomarkers, vaccines or therapeutic targets.

Prior to the development of mass spectrometry-based proteomics, researchers studying protein expression were restricted to antibody-dependent techniques: either by Western Blotting (where a crude protein mixture is separated by gel electrophoresis, transferred to a membrane and then probed with an antibody) or by immunohistochemistry. Although both techniques are still very relevant and widely used today, they are dependent on the availability of antibodies and therefore can only be used where questions relating to the expression of previously known proteins for which antibodies are available are being addressed. The advantage of a mass spectrometry-based study is that protein expression levels can be compared between groups using crude protein mixtures without any preconceptions. Then, identities can be retrospectively assigned to those proteins that are found to be differentially expressed without any hypotheses as to what their identities might be.

4.1.1 Mass spectrometry and identification of proteins

A mass spectrometer extremely accurately (often with sub part per million accuracy) measures the molecular mass of either intact proteins, or more usually its peptides. Peptides rather than proteins are generally used in proteomics because they are smaller (and are therefore more easily vaporised) and because they yield more data; analysing a protein by mass spectrometry determines its mass but analysing its peptides yields its amino acid sequence. Although there is a confusing array of different types of mass spectrometers named with various acronyms, their fundamental method of working can be broken down into three parts; a means of vaporizing and ionising the peptides, a means of separating the peptides by their mass and a means of detecting them. There are two common ionisation methods

("electrospray ionisation" and "matrix-assisted laser desorbtion/ionisation (MALDI)) and several means of separation in different kinds of "mass analyser" (including "time of flight" (ToF)). The work in this project was carried out on a MALDI-ToF/ToF mass spectrometer and a brief description of how it works follows.

In MALDI, the protein to be identified is digested into peptides using trypsin. The peptides are then co-crystallized as a spot on a stainless steel plate with "matrix" (commonly α-cyano-4-hydroxycinnami acid). The plate is inserted into the mass spectrometer and a laser directed at the spot. Photons from the laser are adsorbed by the aromatic rings of the matrix and transferred to the peptides along with a proton, causing the now-positively charged peptides to vaporise. A strong negative electromagnetic field propels the peptide ions into the mass analyser, which consists of a vacuumated flight tube ending in a detector. The trajectory time of a peptide through the flight tube is dependent on its mass-to-charge-ratio (m/z), so the different peptide ions separate in flight and are detected sequentially as they impact the detector. Thus, each protein is recorded as a mass spectrum with m/z on the x-axis and ion intensity on the y-axis. Each peak on the mass spectrum represents one of the peptides derived from the intact protein, its m/z being dependent on its amino acid sequence. As α-cyano-4-hydroxycinnamic acid only protonates each peptide singly, the charge of each ion is 1^+ so the mass of each peptide can be easily calculated from the m/z. This spectrum of ions is unique to each protein and is called a "peptide mass fingerprint" (PMF). In the past, PMFs were routinely used to assign identities to proteins (Pappin et al., 1993). This was done by a computer program loaded with a database of protein sequences. The computer would carry out a

theoretical *in silico,* tryptic digest of each protein in the database and then calculate the molecular mass of each peptide by summing the masses of the peptide's component amino acids. Then, by matching the PMF's experimental masses obtained by the mass spectrometer to the theoretical masses derived by the computer an identity could be obtained. However, over the last decade more sophisticated mass spectrometers have become commonly available which fragment the peptides in a second round of mass spectrometry so their amino acid sequences can also be determined. Also, protein database sizes have increased exponentially such that PMF-derived identities are unreliable because of the consequent high numbers of false-positive protein identities.

The process in which two rounds of mass spectrometry are undertaken and the "precursor" or "mother" PMF ions are fragmented into their "daughter ions" is called "tandem mass spectrometry". When the mass spectrometer is fitted with a MALDI ion source connected to a time of flight mass analyser the process is called MALDI-ToF/ToF mass spectrometry (or MALDI-MSMS). In the second round of MS, peptide ions are ionised from the MALDI plate a second time using the laser and are fired into the mass spectrometer using their electromagnetic charge. This time however, a timed ion selector and a collision cell filled with nitrogen are placed in the trajectory path of the ions. The timed ion selector uses electronic gates to isolate a precursor ion that has been pre-selected from the PMF, usually chosen on account of its high ion intensity (i.e. it is very amenable to ionisation) and size (it needs to contain sufficient numbers of amino acids to yield a potentially diagnostic sequence). The precursor ion then enters the collision cell and fragments on impact with the

nitrogen. Each daughter ion traverses the flight tube, impacts the detector and forms part of what is called the "MSMS spectrum". After one mother ion has been fragmented into daughter ions and the MSMS spectrum obtained, the process can be repeated for other mother ions chosen from the PMF. Once the set of MSMS spectra have been obtained from the PMF each is converted into its amino acid sequence (Johnson et al., 1987). These amino acid sequences can then be matched against those from a protein database and the identity of the previously unknown protein established. As each protein identity is obtained using the amino acid sequence of several peptides, rather than from the PMF (just masses of peptides) a protein identity can be assigned with extremely high confidence.

4.1.2 Study design in proteomics

There are two commonly used but fundamentally different ways that samples are compared in a proteomic experiment: "shotgun proteomics" and two-dimensional gel-based proteomics.

In shotgun proteomics a crude protein mixture is digested into peptides. The peptides are then separated on a high performance liquid chromatography (HPLC) system that is coupled to a mass spectrometer. The peptides are eluted off the HPLC column and are vaporized by electrospray ionisation as they enter the mass spectrometer in solution. Between-sample expression levels are determined, either by differential labelling of the peptides from each sample or by label-free quantification. Label-free quantification works on the premise that the intensity of the peptide signal detected by the mass spectrometer is proportional to the expression level of the intact protein (Elliott et al., 2009). Although shotgun

proteomics has many strengths, fundamental disadvantages are that large amounts of mass spectrometry time are required, subtly different protein isoforms and are difficult to distinguish from each other and a lot of data is lost because the mass spectrometer's cycling speed is too slow to keep up with the pace at which peptides are delivered by the HPLC system. Long HPLC gradients help to separate and elute the tens of thousands of peptides at a speed slow enough for the mass spectrometer to process them, but ultimately it is necessary to separate the peptides in more than one dimension, if more than approximately 500 proteins are to be analysed. Also, multiple biological and technical replicates are required because peptides are not inherently quantitative (they exhibit a range of variable physiological properties (such as charge and hydrophobicity) which lead to large differences in mass spectrometric response (Bantscheff et al., 2007)). Thus, the technique is extremely expensive for research groups who rent time on instruments in a specialist facility. Whether labelled or label-free quantification is chosen, all of the peptides need to be assigned to their parent proteins by a second round of MSMS analysis where each mother peptide is fragmented into its daughter peptides to obtain its amino acid sequence. As all of the peptides from all of the proteins are mixed together at the start of the process, it is difficult to quantify proteins that have a high degree of homology at the amino acid level, because a large proportion of their peptides will be identical, become eluted from the HPLC column at the same time and are therefore indistinguishable.

The principal alternative to shotgun proteomics is two-dimensional gel-based proteomics, where, unlike shotgun proteomics, the proteins are separated and

analysed whilst they are still intact. The crude protein mixture is prepared in a non-ionic buffer and absorbed into a strip that incorporates a pH gradient. When an electrical current is passed through the strip the proteins undergo isoelectric focussing (IEF) and migrate to the point at which they have no net charge (their isoelectric point (*pI*)). The entire strip is then soaked in SDS (which renders all of the proteins negatively charged) and then placed at the top of a polyacrylamide gel. A current is then passed through the gel and the proteins (already separated by charge in the first dimension) now separate in the gel by molecular mass. The result is a gel containing numerous spots, each spot representing a different protein, the coordinates of which are determined by its charge and mass. Multiple IEF strips and gels can be run at once, each one containing a different sample, so when the spot patterns from the different gels are compared differential protein expression between the samples can be seen. Those differentially-expressed spots are then physically cut from the gel, digested by trypsin and identities assigned to them using MALDI-ToF/ToF mass spectrometry.

As identities are only sought for the differentially-expressed spots, the process takes minimal mass spectrometry time compared with a shotgun proteomics experiment and is therefore considerably less costly. Also, as the protein expression analysis is carried out at the protein level rather than at the peptide level, extremely similar proteins can still be visualised as separate entities. The problem of matching peptide intensity levels to protein expression levels is also avoided. The principal disadvantage of the gel-based method is that a significant proportion of the proteome is excluded from the analysis. These include highly hydrophobic

membrane proteins (that cannot be solubilized in the non-ionic buffer), proteins of extreme charge (whose pI falls outside the range of the IEF strip) or proteins of extreme molecular mass (that are either too large to enter the gel or are so small that the gel cannot resolve them). Some users cite technical difficulties, the time-consuming nature and inherent gel-to-gel variation as problems. The latter issue has been resolved by the development of two-dimensional difference gel electrophoresis (2D-DIGE) (Tonge et al., 2001). In 2D-DIGE, gel-to-gel variation is eliminated by multiplexing the samples: each gel contains a pooled internal standard (labelled with a fluorescent CyDye) that is made up from all of the samples in the experiment (so it contains all of the spots in all the gels) as well as the sample under scrutiny (labelled with a different CyDye). As the internal standard is identical in all the gels, it is easy to match the gels in the experiment to each other. The expression level of each spot is then assessed as a proportion of its equivalent internal standard spot in the same gel. These proportions are then compared between the gels and this way gel-to-gel variation is eliminated and highly reproducible results are obtained (Karp et al., 2005, Jackson et al., 2009).

4.1.3 Proteomics and cancer research

The clinical application of proteomics in cancer research is seen as most promising in three distinct areas: the identification of biomarkers to aid in the diagnosis and prognosis and the detection of unique targets that could potentially guide drug development. A large proportion of clinical proteomic work has focused specifically on cancer diagnosis and therapy and there has been an exponential increase in the

number of publications in this area. As expected, most of the research carried out has focused on cancers that have a higher incidence and prevalence.

Biomarker discovery has long been considered a rational approach for the development of diagnostic tests in cancer. Clinically useful cancer biomarkers would provide earlier and better diagnosis of patients (enabling intervention at the early stages of the disease where the chance of it being effective is greatest) and prognostic biomarkers would enable patients to be better monitored during the course of treatment (Schilsky, 2010).

Despite the plethora of work in this area, no reliable protein biomarkers have been developed that return sufficiently high sensitivity and specificity values. The problem is well-illustrated when considering the epithelial ovarian cancer biomarker CA125.

CA125 is potentially expressed in 50-90% of patients with ovarian cancer, depending on the stage, histologic subtype and the study (Moss et al., 2005, Kobel et al., 2008). Although its expression corresponds well with initial therapy, the poor sensitivity of CA125 leads to anxiety and deterioration in the quality of life in asymptomatic patients, such that withholding treatment in the event of isolated rising CA125 levels will not negatively impact overall survival (Rustin et al., 2009).

4.1.4 Proteomics and cholangiocarcinoma

There is a particular need to identify new proteomic targets that can be used as either biomarkers or chemotherapeutic targets in CCA as currently neither chemotherapy nor radiotherapy radically effects patient survival. Furthermore, as early detection with surgical intervention offers the best chance of survival, it follows

that a biomarker capable of detecting disease at the earliest possible stage would also improve survival and outcomes.

The current biomarkers associated with the detection of biliary tree cancers are CEA and Ca 19-9. Their sensitivities however are reported as approximately 70% and 50% respectively, (Scarlett et al., 2006). Specificity of these biomarkers is similarly low. Ca19-9 can be also elevated in pancreatic cancer, gastric cancer, and primary biliary cirrhosis, and is also elevated in smokers.

Ca 19-9 and CEA also seem to have some value as a prognostic marker as expression corresponds well with response to initial therapy.High levels, especially of Ca19-9 appear to be a poor prognostic indicator (Briggs et al., 2009). Overall, it is safe to say there is a pressing need for more reliable markers for CCA.

Despite this evident need, there has been little work describing meaningful quantitative proteomic analyses of CCA. Indicative is a simple database search for the strings [(proteomics OR proteome) AND cholangiocarcinoma]. This search returns a total of 31 studies without any date or study type restrictions. Table 8 Number of proteomic related articles published in PubMed for various cancers

shows the extent to which other cancers have been investigated when applying the equivalent search strings.

Cancer	Number of articles
Breast cancer	1077
Lung cancer	621
Colorectal cancer	593
Prostate cancer	535
Hepatocellular cancer	460
Pancreatic cancer	320

Table 8 Number of proteomic related articles published in PubMed for various cancers

The first study compared the CCA membrane proteome with that from normal biliary tissue (Kristiansen et al., 2008). The samples were selectively enriched for membrane proteins then analysed using a technique similar to shotgun proteomics in that protein quantification was carried out at the peptide level. A total of 52 proteins were found to be up regulated in the CCA samples, including proteins known to be associated with CCA (such as mucins and CEA) and some novel proteins. However, the sample size was small, consisting of only two CCA samples and one normal sample, the former of which were human xenografts grown in athymic mice. Therefore, it is unclear how much of the proteomic differences observed by

Kristiansen *et al* were due to the small sample size or the comparison of a xenograft grown in an immunologically depleted mouse and a human clinical specimen.

The xenograft study was followed by one where paired tumour and normal tissue, taken at the time of radical surgery from two patients with CCA, were analysed by shotgun proteomics (Kawase et al., 2009). A total of 38 proteins were found to be up regulated in the CCA samples, 4 of which were verified by Western Blotting on four further matched CCA and normal biosamples. Although the verified proteins (actinin 1 and 4, protein DJ-1 and cathepsin B) have previously been associated with other cancers, none of them were amongst the potential biomarkers previously highlighted in the Kristiansen *et al* xenograft paper. This is possibly because, again, the sample sizes were too small and/or that the CCA was occupying only a proportion of the tissue in the "tumour" samples, with much of the remaining biosample consisting of stromal tissue, rich in inflammatory cells and fibroblasts.

The stromal heterogeneity "contamination" issue was addressed by a study in which intrahepatic CCA tissue was selectively extracted for analysis using laser capture micro dissection (Dos Santos et al., 2010). In this study, the inherent difficulty of sample paucity associated with laser capture micro dissection was overcome using a specialist technique called "accurate mass and time tagging" where Fourier transform ion cyclone resonance mass spectrometry is coupled to a nano HPLC system enabling minute sample quantities to be analysed (Shen et al., 2004). Dos Santos *et al* collected intrahepatic CCA samples at the time of surgical resection from four patients, isolated the CCA cells and compared their proteome with cholangiocytes from five non-cancerous controls. The proteomic data was obtained

in a shotgun proteomics approach, and the identities of interest verified using a Tissue Microarray made from 59 CCA samples and 22 normal controls. A total of 460 proteins were identified with an acceptable degree of confidence, but it is notable that there was significantly more proteomic between-patient heterogeneity in the specifically-selected intrahepatic CCA cells than in the cholangiocyte controls. This result demonstrates the absolute requirement for sample sizes to be larger than those of the work published to date if anything other than superficial data is to be obtained. When the CCA samples were compared with the controls, Don Santos *et al* found 39 of the 460 proteins to be differentially expressed, three of which (vimentin and carbonic anhydrase II) were verified using the tissue microarray. Neither of these proteins had been identified in the earlier Kawase *et al* (2009) paper, but they are both identified as being differentially expressed in Kristiansen *et al* (2008). However, vimentin was downregulated in the Kristiansen paper and up regulated in the Don Santos study and the opposite was the case for carbonic anhydrase II. Thus, it is impossible to draw meaningful conclusions from these two studies.

The most recent study analysed snap frozen samples from 4 patients with intrahepatic CCA (Darby et al., 2010). This study performed 2D DIGE using an internal standard prepared with equal volumes of the CCA and control samples, thus eliminating technical error. The experiment used large 24cm gels each of which contained a differentially labelled CCA, liver tissue and internal standard sample. The major shortfalls of this study were its small patient sample and the use of normal liver tissue as a control. It is unclear how much of the differences observed represent a comparison of biliary vs. liver tissue as opposed to tumour vs. normal tissue. A

total of 138 different proteins were identified by mass spectrometry to have different expression levels, of which 70 were over expressed and 68 under expressed in the tumoural samples. The authors concentrated their discussion on the roles of protein 14-3-3, periostin and alpha smooth muscle actin.

Rather than interrogate tissue samples, an alternative avenue of research is to analyse bile. Bile should theoretically be enriched in CCA-derived proteins and is obtainable using endoscopic retrograde cholangiopancreatography so is potentially useful in diagnosis. In the first proteomic analysis of bile, 87 proteins were identified from a sample taken a CCA patient, including carbonic anhydrase and mucin 2 (Kristiansen et al., 2004). This has been followed by a recent study in which bile from 4 CCA patients was analysed (Farid et al., 2011), with nearly 500 proteins being identified in each patient with confidence (i.e. with at least two significant peptides), including mucin 1 and vimentin. However, there was only a relatively small degree of overlap in the list of proteins present, such that only 185 proteins were present in all four patients. Thus, it appears likely that bile, as is the case with serum, will prove to be a highly variable medium in which it will be difficult to search for biomarkers. However, the practical difficulties in purifying the relatively low protein content of bile and removing the bile salts, bile acids and lipids that could prejudice proteomic research have clearly been resolved. Indeed, a protocol has recently been published in which over 2500 proteins were identified in the bile of calculus (gallstone) cholecystitis patients (Barbhuiya et al., 2011).

4.1.5 Proteomic analysis of CCA cell lines

The use of cell lines in cancer proteomic research confers advantages and disadvantages along similar lines to those discussed earlier in cytotoxic drug evaluation (see section 1.2.1). Advantages such as low cost, unlimited supply and sample purity make them very attractive for initial evaluation. However, the in vitro expression of proteins and differences from the original tumour tissue several after subculture are limitations that must be taken into account when interpreting the results.

The first proteomic study of a CCA cell line was a 2D gel study where the Thai CCA HuCC-A1 cell line was compared to two hepatocellular carcinoma cell lines (Srisomsap et al., 2004). Differential expression of cytokeratins and vimentin were found. This was followed by a more in-depth study where the crude protein mixture from the same cell lines were separated into two fractions using different detergents and then analysed by 2-DE (Srisomsap et al., 2007). A greater range of differentially expressed proteins were found, with the CCA cell line being enriched in cytoskeletal proteins and proteins involved in protein turnover.

The most important drawback of both these cell line studies was that the comparison made was between models from two cancers (biliary and liver). Making the distinction between HCC and CCA is a frequent clinical question. However, important questions such as differences between tumour and normal tissue could not be addressed.

4.1.6 Aim

The aims of this section of the study were two-fold. Firstly to use 2D-DIGE to compare proteomes between an immortalised cholangiocyte cell line and three CCA cell lines to determine how different they were. Secondly, to assign identities to differentially expressed proteins using MALDI-ToF/ToF mass spectrometry.

4.2 Methods

4.2.1 Lysis buffer preparation

The following were added to a sterile 50ml tube:

- 7M urea (21.02g) (Sigma, Cat No. U6504)
- 2M Thiurea (7.61g) (Sigma, Cat No. T8656)
- 30mM Tris Base (0.18g) (Sigma, Cat No. T1503)
- 4% CHAPS (2g) (Sigma, Cat No. C9426)

Using double distilled sterile water the above was made up to 50mls and placed on a roller mixer for solubilisation.

4.2.2 Protein extraction from cell lines

Cell lines used for this section of the study were: H69, M213, SkChA1 and HUCC-T1. H69 is an immortalised cholangiocyte, M213 is an intrahepatic CCA of Thai origin, SkChA1 is an intrahepatic CCA of western origin and HUCC-T1 is an extrahepatic CCA. Cells were cultured to sufficient quantities in tissue culture flasks as described (see

section 3.2.1). Four biological replicates of each cell line were commenced preferably from stored cells which were at different passage numbers. The total surface area for each version of each cell line amounted to about 675 cm^2 (9 x T75 flasks). Cells were harvested when they reached approximately 80% surface confluence. Once lifted, the cells were pooled into a centrifuge tube and washed 4 times with sterile PBS to remove all traces of FCS, after which 4ml of lysis buffer containing a mammalian general purpose protease inhibitor cocktail (GE Healthcare) was added. Lysis was aided by agitation then the mixture was passed through a 5ml syringe attached to a 21 gauge needle five times to shear any DNA strands. The samples were then aliquotted into 1.5ml centrifuge tubes and centrifuged at 16,100g for 1 hour at 20^{o}C. The supernatant was collected and both the pellet and supernatant aliquots stored at -20^{o}C until further use.

Thus, the starting material for the experiment consisted of four biological replicates from each of the four cell lines (16 preparations in total).

4.2.3 2-D clean up

Samples were purified and concentrated, by treating with a 2-D Clean-Up Kit (GE Healthcare, Cat No. 80-6484-51). The intention of this step was to reduce impurities such as nucleic acids, salts and buffers. A protein sample in 280µl of lysis buffer was added to 840µl of precipitant and incubated on ice for 15 minutes. A further 840µl of co-precipitant was added and mixed. Samples were then centrifuged at maximum speed for 5 minutes. The supernatant was removed, the samples centrifuged again and the remaining supernatant aspirated. A layer of 40µl of co-precipitant was added to each sample and left on ice of 5 minutes. Samples were centrifuged for 5

minutes at maximum speed and then the wash was removed and discarded. Samples were dispersed in 25µl of de-ionised water and then mixed with 1ml of wash buffer. After 30 minute incubation at -20^{o}C they were centrifuged for 5 minutes and the supernatant removed and discarded. The remnant protein pellet was re-suspended in pre-prepared lysis buffer.

4.2.4 Determination of protein concentration

It is crucial in 2D-DIGE that the protein concentration of each sample is identical. Concentrations and total protein yield were measured on a Nanodrop spectrophotometer (Thermo Scientific) using the Pierce 660nm protein assay (Thermo Scientific, Cat No 22660) against standards consisting of albumin also solubilized in lysis buffer. The Nanodrop software automatically calculates sample concentrations. One µl of sample was mixed with 15µl of Pierce 660. Readings were performed 50 minutes after mixing. Protein concentrations were then adjusted to 1283 µg/ml (the concentration of the most dilute sample) by adding more lysis buffer and then reassessed to ensure that they are all the same.

4.2.5 Sample Labelling

The pH of each sample was established using pH 7.5 - 9.5 indicator strips and adjusted to pH 8.5 using 50mM NaOH. An aliquot containing 50 µg protein from each sample was then labelled with 400 pmol Cy5 DIGE Fluor minimal dye (GE Healthcare) according to the manufacturer's protocol. An internal standard was made by pooling 50 µg of each sample (800 µg in total), then labelled it with 6.4 nmol Cy3 (also GE Healthcare). The labelling reactions were carried out on ice for 30 min then

terminated by adding 1 ml of 10 mM lysine per reaction. Sixteen sample mixtures were then prepared, such that each mixture contained a mixture of a 50 µg Cy5-labeled sample and 50 µg of the Cy3-labeled internal standard (100 µg in total).

All 16 samples had been processed simultaneously up to this point.

4.2.6 2D-DIGE experimental design

Although it would be ideal for the 2-DIGE of all 16 samples to be carried out simultaneously to ensure absolute reproducibility, this was not possible because the maximum number of samples that the IEF and gel electrophoresis equipment can handle simultaneously is twelve. Therefore, it was necessary to split the work into two batches of eight samples, with the second batch running a day behind the first. To avoid batch effect, each set of eight samples consisted of two replicates from each cell line.

4.2.7 Quality assessment

In order to establish that the extraction process and the sample clean-up had been successful and to maximise the chances of success for the forthcoming isoelectric focussing, gel casting and gel electrophoresis, two quality assessment (QA) tests were carried out. Each QA test used 100 µg of an unlabelled sample and one of the gels from each casting. The only divergence from the forthcoming protocol is that because the QA test samples were not fluorescently labelled the gels were visualised by removing them from their glass plates and staining them with Coomassie Biosafe stain (BioRad) as per the manufacturer's protocol.

4.2.8 Gel casting

Gels were prepared in a 2DE Optimizer (NextGen Sciences), which casts twelve 24 cm gels simultaneously to ensure homogenicity. The Optimizer was programmed to cast gels with an acrylamide gradient of 9 to 16% as previous work had demonstrated that this gradient provides a good separation of proteins from 5-200 kDa. The reagents used were as follows: 692ml of double distilled water, 258 ml of Acrylamide 37.5:1 solution, (Sigma Cat No. 01709), 59ml of ammonium persulphate 1% wt/vol (Sigma Cat No. A9164), 157 ml of Glycerol 60% vol/vol (Sigma, Cat No G5516), 44ml of TEMED 1% vol/vol (Sigma Cat No. T9281) and 157 ml of Tris-SDS solution (2.25M Tris at pH 8.8 and 0.6% SDS). The Tris SDS was made up of Trizma base (230.85g/l, Sigma Cat No. T1503), Tizma HCL (55.35g/l, Sigma Cat No. T3253) and SDS (6.0g/l Sigma Cat No. L4390).

Gels were cast in low-fluorescent glass plates using an Ettan Dalt Twelve gel caster (both GE Healthcare), with the casting volume programmed so that the gel mixture filled the gap between the plates but leaving a 2cm gap at the top. This space was filled with a 0.5% SDS gels solution and the gels were then left overnight to polymerize at 4°C.

4.2.9 Isoelectric strip rehydration

Each sample (now mixed with the internal standard and labelled) was mixed with rehydration solution (8M urea, 2% CHAPS, 280mM DTT, 0.05% bromophenol blue and 0.5%, pH 3-11 IPG buffer (GE Healthcare)) to make a final volume of 450 µl. This was pipetted evenly along the length of a 24 cm, individual ceramic strip holder, between the electrodes. A 24 cm, pH 3-11 non-linear Immobiline Drystrip (GE Healthcare, cat 17-6003-77) was gently laid on top of the sample and then covered in Immobiline DryStrip Cover fluid (GE Healthcare, Cat No. 17-1335-01). The strip holder's cover was replaced and the strips were left overnight in the dark for passive rehydration.

4.2.10 Isoelectric focussing

Two paper wicks (BioRad, Cat No. 1654071) soaked in 20µl of ddH_2O were placed between the electrode poles of the IEF strip and the strip holder. The strip holders were then placed onto the bed of an Ettan IPGPhor 3 isoelectric focussing system (GE Healthcare) and IEF carried out according to the following protocol: 500V for 1 hour, 1000V for 1 hour, 8000V for 5 hours, 8000V for 70000Vh and 500V until discontinuation. As described above, eight strips were focussed simultaneously.

4.2.11 Sample reduction and alkylation

Immediately following IEF the strips were removed from their holders and soaked for 15 mins in 4 ml of equilibrium solution (6M Urea, 75mM Tris pH8.8, 30% glycerol, 2%

SDS and 0.01% bromophenol blue) containing 1% DTT and then for a further 15 min in equilibrium solution containing 4% iodoacetamide.

4.2.12 Gel electrophoresis

Immediately following equilibration the SDS solution was aspirated from the top of the gels and each IEF strip slotted into the space. A stainless steel spatula was used to ensure no air bubbles were visible at the strip/gel border. A 1% molten agarose solution was pipetted onto the strip, ensuring a good seal. The gels were then loaded into an Etttan Twelve Gel Electrophoresis system (GE Healthcare) filled with TGS (Tris/Glycine/SDS) running buffer (BioRad, Cat No 161-0772). Electrophoresis was carried out at 15W for 1 hour followed by 180W until the bromophenol blue dye ran to the bottom of the gel.

4.2.13 Image acquisition

The gels were preserved at 4^{o}C in a dark enclosure until scanning. They were imaged on a Typhoon Trio laser scanner (GE Healthcare). Scanning was performed whilst the gel remained between its glass plates to prevent drying and shrinkage. Cy3 scanning was performed at 532 nm excited fluorescence and Cy5 at 633 nm. A preliminary quick and low resolution (1,000 µm pixel size) scan was performed to adjust the PMT power (in Volts) and bring the darkest spots to between 70 and 80,000 (arbitrary units). The purpose of this is to bring the highest intensity signals into the linear range of the instrument. Once the power setting was determined, gels were scanned at 100 µm pixel size resolution and were saved in .gel format.

4.2.14 Gel analysis

Analysis was performed using the Progenesis Same Spots© software, v. 4.0 (Nonlinear Dynamics). A reference gel was selected on the basis of the best Cy3 (internal standard) image in terms of spot differentiation and gel distortion. All fifteen other Cy3 images were aligned to this reference. Gel-matching was carried out manually by placing approximately 150 anchor spots per gel. Then, the automatic alignment function of the software was used for the remaining spots. The software's 'transition view' window was used as a quality check prior to accepting an automatic alignment. Each automatically-matched spot was inspected individually using the software's 2 and 3 dimensional montage function. The split, merge and delete functions were used to correct the minor inaccuracies performed by the automatic function of the software.

Spot matching between the gels is straightforward because it is carried out using the Cy3 internal standard which is identical on each gel. As the internal standard is made from all of the samples in the experiment, every spot on every gel will be represented in it, and because the Cy3 and Cy5 fluorophors have the same molecular mass and charge, the Cy5 sample spot will have exactly the same coordinates in each gel as its equivalent Cy3 reference spot. Therefore, once the internal standard spots have been matched between the gels, the Cy5 sample spots are also matched.

4.2.14.1 Gel normalisation

After spot matching, normalisation was performed to calibrate data between different gels. Experimental variation, such as sample quantity, sample labelling and gel image capture can affect abundance measurement. This variation was normalised by comparing the intensity of the internal standard on each gel to the reference gel, thus giving a gain (or loss) factor. The sample measurement can then be adjusted by multiplying the results by this factor. This function is performed within SameSpots software.

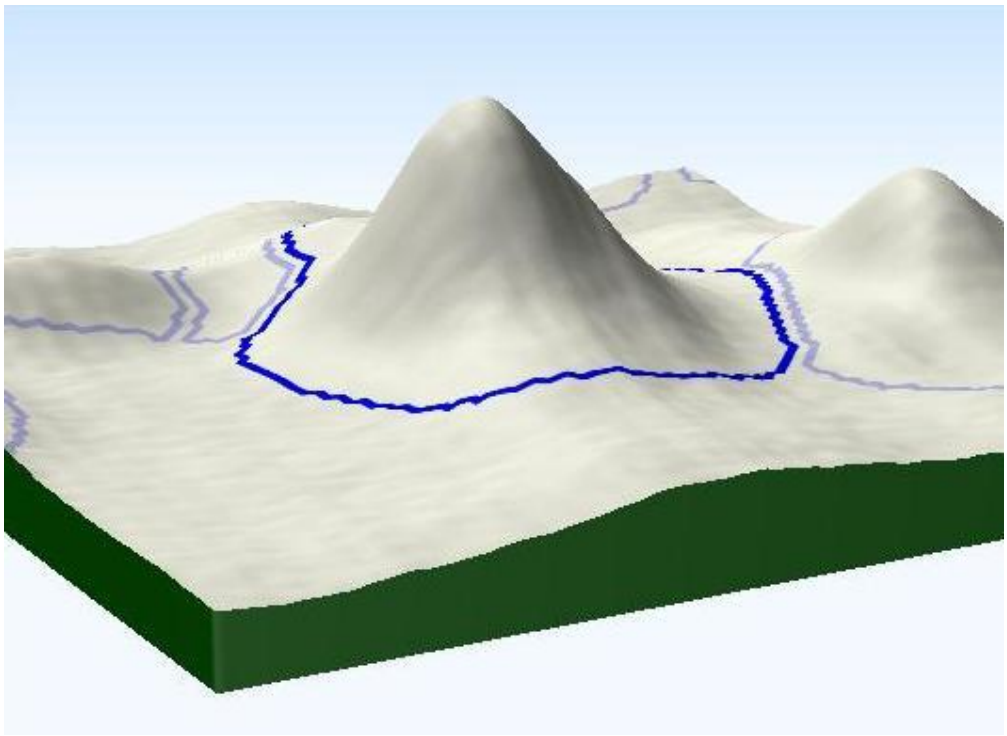

Figure 4-1 Same Spots 3 dimensional analysis

Each spot in the gel represents a different protein, with the protein's expression level determining the spot's volume. By comparing the volumes of every spot in every gel, differences in protein expression between the cell lines are established.

Once spot matching had been completed, the spots were ranked according to the greatest fold-change across the groups at 95% confidence level. Spots that were

differentially expressed in any of the cell lines compared with any other cell line were identified by the software and ranked by p-value from one way ANOVA analysis.

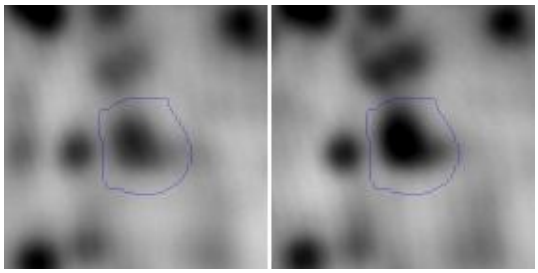

Figure 4-2 Example of a differentially-expressed spot from Same Spots software.

The highlighted spots in the two gels have been matched so they represent the same protein from two cell lines. The spot in the right-hand image (HUCC cells) has a greater volume than the left hand image (H69 cells) as it stains more heavily; therefore, the protein is expressed at a higher level in HUCC than H69. In order to assign an identity to the spot it must be cut from the gel and analysed by mass spectrometry.

Figure 4-2 illustrates an example of spot matching as processed by Same Spots. Two matched spots with different intensities (i.e. spot volumes) can be seen. After MALDI-MSMS analysis this spot (spot no 52) was identified as the protein lactoylglutathione lyase. The image on the left is derived from protein from H69 cells, whist the one on the right from HUCC cells. It can be seen that the intensity of the spot on the right is more intense, and this difference was calculated as statistically significant taking all four biological replicates into account.

4.2.15 Preparative gels for mass spectrometry

As each of the gels used for the analysis “only" contained 100 µg of protein, four preparative gels were made (one for each cell line), each of which contained 500 µg of protein. These much higher protein loads significantly increase the chances of gaining protein identities by mass spectrometry. Samples were prepared as

previously described, except the total voltage for IEF was increased by 20% to allow for the increased protein load: 500V for 1,500Vh, 1,000V for 800Vh, 10,000V for 16,500Vh, 10,000V for 26,640Vh and then 500V until discontinuation.

After electrophoresis, the gels were removed from their glass plates, fixed for 2 hours in a 40% methanol, 10% acetic acid solution then stained with Biosafe Coomassie (BioRad). The gels were imaged using a Fujifilm LAS-3000 imager and the differentially expressed spots identified by matching the spot patterns from the preparative gels to those from the SameSpots images.

4.2.16 Excising spots

Spots identified as differentially expressed were cut from the preparative gel using a spot picker (Gelcompany). If the spot was small and well circumscribed, a 1.5mm diameter spot picker was used whilst if the spot was larger a 3.0 mm one was used. Gel pieces were placed in individual protein low binding eppendorf tubes (Eppendorf, cat no 022431081) preserved in 400µl of gel fixative solution (40% methanol, 10% acetic acid) and assigned a Spot Number.

4.2.17 Preparing spots for MALDI=ToF/ToF analysis

The fixative solution was carefully removed and each gel piece and washed with 100µl of 25mM ammonium bicarbonate. Each sample was then washed twice in 100µl 25mM ammonium bicarbonate in 50% (v:v) which was then replaced with 100% aqueous acetonitrile. After 5 mins the acetonitrile was removed and the

sample was dried in a vacuum concentrator for 20 minutes (ThermoSavant SC210A SpeedVac Plus).

Trypsin was used to digest the protein samples. Trypsin was prepared as follows: an aliquot of 100µl of Trypsin Buffer as supplied by the manufacturer (Promega, Cat No 608-274-4330) was added to 20µg of sequencing grade modified porcine trypsin (Promega Can no V511A) to make stock trypsin solution. This was then diluted 1:4 in 25mM ammonium bicarbonate to make the working concentration of 0.02µg/µl. Each sample was covered in 10µl of trypsin and allowed to re-swell for 5-10 minutes. In the event that the trypsin did not cover the whole gel piece, a topping of 10µl of 25mM ammonium bicarbonate was added. The digests were incubated at 37^{o}C overnight.

A 1µl aliquot of each peptide mixture was applied directly to the ground steel MALDI target plate, followed immediately by an equal volume of a freshly prepared matrix solution (5 mg/ml solution of 4-hydroxy-α-cyano-cinnamic acid (Sigma) in 50% aqueous (v:v) acetonitrile containing 0.1% , trifluoroacetic acid (v:v)).

4.2.15 MALDI-ToF/ToF mass spectrometry

Positive-ion MALDI mass spectra were obtained using an Ultraflex III MALDI-ToF/ToF mass spectrometer (Bruker Daltronics). MS spectra were acquired over a mass range of m/z 800-4000, externally calibrated against an adjacent spot containing 6 peptides (of 904.681, 1296.685, 1750.677, 2093.086, 2465.198 and 3657.929 Da)

For each spot, the ten strongest PMF peaks with a signal-to-noise ratio > 30 were selected for MS/MS fragmentation. Bruker flexAnalysis software (version 3.3) was

used to perform the spectral processing for both the MS and MS/MS spectra.Tandem mass spectral data were submitted to database searching using a locally-running copy of the Mascot program (Matrix Science Ltd., version 2.3), through the Bruker ProteinScape interface (version 2.1). The search parameters used were: Enzyme, Trypsin; Fixed modifications, Carbamidomethyl (C); Variable modifications, Oxidation (M); Peptide Mass Tolerance, 250 ppm; Fragment Mass Tolerance, 0.5 Da; Instrument type, MALDI-TOF-TOF. Identities were sought against the IPI Human database (version 3.87). IPI human is a database of human proteins, currently consisting of > 90,000 minimally redundant yet maximally complete proteins (one sequence per transcript) selectively-sourced from other databases such as SwissProt and Trembl (Kersey et al., 2004). The search criteria allowed for Carbamidomethyl (C) as a fixed peptide modification and oxidation (M) as a variable modification. One missed tryptic cleavage was allowed for and the mass tolerances were 250 ppm (MS) and 0.5 Da (MSMS).

Only spot identities with a total ion score of over 95% were accepted.

All proteomic work was carried out by me under laboratory supervision of Dr William Mathieson (Imperial College London). Mass spectrometry was carried out at the University of York's Centre of Excellence in Mass Spectrometry under the supervision of Adam Dowle (University of York).

4.3 Results

4.3.1 Test Gel I

The first 24cm test gel demonstrated horizontal spot-streaking in the basic region of the gel.

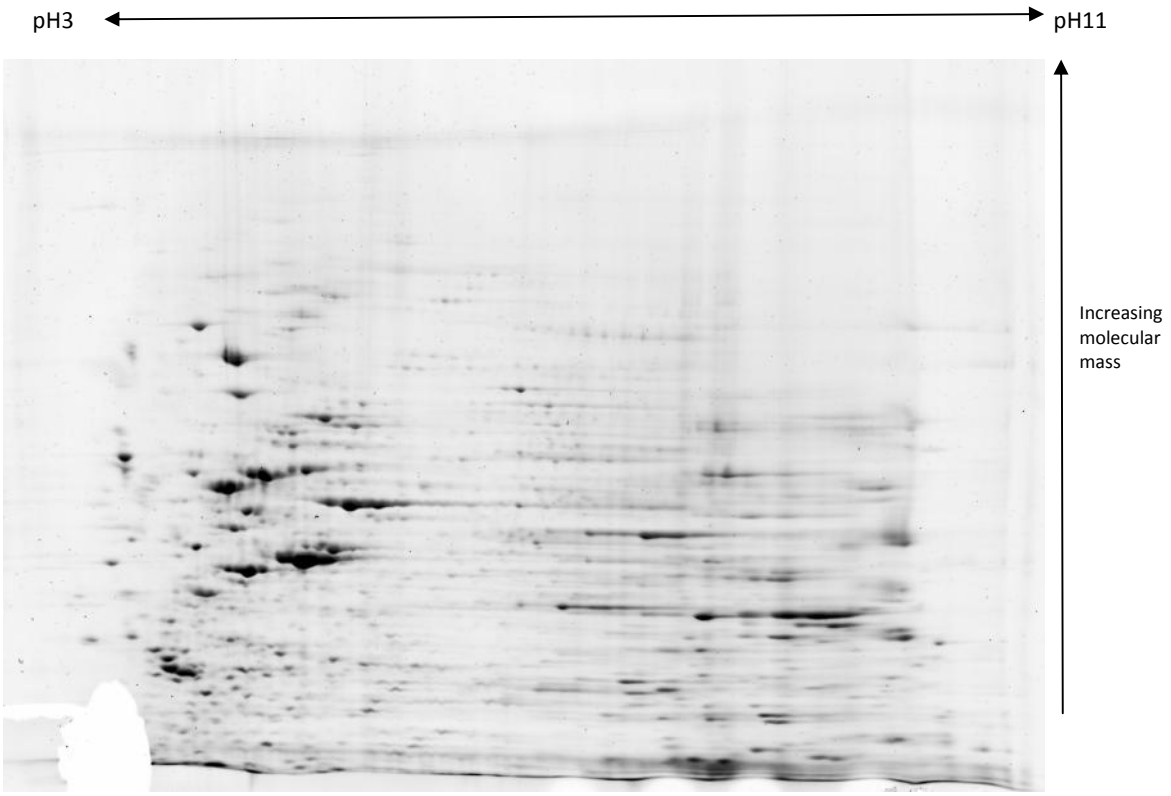

Figure 4-3 M213v2, 24 cm gel.

As the streaking was horizontal it shows that the IEF of the proteins had not been successfully completed. As the streaking is restricted to the basic region of the gel it was likely to be related to the random re-formation of disulphide bonds in the basic proteins only. DTT was incorporated into the buffer prior to IEF to cleave disulphide bonds. However, DTT can migrate from the basic regions of the IEF strip during focussing, enabling the disulphide bonds to reform randomly, leading to this

streaking pattern in the basic region. To test this hypothesis two test gels were (see section 4.3.2) carried out, one with an alkylation step so that reactive cysteine groups will be capped prior to IEF and unable to reform disulphide bonds.

4.3.2 Test Gel II

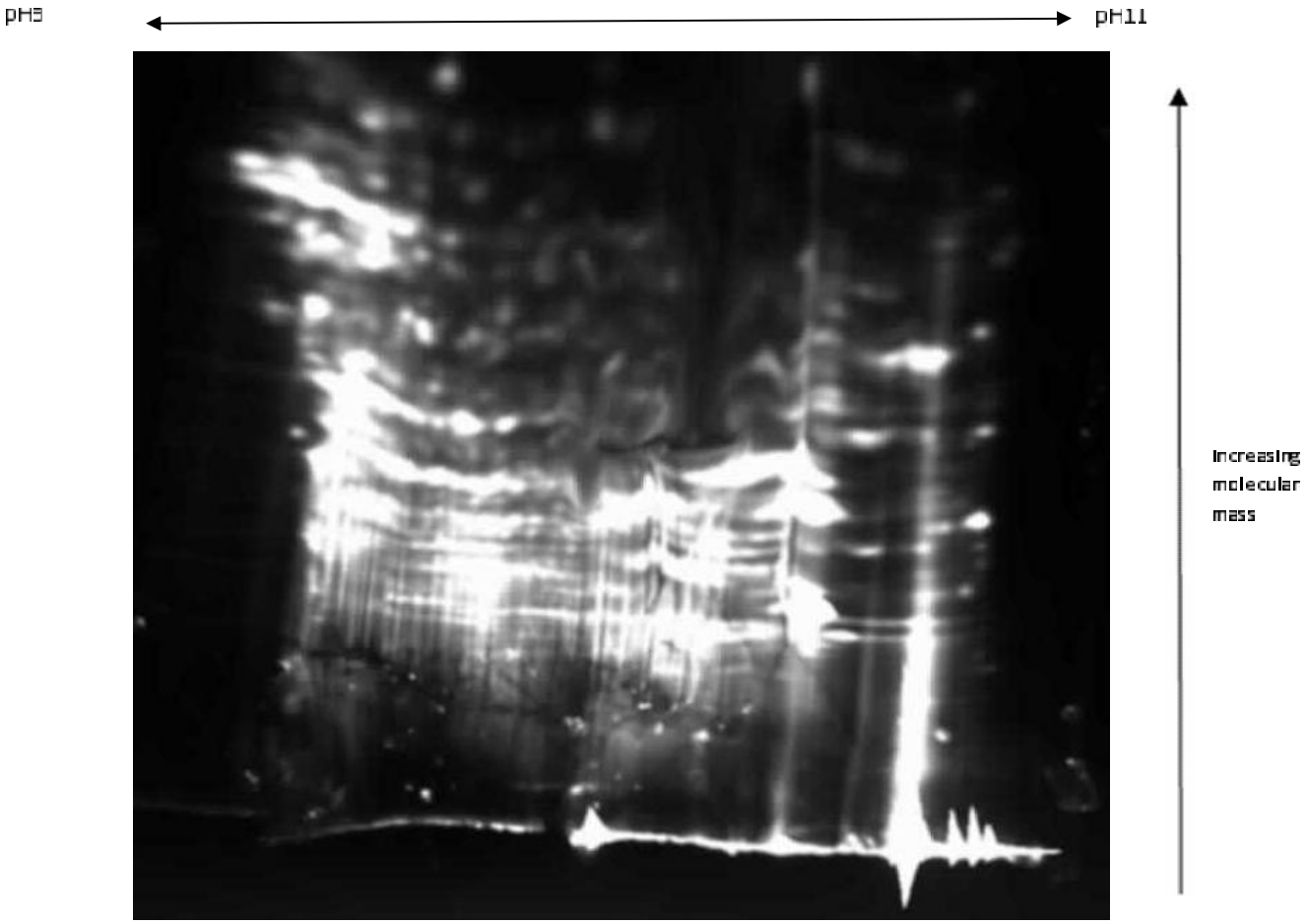

Figure 4-4 M213v3, 7cm gel, without Reduction/Alkylation step Cy5

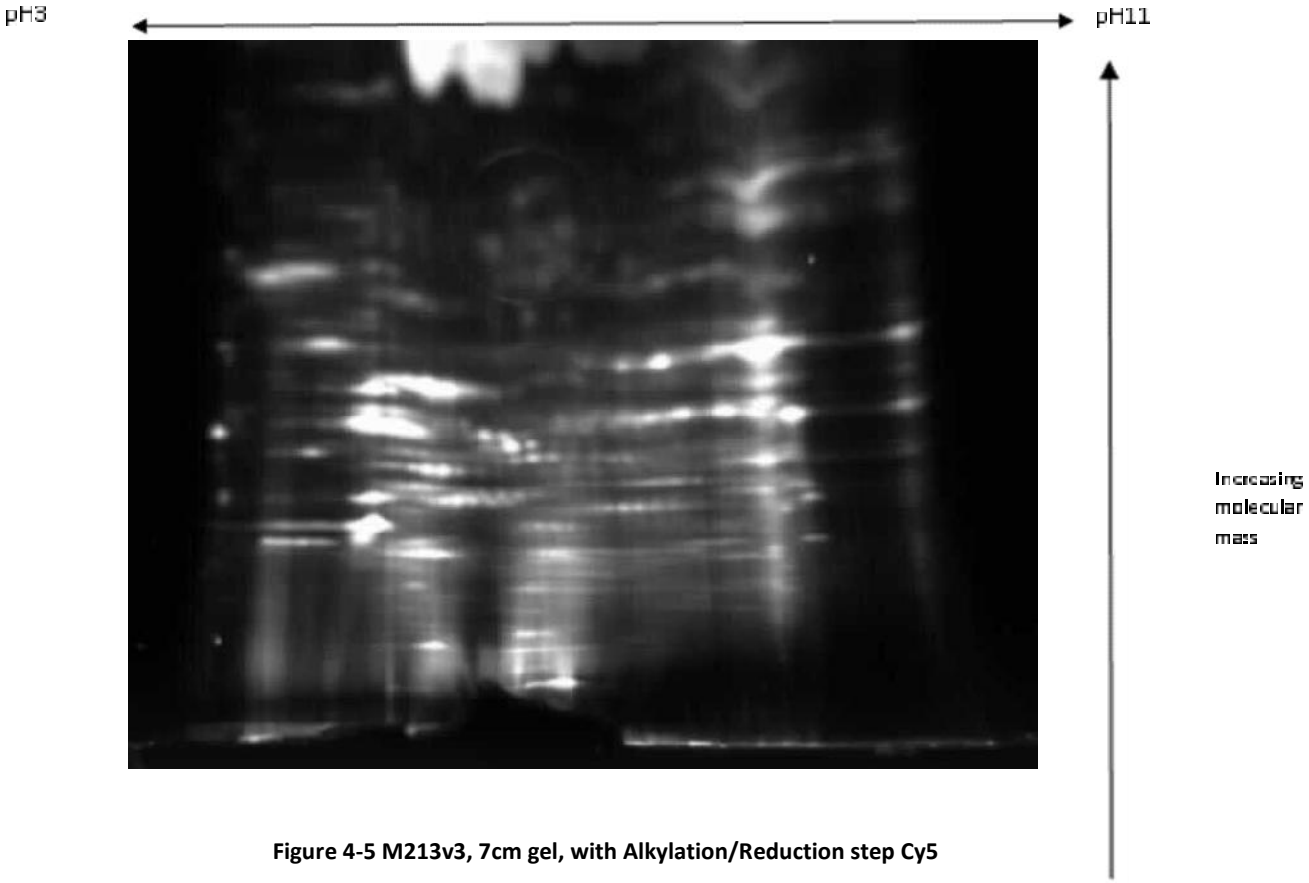

Figure 4-5 M213v3, 7cm gel, with Alkylation/Reduction step Cy5

4.3.3 Gel electrophoresis and analysis

The SameSpots automatic spot-detection software identified a total of 1380 spots in the gels. However, on manual inspection it became apparent that some spots identified as single actually represented two or more individual spots. These were split manually using the relevant software function. The software misinterpreted some artefacts such as folds or specks on the gel as spots; these were manually deleted. A few larger spots were identified as more than one individual spot and were manually merged. After the above process, the study identified a total of 1110 spots that were considered genuine.

Although the normalized volume of each spot varied between gels, all 1110 spots were detected in all of the gels. Initially, comparisons were made between the different cell lines. Hence, although there was inter-sample difference in the level of protein expression there were no instances where a protein was detected in one cell line but not in another. Cell line H69 was taken to be the reference cell line, representing normal cholangiocytes.

In order to compare the spot patterns between cell lines, the normalized spot volume for each spot was averaged by one-way ANOVA and any spots in any cell line compared with H69 with a statistically different volume were highlighted. Each of the three CCA cell lines was compared to the reference cell line for number and magnitude of difference. Scatter plots were generated to illustrate the distribution of gel spots, for the complete proteome and for selected spots deemed to be significant in the analysis. In the ANOVA analysis, a p value of <0.01 was used as a threshold for significance.

4.3.3.1 Comparison of H69 and HUCC cell lines

When compared to H69, the HUCC cell line, representing intrahepatic cholangiocarcinoma, had 168 spots that were expressed differently at a statistically significant level. Of these 100 (9.01%) were up regulated and 68 (6.13%) were down regulated. A total of 942 (84.86%) spots were not different to a statistically significant level.

The above figures only represent the numbers of spots and no distinction in terms of protein abundance is accounted for. Therefore analysis of the volume of the

differentially expressed spots was performed. In terms of spot-volume, the up regulated proteins in HUCC represented 10.98% of the total proteome, whilst the down regulated ones 4.84%.

Figure 4-6 is a scatter plot plotting the normalised volume of the all individual spots for H69 and HUCC. The Pearson correlation coefficient (*r value*) was calculated at 0.05785 with a p value of 0.0540 using Microsoft Excel 2010. The r value suggests that there is no correlation; however, this is not statistically significant.

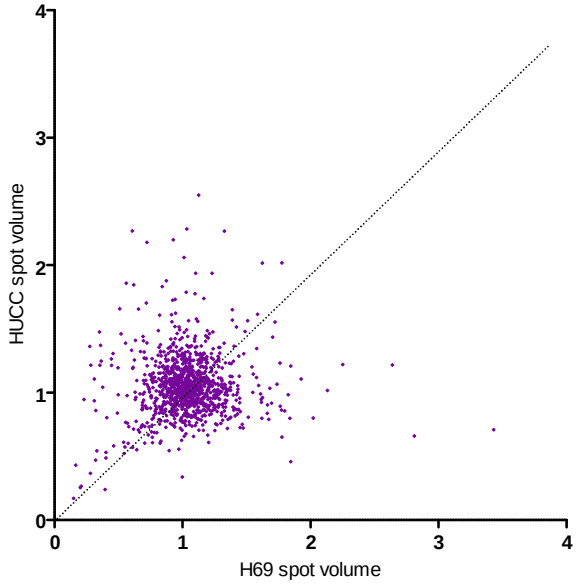

Figure 4-6 H69 v HUCC Scatter plot of all 1110 spots identified

Figure 4-7 is a scatter plot of the 168 differentially expressed spots. Spots lying above the x=y boundary (dotted line) represent up regulation in HUCC compared to H69 whilst those below represent down regulation. Visual inspection suggests that more spots are up regulated than down regulated (100 vs 68).

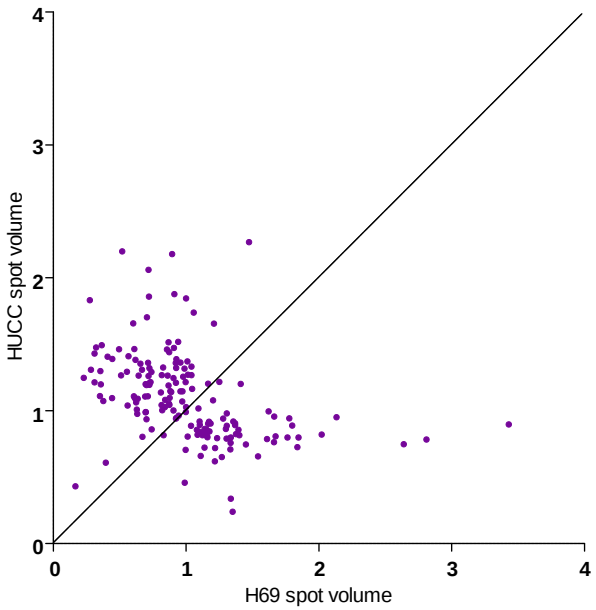

Figure 4-7 H69 v HUCC, scatter plot of differentially expressed spots

4.3.3.2 Comparison of H69 and SkChA1 cell lines

In cell line SkChA1, 293 spots were differentially expressed. Of these, 189 (17.03%) were up regulated and 104 (9.37%) were down regulated. There were 817 (73.60%) spots that were not differentially expressed. The up regulated proteins represented 24.51% of that cell line's proteome volume and the down regulated ones represented only 6.20% of the total volume. The *r value* in this comparison was -0.3664 with a *p value* of < 0.0001. This r value suggests a better correlation than that of HUCC. The p value confirms that this is also to a statistically significant level.

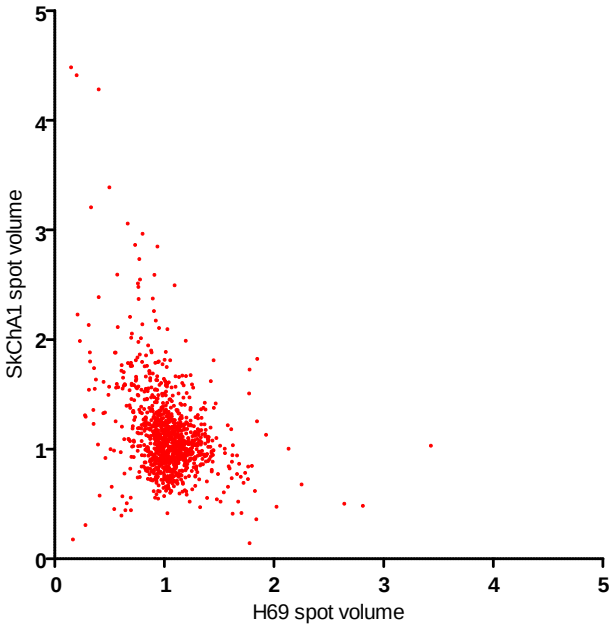

Figure 4-8 H69 v SkChA1 Scatter plot of all 1110 spots identified

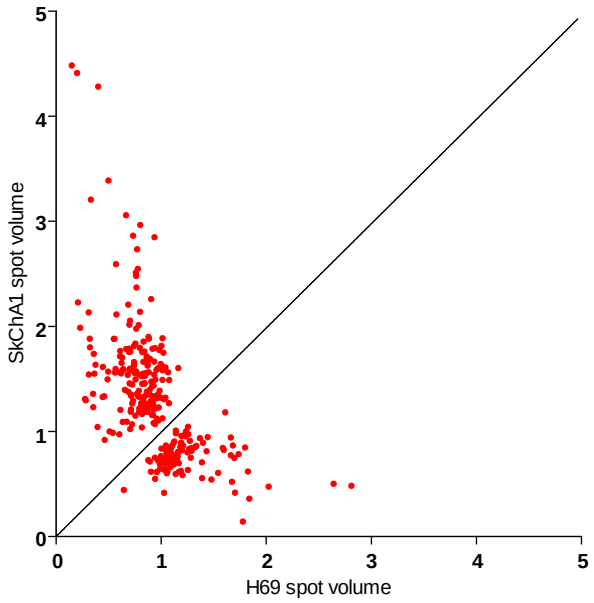

Figure 4-9 H69 v SkChA1, scatter plot of differentially expressed spots

4.3.3.3 Comparison of H69 and KKU M213 cell lines

In cell line M213, 184 spots were differentially expressed. Of these, 119 (10.72%) were up regulated and 65 (5.86%) were down regulated. There were 926 (83.42%) spots that were not differentially expressed. The up regulated proteins represented 13.75% of that cell line's proteome volume and the down regulated ones represented only 4.20% of the total volume. . The *r value* in this comparison was -0.1667 with a *p value* of < 0.0001. This r value suggests a stronger correlation than that of HUCC but not as strong as that of SkCHA1.

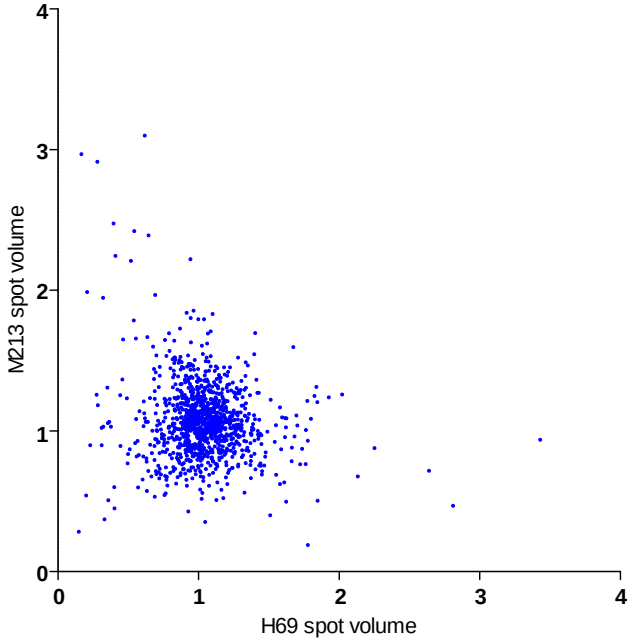

Figure 4-10 H69 v KKU M213 Scatter plot of all 1110 spots identified

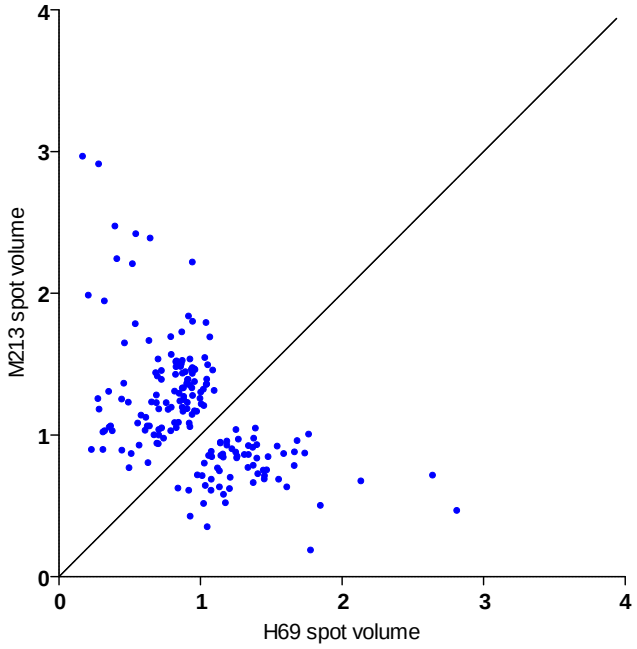

Figure 4-11 H69 v KKU M213**, scatter plot of differentially expressed spots**

4.3.4 Gel spot picking

A total of 96 differentially-expressed spots were visualized in the preparative gels and excised for MALDI-MSMS analysis. Of these, 74 spots were harvested from a gel prepared using cell line M213, 7 from cell line SkChA1 and 14 from H69. There was overlap of 5 spots giving a total of 91 unique spots with 5 replicates (see Figure 4-12).

The table below summarises the number of differentially expressed spots and their significance in the cell line comparisons. Spots were picked manually, (see section 4.2.16) so they had to be visible to the naked eye. Image acquisition using a laser scanner with subsequent software analysis (see section 4.2.13) has a superior sensitivity and resolution to the naked eye inspection of the gels. It is estimated that

Coomassie blue, used in staining proteins can detect about 25% of proteins detected in DIGE (Patton, 2002). Having said that, it is generally accepted that if a protein spot cannot be visualised by Coomassie-blue staining it is unlikely to yield sufficient protein for a MALDI-derived identity. As a result only 96 of the proteins detected were eventually picked for identification by MALDI.

	Number of differentially expressed spots	No of spots picked and analysed by MALDI	No of IDs obtained
Up regulation in all 3 cell lines	26	10	6
Down regulation in all 3 cell lines	8	1	1
Up regulation in HUCC and SkChA1	33	9	4
Down regulation in HUCC and SkChA1	8	2	2
Up regulation in HUCC and KKU M213	7	1	1
Down regulation in HUCC and KKU M213	7	3	2
Up regulation in SkChA1 and M213	31	1	1
Down regulation in SkChA1 and M213	9	2	1
Up regulation in HUCC only	37	2	2
Down regulation in HUCC only	28	4	3
Up regulation in SkChA1 only	56	4	4
Down regulation in SkChA1 only	98	13	12
Up regulation in KKU M213 only	29	6	6
Down regulation in KKU M231 only	43	4	2
Down regulation in HUCC and SkChA1, up in M213	1	0	-
down regulation in HUCC, up regulation in SkChA1	1	0	-
Down regulation in HUCC, up regulation in SkChA1 and M213	1	0	-
Down regulation in SkChA1, up regulation in KKU M213	10	1	1
Up regulation in HUCC and SkChA1 but down regulation in KKU M213	1	1	1
Up regulation in HUCC down regulation in KKU M213	6	0	-
Up regulation in SkChA1 down regulation in KKU M213	5	3	3

Table 9 Protein expression as compared to H69 cell line

The figures that follow illustrate the gels used for spot picking including the locations of each of the 96 spots that were picked.

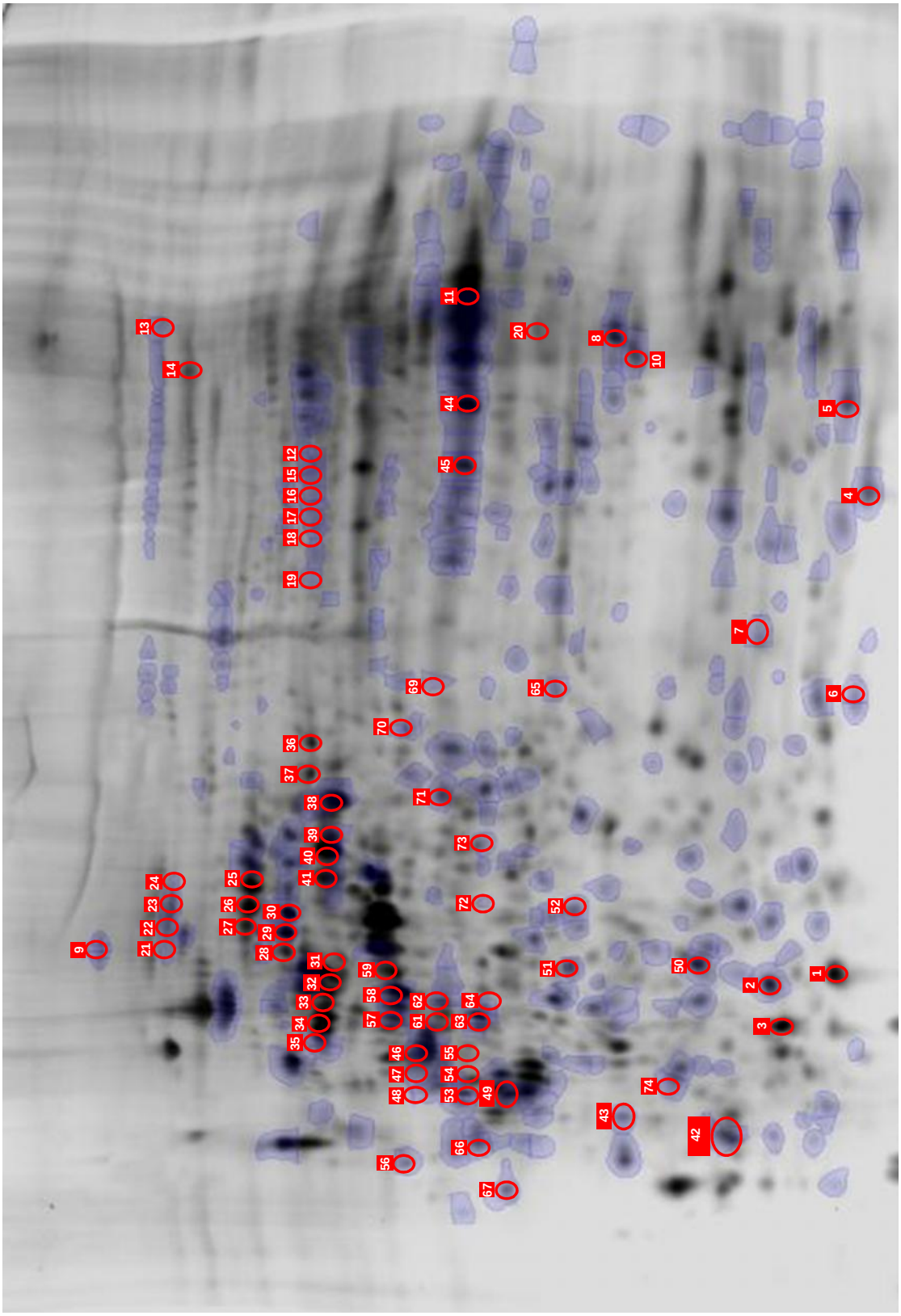

Figure 4-12 Spots (74 in total) harvested from a gel prepared using cell line M213

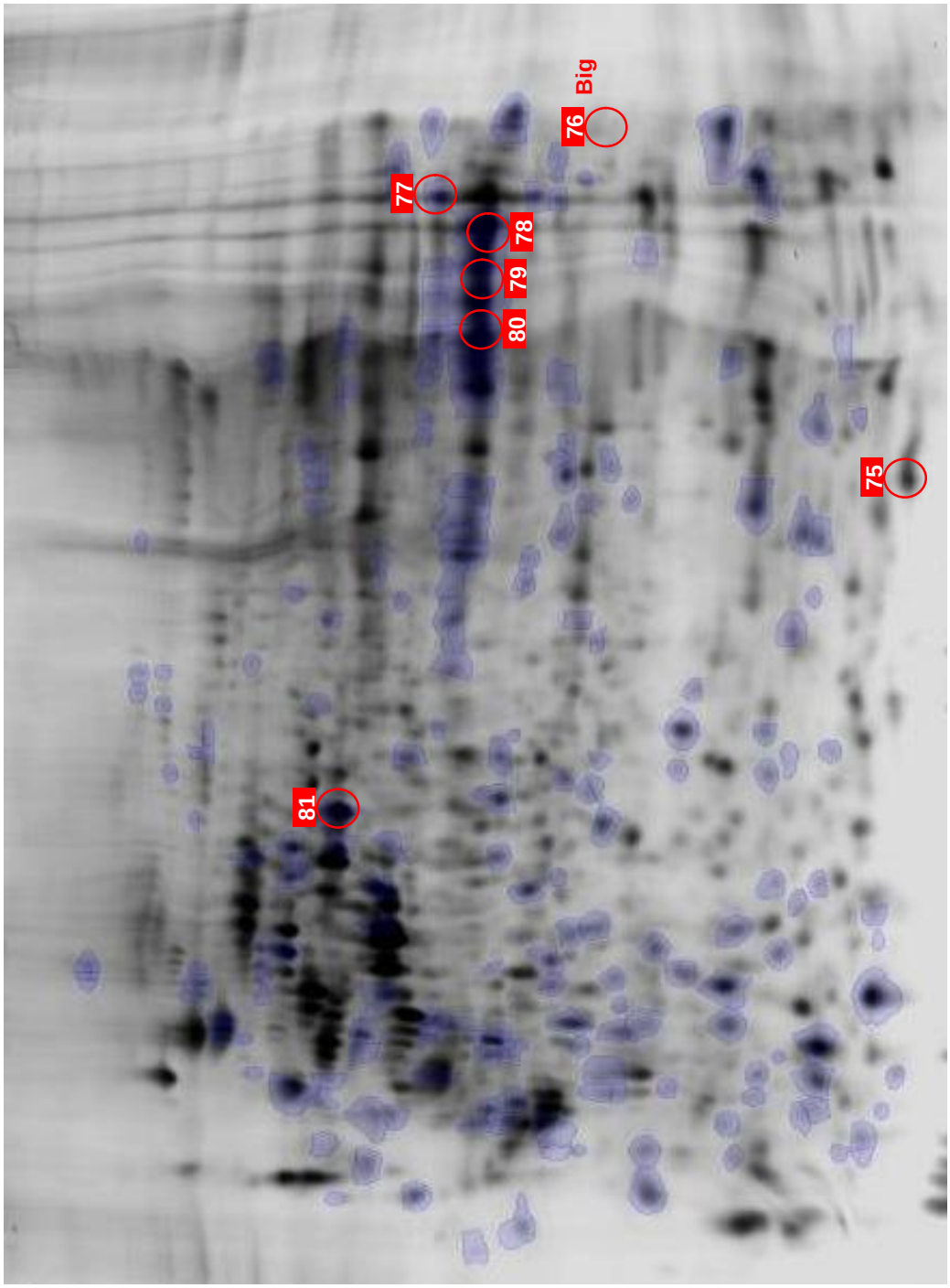

Figure 4-13 Spots (6 in total) harvested from a gel prepared using cell line M213

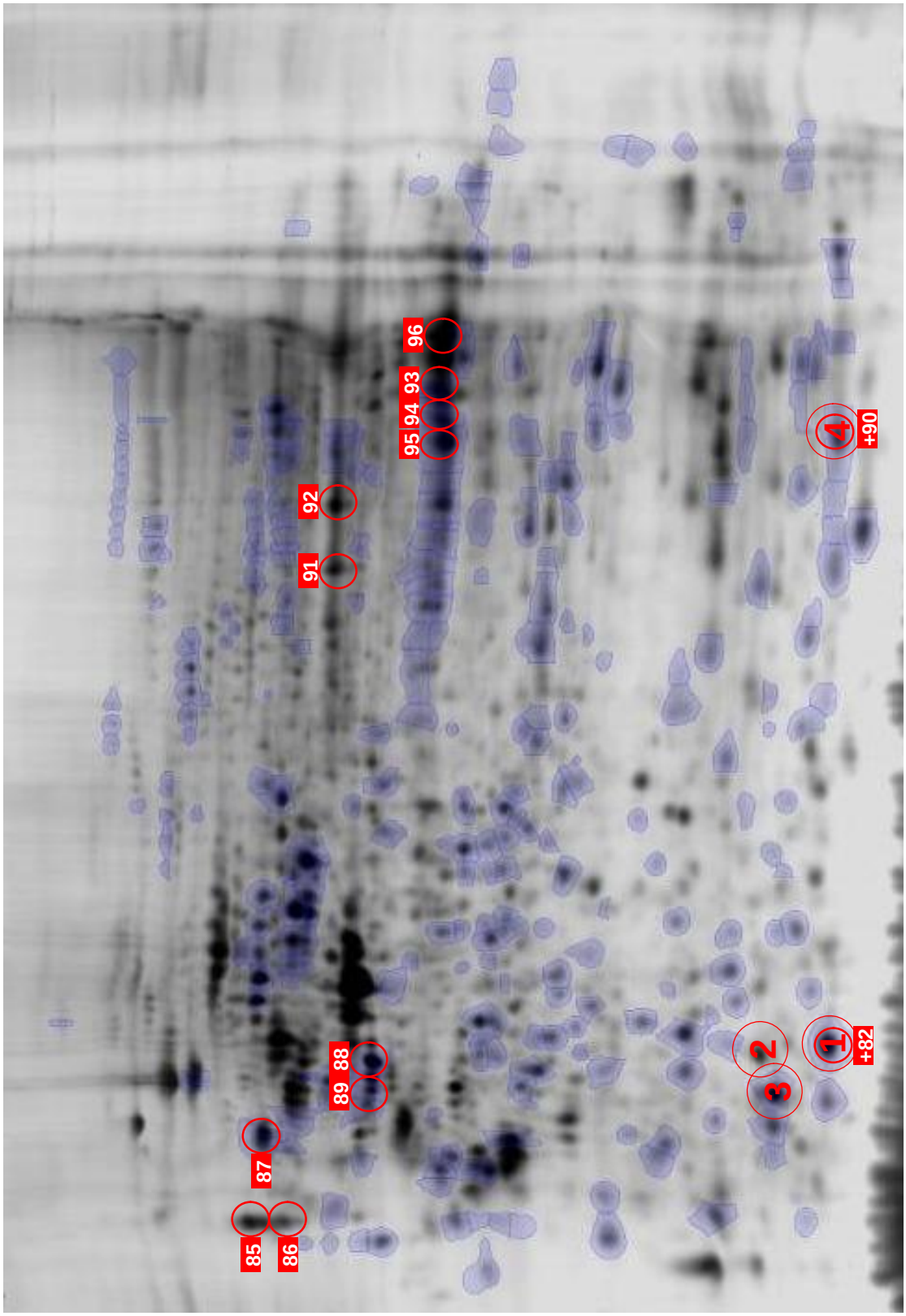

Figure 4-14 Spots (15 in total – some replicates) harvested from a gel prepared using cell line SkChA1

MALDI TOF/TOF and database analysis generated confident identities for 52 proteins. Each identity was scrutinised in detail separately and was only accepted as genuine if the Mascot protein score was sufficiently high to be confident that the identity is genuine at 95% confidence interval and > 1 peptide was fragmented to obtain that identity. In cases where only one peptide had been fragmented the protein's identity was only accepted if the spot on the gel was at the appropriate place (i.e. the charge and molecular weight were correct). Some proteins were detected more than once, so the different spots represented isoforms of the protein. Overall, the total number of unique proteins was 29, listed in Table 10.

Protein identity	IPI human accession number	No of peptides	Mascot Protein score	HUCC	SkChA1	KKU M213
14-3-3 protein sigma	IPI00013890	3	256	+	o	+
ACTB Actin, cytoplasmic 1	IPI00021439	3	289	o	-	o
Alpha-enolase	IPI00465248	5	348	o	-	o
Alpha-enolase	IPI00465248	3	143	o	+	-
Annexin A2	IPI00418169	3	186	+	+	+
Annexin A2	IPI00418169	6	494	+	+	o
ATP synthase subunit beta, mitochondrial	IPI00303476	9	789	+	o	o
Calmodulin	IPI00075248	5	373	o	-	o
Calreticulin	IPI00020599	3	216	-	o	-
Calreticulin	IPI00020599	1	90	o	o	+
Complement component 1 Q subcomponent-binding protein, mitochondrial	IPI00014230	2	147	-	-	o
Elongation factor 1-delta	IPI00023048	2	163	o	o	+
Endoplasmin	IPI00027230	9	635	o	o	+
Endoplasmin	IPI00027230	9	635	o	+	-
Endpolasmin	IPI00027230	9	635	o	+	+
Galectin-1	IPI00219219	2	131	o	o	+
Gamma-synuclein	IPI00297714	3	317	o	+	o
GAPDH	IPI00219018	3	338	-	o	o
GAPDH	IPI00219018	3	286	o	-	o
Heterogeneous nuclear ribonucleoprotein A1	IPI00215965	7	363	-	-	o
HSPA8 Isoform 1 of Heat shock cognate 71 kDa protein	IPI00003865	6	506	o	-	o
Isoform 2 of Annexin A2	IPI00418169	5	285	+	+	o

Lactoylglutathione lyase	IPI00220766	1	96	+	+	-
Nucleophosmin	IPI00220740	1	106	-	-	-
Nucleophosmin	IPI00220740	1	110	o	-	-
Nucleophosmin	IPI00220740	1	99	o	+	o
Nucleophosmin	IPI00220740	3	323	o	O	-
Nucleoside diphosphate kinase B	IPI00026260	4	261	o	-	O
Peroxiredoxin	IPI00027350	2	167	o	-	O
Prostaglandin E synthase 3	IPI00015029	2	178	+	+	O
Protein S100-A6	IPI00027463	1	87	+	+	O
Protein SET	IPI00072377	2	180	o	+	O
PSME2	IPI00384051	3	172	o	-	O
Rho GDP-dissociation inhibitor 1	IPI00003815	5	334	o	+	-
Thioredoxin	IPI00216298	4	247	o	o	+
Translationally controlled tumour protein II	IPI00009943	3	258	+	+	+
Tubulin alpha-1C chain	IPI00218343	5	313	o	o	-
Tubulin alpha-1C chain	IPI00218343	7	723	o	+	O
Tubulin beta-2C chain	IPI00007752	6	477	-	o	-
ubiquitin	IPI00179330	2	92	o	-	+
o: no difference, -:down regulated, +: up regulated						

Table 10 Summary of identified proteins

4.4 Discussion

The selection of cell lines for this study was made on the following basis: H69, a cell line derived from immortalised normal cholangiocytes would act as a control. The selection of cholangiocarcinoma variants represents pathologically distinct cholangiocarcinoma subtypes : one derived from a patient with intrahepatic disease (HUCC-T1), one from an extra hepatic (SkChA1) and one derived from a patient who suffered from the Far Eastern intrahepatic variety (M213).

Protein from cell lines is relatively inexpensive and easy to harvest and in theory can provide an unlimited supply. Furthermore it is relatively homogenous with none of the between-sample differences that characterize clinical material. Consequently, the decision was made to proceed with four biological replicates (i.e. different passage numbers) of each of the four cell lines. Hence, 16 gels were run in total in order to add power to the statistical significance of the findings.

4.4.1 Alpha enolase

Enolase is a protein with a mass between 82 and 100 kDa, depending on the isoform. It is a glycolytic enzyme and responsible for catalysing the conversion of 2-phosphoglycerate to phosphoenolpyruvate in the Embden Meyerhof Parnas glycolysis pathway. In humans, 3 enolase isozymes can be found, which are tissue-specific: alpha, beta and gamma enolases. Alpha enolase (enolase 1) is found in many tissues but mostly in the liver while beta enolase is abundant in muscles and gamma enolase (often referred to as neuron specific enolase) is exclusively found in neuronal tissue (Pancholi, 2001).

The presence of high glucose metabolism (and by consequence glycolysis) in cancer is exploited in routine clinical practice today through the use of fluorodeoxyglucose positron emission tomography (FDG-PET). This observation, often referred to as the Warburg effect, was first recognised in 1920 (Bensinger and Christofk, 2012).

Although enolase forms but one link in the glycolysis cascade, expression levels of its three isoforms in cancer have been widely investigated. Over-expression of alpha enolase has been reported in many cancers including lung, breast, colorectal and hepatocellular cancer.

Takashima et al analysed the proteomes of 26 samples from hepatitis C associated HCC patients and reported an association of alpha enolase levels with hepatocellular tumour progression and with poor prognostic features such as tumour size and tumour vascular invasion (Takashima et al., 2005). The group championed the use of this protein as a prognostic biomarker.

Three years later, the same group analysed specimens from 60 patients who had had liver resections for HCC with curative intent. Samples were analysed using arrayCGH (a technique that quantifies genomic aberrations). They showed that a variety of glycolysis related genes were up regulated, including alpha enolase. High levels of this enzyme, however, clustered to a subgroup of patients who had a worse prognosis and less overall survival (Hamaguchi et al., 2008). The authors went on to suggest that apart from acting as a potential biomarker, alpha enolase could provide a suitable oncological target in view of its differential expression but more importantly in view of its specificity to liver tissue. This tissue specificity, hypothetically at least, could minimise the side effects of targeting the enzyme. They

therefore had an in-vitro arm to their study where an HCC cell line (HLE) was exposed to siRNA (small interfering RNA) inducing a transient knockdown of alpha enolase transcription. This transfection resulted in a marked decrease in both the mRNA and protein levels of alpha enolase and significantly inhibited the proliferation of this cell line. This was observed in both a glucose rich and glucose depleted environment. Cell cycle analysis showed that cells remained in the pre-mitotic phase (G2).

With respect to CCA the only publication relating to alpha enolase levels was published in 2012 by Yonglitthipagon et al (Yonglitthipagon et al., 2012). This study performed a proteomic analysis of various CCA cells, one of which was KKU M213. Their results showed that alpha enolase was up regulated in all CCA cell lines including KKU M213 when compared to the H69 cells. This study also performed immunostaining on 301 paraffin embedded samples from patients with resected CCA to determine alpha enolase expression. Patients, whose cancers expressed high levels of alpha enolase, had significantly poorer survival rates than patients with low expression.

I have identified 6 differentially expressed spots corresponding to alpha enolase (spots 12, 15, 16, 17, 18 and 19). Their gel location shows that all six are on the same horizontal line indicating that the isoforms have the same molecular mass but different charges. These post translational modifications are probably caused by events such as methylation, acetylation and phosphorylation rather than glysosylation (which would alter the molecular mass). Five of the six spots, however, were down regulated in SkChA1, a cell line harvested from a western patient with EH

CCA. Exactly why this result contradicts some of the findings in the published literature is not obvious. Certainly, there are studies where alpha enolase has been shown to be down regulated, but these were in other cancers, namely HCC and non-small cell lung cancer (Kim et al., 2003, Chang et al., 2003). Another factor that should be considered is the patient profiles used in the quoted studies. Yonglitthipagon et al studied alpha enolase expression exclusively in patients from the Far East with intrahepatic CCA. SkChA1 is a cell line derived from a patient with a very different profile.

One of the six spots corresponding to alpha enolase (spot 18), was down regulated in cell line KKU M213 whilst up regulated in SkChA1 as compared to H69. This contradicts the findings of Yonglitthipagon et al (2012) who reported up regulation of alpha enolase in this particular cell line. The culture technique for this cell line was the same in both studies, however, the methodology for protein analysis differed considerably. Yonglitthipagon et al used 2 dimensional polyacrylamide gel electrophoresis (2D PAGE) with 7cm IPG strips. This technique allows for a greater margin of error than DIGE on 24 cm IPG strips as used in this study. Standard 2D PAGE requires multiple technical replicate gels to allow for gel-to-gel variation when comparing samples, as only one sample can be run on each gel. However, in DIGE, each sample is compared with a common internal standard that has been run on the same gel (but labelled with a different dye). Thus, experimental variations such as gel-to-gel differences become irrelevant. Also, our use of the common and identical internal standard also allows for more accurate spot-matching between the gels.

Finally, our use of 24cm (instead of 7cm) gels allows for a much improved resolution both in imaging and in spot picking.

Apart from its cytosolic function as a glycolytic enzyme, it is becoming increasingly evident that alpha enolase can also act as a cell membrane receptor. In head and neck cancer and non-small cell lung cancer it functions as a strong plasminogen-binding receptor enhancing local fibrinolysis and contributing to tumour invasion (Tsai et al., 2010, Chang et al., 2006). It is possible that only the intracellular isoform was solubilizable in the lysis buffer so no comment can be made on the role of alpha enolase as a cell membrane receptor in CCA cells lines.

4.4.2 Annexin A2

Annexins are intracellular proteins, classified into 5 groups: A to E. Only group A is found in mammals and consequently humans. They participate in a diverse range of physiological activities including signal transduction, anticoagulation, endocytosis and exocytosis, anti-inflammatory, cell proliferation, differentiation and apoptosis. There are 12 subcategories of annexins found in humans, annexin A1 to A11 and annexin A13. The annexin A2 protein, encoded by the ANXA2 gene, can exist as a monomer, heterodimer or heterotetramer with protein p11, a member of the S100 group of proteins. Annexin A2 fulfils different biological functions which depend on which of the above forms it takes. The monomeric form is mostly distributed in the cell cytoplasm, nucleus and extracellular surface whilst the heterotetrameric form forms a common receptor for tissue type plasminogen activator (Zhang et al., 2012b).

Annexin A2 has multiple functions. It has a role in membrane structure, membrane transportation, exocytosis and signal transduction. It is also involved in cell proliferation, cell differentiation and apoptosis (Chiang et al., 1999).

Accumulated evidence has identified annexins as protagonists in neoplasia. Much of the focus has been on their role as diagnostic and prognostic markers, however, their regulation as a potential therapeutic target is also gaining favour.

By simple association, annexin A2 has been shown to have altered levels in most common cancers. It is up regulated in colorectal, breast, renal cell, hepatocellular and pancreatic cancers (Ji et al., 2009). It is also down regulated in prostate cancer, oesophageal squamous carcinoma, nasopharyngeal carcinoma and sinonasal adenocarcinoma (Zhang et al., 2012b).

Tian et al, used 2 dimensional gel electrophoresis (2 DE) to look at differentially expressed proteins in pancreatic cancer (Tian et al., 2008). They compared 8 samples from fresh tissue (stored at -80^{o}C) pancreatic ductal adenocarcinoma with normal adjacent pancreatic tissue. They identified 30 spots of interest which were analysed by tandem mass spectrometry. Annexin A2 was up regulated in all the cancer samples.

Yu et al, analysed fresh samples from 40 patients with resected HCC by cDNA microarrays. All tumour samples expressed higher levels of annexin 2, when compared to normal liver tissue acquired at the same resection (Yu et al., 2007). Two years later, the same group published a further study where RNA levels were scrutinised. Annexin A2 expression levels were again significantly higher in 40 HCC

samples when compared to adjacent normal liver tissue. The mean difference was a 2.44 fold increase in annexin A2. Furthermore, higher levels of annexin A2 were associated with higher grade tumours. In the same study, the group analysed serum samples from patients with HCC and compared these to serum taken from patients with acute hepatitis, chronic hepatitis, patients with hepatic cirrhosis and normal patients. An ELISA technique was used and showed that on average, annexin A2 levels were highest in the patients with HCC, followed by patients with cirrhosis. The group with the lowest average was the one with normal controls. However, even though each group had 55 to 86 patients there were some normal controls who had higher levels of serum annexin A2 than HCC patients. Therefore this test is not specific enough to be considered diagnostic (Ji et al., 2009).

The studies reporting annexin A2 levels in CCA are few, the most recent one coming from the same group that reported high levels of alpha enolase in CCA and discussed above (see section 4.4.1). Yonglitthipagon et al (2010) reported up regulated levels of annexin A2 in two of the four cell lines investigated, one of which was KKU M213. The methodology used was identical to the aforementioned one described in the discussion of alpha enolase. Yonglitthipagon et al's study also investigated samples from 301 patients with IH CCA. Tissue microarray and immunohistochemistry were used to determine expression changes of annexin A2 in normal bile duct, hyperplastic bile duct and cholangiocarcinoma tissue. Biopsies from 76.2% of patients stained positively for annexin A2. Staining positively for annexin A2 did not correlate with tumour size or histological subtype, however, it correlated with poor prognosis (Yonglitthipagon et al., 2010).

In my study, annexin A2 was up regulated in all three CCA cell lines when compared to the cholangiocyte H69 cell line. This not only correlates with the study by Yonglitthipagon et al (2010) in confirming that the KKU M213 expresses higher levels of annexin 2, but it also reinforces the evidence that this protein is widely over-expressed in CCA. Yonglitthipagon et al also suggested in their study that CCA caused by the liver fluke *Opisthorchis viverrini* expresses high level of annexin A2. As there are no studies investigating the expression of annexin A2 in patients with western hemisphere variety of CCA, or patients with EH CCA, no reliable comment can be made generally about annexin A2 and CCA. The results of my study strongly suggest that annexin A2 is up regulated in all CCA, irrespective of location or aetiological factor. This of course needs to be validated by further investigation of patient samples.

The implications of identifying annexin A2 as a differentially-expressed protein in CCA are not limited to biomarker discovery. In vitro and in vivo animal studies have succeeded in regulating tumour proliferation by modifying annexin A2 expression. Braden et al published a study in 2007 describing a novel combination agent active against in vitro prostate cancer cells (Braden et al., 2007). The agent combined a plasmid, which upon cellular transcription produces siRNA against annexin A2, with a nanoparticle: poly(D,Llactide-co-glycolide) (PLGA). PLGA is a biodegradable and biocompatible polymer, which undergoes slow intracellular hydrolysis releasing the plasmid at a sustained rate. The same group followed up this study in 2009 by exposing a prostate cancer mouse model to the same combination agent (Braden et al., 2009). Nude mice were inoculated with the DU 145 prostate cancer cell line,

producing a xenograft model. Following exposure with the above agent (agent was directly injected into the tumour tissue), survival and tumour growth patterns were favourable for the treated cohort. Annexin A2 levels (measured by qrtPCR) were much lower in the treated group.

4.4.3 Protein 14-3-3 sigma

The 14-3-3 proteins, first identified in 1967, make up a family of highly conserved acidic molecules. Their name, 14-3-3, refers to the elution and migration pattern of these proteins on chromatography and gel electrophoresis. The 14-3-3 proteins eluted in the 14th fraction of bovine brain homogenate and were found on positions 3-3 of subsequent electrophoresis (Aitken, 2006).

There are seven known mammalian isoforms (β, γ, ε, ζ, θ, η and σ) each encoded by a different gene. 14-3-3 proteins self-assemble into homo and heterodimers, with some family members preferring to homodimerize (sigma and gamma) and other family members (epsilon) preferring to heterodimerize. The dimers can interact with a diverse range of cellular proteins which include biosynthetic enzymes, cytoskeletal proteins, transcription factors, signalling molecules, apoptosis factors and tumour suppressors (Morrison, 2009). The interaction takes place at specific serine and threonine residues and can result in: i) conformational change in the target molecule or ii) masking or exposure of the functional shape that regulates the intracellular localisation of the target molecule or iii) changes in the phospohorylation state or stability of the target molecule.

Several human diseases exhibit over or under expression of protein 14-3-3 in its different isoforms. For example, elevated levels of 14-3-3 proteins are found in the cerebrospinal fluid of patients suffering from Creutzfeldt-Jakob disease (Takahashi et al., 1999). Of particular interest however, is that the sigma isoform has been linked to a variety of cancers: breast, stomach, bladder, prostate and oral squamous cell carcinoma.

Wu et al (2012) studied the expression of all the 14-3-3 protein isoforms in 86 CCA patients. Using immunohistochemistry they quantified the level of expression and compared the results to adjacent normal biliary tissue (Wu et al., 2012a). Most of the 14-3-3 protein isoforms were up regulated in CCA. The sigma isoform correlated positively with local lymph node and distal metastasis as well as poorer overall survival.

My study demonstrated an up regulation of protein 14-3-3 in cell lines HUCC and KKU M213. It is consistent with the findings of Wu et al (2012). Functionally, the sigma isoform is associated with control of the G2/M checkpoint in the cell cycle. It is part of the downstream signalling pathway of p53 and the action of the sigma isoform is to promote apoptosis. Hence, it would be expected that up regulation of this isoform would have the opposite effect to what Wu et al have reported, i.e. that it would be a good prognostic marker. However, protein 14-3-3 sigma has also been shown to have another effect, in inhibiting the pro-apoptotic proteins BAD and BAX (Wu et al., 2012a). As discussed in section 3.4, apoptosis is a balance of pro and anti-apoptotic signals. The imbalance described above could explain its role in CCA tumourigenesis. This checkpoint imbalance could also be potentially exploited in

cancer therapeutics. It has been suggested that by modifying the levels of 14-3-3 sigma, susceptible cells could be tipped into a pro-apoptotic route therefore increasing the efficacy of chemotherapy, in a similar fashion to PK11195 (Samuel et al., 2001).

4.4.4 Translationally controlled tumour protein

The translationally controlled tumour protein (TCTP) was first identified in the late 1980s. It has a molecular mass of 19kDa and is found in a wide range of eukaryotic organisms, such as animals, plants and fungi, indicating that its evolutionary origins lie in the distant past. TCTP is hydrophilic and its intracellular localisation is predominantly in the nucleus and cytoplasm. TCTP is one of the 20 most abundantly expressed proteins in normal cells and its function is described as *"a heat stable, calcium binding, antioxidant protein that negatively regulates apoptosis and causes release of histamine from basophils*" (Gnanasekar et al., 2009).

Although expression levels vary, it is expressed in hundreds of tissues (Bommer and Thiele, 2004). Mitotically active tissues express higher levels of TCTP. Of the variety of functions of TCTP, of most interest is its property of protecting cells from death. Although exactly how this is achieved remains to be identified, several mechanisms have been proposed:

TCTP is considered a chaperone protein. It can protect a variety of heat denatured proteins such as lysozymes and luciferase (a bioluminescence protein) from thermal or chemical shock effects. It is also over expressed in cells exposed to such stimuli (Gnanasekar et al., 2009). By reducing this stress response, Nagano et al propose

that TCTP protects cells from its pro apoptotic effects (Nagano-Ito and Ichikawa, 2012). TCTP is a calcium ion scavenger, again resulting in apoptosis inhibition. Finally, TCTP can protect cells from hydrogen peroxide-induced oxidative stress and subsequent apoptosis (Gnanasekar and Ramaswamy, 2007).

Of particular interest is that TCTP can protect cells from apoptosis induced by cytotoxic agents such as treatment with etoposide and 5FU. It achieves this by interfering with the Bcl-2 family of proteins (especially MCL-1 and Bcl-xL), readjusting the apoptosis equilibrium in favour of cell survival (see sections 1.1.6 and 3.4) (Liu et al., 2005). In addition TCTP can inhibit apoptosis through the p53 route. This is achieved by TCTP binding to p53, destabilising the protein (Rho et al., 2011). It also represses p53 transcription (Amson et al., 2012).

In my study, TCTP was over expressed in all three CCA cell lines when compared to the immortalised cholangiocyte cell line H69. Srisomap et al did not show the same result in their study of the HUCC cell line although they did show differential expression in HCC cell lines (Srisomsap et al., 2004). None of the proteomic studies on human CCA showed up regulation of TCTP in patient samples. It is clear, however, that much research has been targeted at TCTP and its effect on other cancers. Recently a group from France have patented an antisense oligonucleotide targeting TCTPA mRNA and have shown its effect in preclinical studies (Baylot et al., 2012).

4.4.5 Nucleophosmin

Nucleophosmin, also known as nucleolar phosphoprotein B23 and numatrin, is a ubiquitously expressed nuceolar phosphoprotein encoded by the NPM1 gene. It has

a molecular weight of about 37 kDa and although it is mainly found in nucleoli it shuttles between the nucleus and cytoplasm. Nucleophosmin is a multi-functional protein involved in many cellular activities and has been related to both growth promoter and growth suppressive roles (Grisendi et al., 2006). It is not surprising therefore that it has been linked to the uncontrolled growth of cancer cells and carcinogenesis although its exact role therein remains controversial.

Nucleophosmin is over or under expressed in various solid tumours. For example in colorectal, gastric, prostatic, hepatocellular and ovarian carcinomas it is over expressed and has been championed as a tumour marker (Grisendi et al., 2006).

On the other hand, reduced NPM protein expression was associated with poor prognosis in a recent study on breast cancer (Karhemo et al., 2011). This study included samples from 1160 patients with biobanked tissue core biopsies and with follow-up data of approximately 9.5 years. Samples were examined by immunostaining and q-RT-PCR. The lower the expression of nucleophosmin, the poorer the prognosis (measured outcome was distant disease free survival).

My study showed 4 spots corresponding to nucleophosmin that were differentially expressed amongst the different cell lines examined. All four had a strong Mascot protein score indicating that they are reliable results and all corresponded to the correct location for nucleophosmin on the electrophoresis gel. The overall observation was that the expressed levels of nucleophosmin were less in tumour cell lines than the control cholangiocyte. This observation corresponds with that of the published study in breast cancer but not with other cancers mentioned. None of the studies outlined in section 4.1.4 reported any variability in this protein.

4.4.6 Complement component 1 Q subcomponent-binding protein, mitochondrial

Complement 1q binding protein (also known as p32) is a 33kDa protein first described in 1981. Currently there are conflicting reports regarding the function of p32 and its potential role in the progression of cancer.

In an in vitro setting, Ithana et al 2008 demonstrated that p32 is an essential mitochondrial mediator of apoptosis by binding to the ARF tumour suppressor protein. Knockdown of p32 by siRNA, resulted in apoptosis inhibition (Itahana and Zhang, 2008). In contrast, other studies have demonstrated a pro survival benefit with increased levels of p32 and an overexpression of p32 in a variety of human solid tumours, although not CCA (McGee et al., 2011).

Another interesting observation is that Kamal et al showed that p32 is required for cisplatin-induced apoptosis (Kamal and Datta, 2006). Although our previous experiments (see section 3.3.2.2.1) looked at the effect of cisplatin on all the cell lines, these were done in a different context. Therefore comparisons cannot be directly drawn between over or under expression of p32 in the cell lines and their response to cisplatin monotherapy.

This study showed that p32 was down regulated in cell line HUCC and SKCha1 whilst there was no significant difference between KKU M213 and the normal cholangiocyte cell line.

4.4.7 Endoplasmin

Endoplasmin, also known as Heat Shock Protein 90 B1 (HSP90B1) is a housekeeping chaperone molecule with a molecular mass of 90kDa. It belongs to the larger family of HSP90 proteins - one of the commonest expressed protein groups. Heat shock proteins can aid cell survival by refolding and stabilizing stress-affected denatured proteins. The cellular stresses include heat, lack of nutrients, hypoxia and in the case of cancer cells, immunological mediated stress. The HSP90 proteins are able to 'buffer' cells from these stresses allowing them to survive in an otherwise inhospitable environment (Grbovic et al., 2006, Takayama, 2003, Neckers and Workman, 2012). Furthermore they are involved in signal transduction and other key pathways of particular relevance in malignancy. HSP 90 is regarded as essential for tumourigenesis and is over expressed in cancer cells (Neckers and Workman, 2012).

Being a housekeeper protein, HSP90 was initially dismissed by the pharmaceutical industry as not a suitable target for cancer therapeutics, in view of the perceived potential side effects and toxicity this approach might generate. However, following work by academic non-profit organisations and the United States National Cancer Institute, this view has changed and at present 17 agents are at an advanced stage of development by pharmaceutical companies and have entered clinical trials (Neckers and Workman, 2012). To date, none have been licenced for use or completed phase III trials and none have been on patients with CCA.

The expression of HSP90 in patients with CCA was recently investigated by a group in Thailand (Boonjaraspinyo et al., 2012). Fifty frozen patient samples were analysed by PCR and tumorous tissue was compared to adjacent normal tissue. There was up

regulation of HSP90 in 76% of patients. When clinicopathological correlation was performed there was no relation of HSP90 levels with patient survival.

Another group from Taiwan recently published a preclinical study of HSP90 inhibitor action in CCA (Chen et al., 2012). Samples from 8 patients with intra hepatic CCA samples were initially analysed for gene expression using microarrays. The results were used to query the Broad Institute connectivity map, for identification of potential active drugs. In the top 5 drugs returned, 3 were HSP90 inhibitors: 17-AAG (tanespimycin), geldanamycin and alvespimycin. The researchers then exposed in-vitro cell lines (one of which was HUCC-T1) to 17-AAG and NVP-AUY922. They also exposed a rat CCA model (chemical induced CCA) to NVP-AUY922 treatment. They observed that both HSP90 inhibitors caused inhibition of in vitro cell proliferation. They also observed that NVP-AUY922 resulted in a partial response in the rat model, as assessed by FDG PET scanning. A smaller study from China, studied the effect of 17-AAG on two cell lines in vitro. They observed that it induced apoptosis in both the cell lines used, however HSP90 levels were not evaluated (Zhang et al., 2012a).

My study found that endoplasmin was up regulated in cell lines SkChA1 and KKU M213 but not in HUCC. Considering that KKU M213 is a cell line derived from a Thai patient this observation is not surprising. SkChA1, a cell line derived from extrahepatic CCA of a western patient may indicate that endoplasmin is up regulated in this group too. However, there are no published studies on patient samples to correlate this finding.

4.4.8 Lactoylglutathione lyase

Lactoylglutathione lyase, also known as glyoxalase 1 is an essential enzyme in the pathway that detoxifies methylglyoxal, itself a by-product of glycolysis. Accumulation of methylglycoxal can induce cell apoptosis although the mechanism has yet to be elucidated (Thornalley and Rabbani, 2011). Hence, cells with a high glycolytic rate have a higher detoxification requirement to ensure survival. It is not surprising therefore that glyoxalase 1 has been found to be overexpressed in a variety of cancers, such as breast, gastric, colon, prostate and melanoma (Thornalley, 2008). Expression levels have been linked to survival, with over expression associated with a poorer prognosis (Cheng et al., 2012). Furthermore, high expression has been linked to multi drug resistance in cancer chemotherapy (Sakamoto et al., 2000).

The role glyoxalase 1 levels will play in oncology has yet to be established. From the published studies to date it has yet to find its way in clinical practice, however, it seems it might have the potential to be used as a prognostic marker and to guide chemotherapy. Inroads are being made to its use in therapeutics, with the preclinical development of glyoxalase 1 inhibitors (Thornalley and Rabbani, 2011).

To date, there are no published studies relating to the expression of glyoxalase in patients with CCA. This study found glyoxalase 1 up regulated in cell lines HUCC and SKChA1. These results, link CCA cell lines to overexpression of components of the glycolysis pathway, in a similar fashion to alpha enolase expression, discussed previously (see section 4.4.1).

4.4.9 Protein S100A6 (calcyclin)

Protein S100A6, also known as calcyclin, is a 10.5kDa, 90 amino acid protein belonging to the S100 protein family group. This group consists of about 20 low molecular weight calcium binding proteins. S100A6 is predominantly a cytoplasmic protein and binds calcium through its two EF-hand motifs (Schafer and Heizmann, 1996). It is thought to mediate calcium signals in normal and transformed cells. Cellular processes mediated include mobility, adhesion, proliferation and differentiation. Its alternative name '*cal-cyclin'*, was coined to reflect its binding properties to calcium and its involvement in the cell cycle (Lesniak et al., 2009).

Alteration in calcyclin levels have previously been observed in a variety of tumours. Increased levels have been reported in many alimentary tract cancers such as colorectal, gastric and pancreatic cancers and levels of expression have been linked to cancer stage (Komatsu et al., 2000). Reduction in S100A6 was observed in hepatocellular carcinoma so level determination has been proposed as a method of differentiating primary with metastatic tumours to the liver (Kim et al., 2002, Melle et al., 2008).

Kim et al (2002) compared tissue samples from CCA and HCC patients for S100A6 mRNA and protein expression. Comparisons were made between 3 CCA and 6 HCC biopsies for S100A6 mRNA expression by Northern blotting. Protein expression was assessed by immunohistochemistry staining of samples from 18 CCA and 20 HCC patients. The mRNA comparison revealed that all CCA samples expressed calcyclin transcripts whereas none of the control or HCC ones did. Protein comparison showed that all CCA samples stained positive to S100A6 to varying degrees. In the

HCC samples only 2 out of 20 stained positive, both cases being stage IV HCC disease. The authors concluded that S100A6 levels could be employed as an extra marker to help distinguish CCA from HCC (Kim et al., 2002).

This study demonstrated increased expression of calcyclin in two cell lines, HUCC and SkChA1. This correlates with the only published work identified on this protein in patients with CCA. Calcyclin expression has yet to become a routine laboratory test. As such, although the results suggest that the observation by Kim et al is supported, further evidence and proliferation of calcyclin testing would be necessary for the above authors' conclusions to be adopted.

4.4.10 Galectin-1

Galectin-1 belongs to a family of 15 proteins, the galectins, which are a subgroup of lectins. They are defined by the presence of carbohydrate recognition domains specific to β-galactosides and were first described as a separate entity in 1994 (Barondes et al., 1994). Galectin 1 is a multifunctional protein encountered both in the intra and extra cellular space and has been linked to various tumour related processes. It is involved in apoptosis regulation but also in tumour migration, invasion and angiogenesis.

Galectin 1 leads to the above through its signalling properties. It can bind to CD43 and initiate T-cell mediated apoptosis, in a caspase independent mechanism. Through binding to laminin, fibronectin, lysosomal-associated membrane proteins (Lamp 1 and 2) and carcinoembryonic antigen (CEA) it promotes cell adhesion. It also

binds to neuropilin 1 promoting endothelial proliferation, adhesion and migration (Barrow et al., 2011).

Within the family of galectins, galectin -1 has been the most extensively investigated, especially in its relation to cancer. It is over expressed in patient specimens or cell lines derived from most common tumours. Expression in colorectal, breast, prostate, lung, pancreatic, HCC and renal cell carcinomas have been shown to be up regulated through a variety of experimental methods (Demydenko and Berest, 2009). Furthermore it is over expressed in vascular endothelial cells (Thijssen et al., 2007).

Watanabe et al demonstrated that circulating serum galectin 1 levels measured by ELISA were significantly higher in patients with colorectal cancer at an early stage compared to those in controls. Furthermore, galectin 1 levels fell significantly after surgical resection. They suggested that further evaluation could result in galectin 1 serum testing being a screening test for colorectal cancer (Watanabe et al., 2011).

Shimonshi et al examined expression patterns of galectin in patients with IH CCA, using immunohistochemistry. Formalin fixed samples from 20 normal bile ducts were compared to 40 resected samples from patients with CCA and 15 with biliary dysplasia. All the normal, hyperplastic and dysplastic biliary epithelium samples did not express galectin 1, whereas 73% of the IH CCA samples were positive. Expression within this group correlated with tumour stage with higher expression in samples with higher grade tumours, such as patients with vascular and lymphatic invasion. Examination of galectin in stromal tissue and surrounding vasculature revealed a stepwise progression of expression with a weak signal in normal tissue, mild in dysplastic and strong in CCA samples (Shimonishi et al., 2001).

More recently, Wu et al validated the above findings by examining galectin 1 level expression in a cohort of Thai variant IH CCA samples (Wu et al., 2012b). Comparisons were made between 78 pairs of CCA and adjacent normal liver tissue. Both FFPE and FF samples were analysed for mRNA and protein level expression. mRNA was quantified using real time PCR and protein expression by immunostaining. Both at the genetic and proteomic level galectin-1 was over expressed. Higher galectin expression was also associated with advancing stage and poor survival (Wu et al., 2012b).

The extracellular distribution of galectin 1 has led to its proposal as a possible therapeutic target in cancer. Several agents have been developed, some in academic units but some by the pharmaceutical industry. Nine agents at various stages of development have been presented in a recent review (Ito et al., 2012). GM-CT-01 (trade name: Davanat) is an agent prepared from guam gum, a plant extract. It acts as a galectin 1 inhibitor and is currently the agent furthest in development. It entered a phase II trial in combination with 5-FU for patients with biliary tree tumours in 2006, however the study was terminated due to "financing and re-organisation" issues (NCT00386516).

This study identified over expression of galectin-1 in cell line KKU M213, the cell line derived from Thailand. This observation correlates with Wu et al providing supporting evidence that galectin-1 could be of potential value as a diagnostic tool or a therapeutic target in patients with CCA.

4.4.11 Thioredoxin

Thioredoxin-1 is a small cytoplasmic protein (12 kDa) present in all organisms that was first reported in 1964 (Laurent et al., 1964). It is part of the ubiquitous thioredoxin/thioredoxin reductase redox couple and as part of this system has been linked to numerous cell processes including the regulation of cell growth and death - and its dysregulation. Not surprisingly therefore, it has also been linked to tumourigenesis. Furthermore it has been shown to inhibit both spontaneous and drug induced apoptosis (Powis and Kirkpatrick, 2007).

The overexpression of either thioredoxin or its reductase, has been reported in a variety of human tumours including pancreatic, prostate, breast, thyroid, non-small cell lung carcinoma, malignant melanoma and mesothelioma (Yoon et al., 2006). Increased levels can be detected by ELISA in plasma and appear to correlate with poorer outcomes. It has therefore been investigated as a rational druggable target.

PX-12 is the first thioredoxin-1 inhibitor to reach clinical trial evaluation. It is a small irreversible inhibitor of thioredoxin -1 (Kirkpatrick et al., 1998). Two phase I studies in patients with solid tumours, demonstrated its safe use although they both reported cough and halitosis as dose related side effects (Baker et al., 2012, Ramanathan et al., 2007). A recent phase II trial using PX-12 as monotherapy in patients with stage IV pancreatic adenocarcinoma was terminated early after recruiting 16 patients, as all patients showed disease progression within 4 months of commencing treatment (Ramanathan et al., 2011).

Thioredoxin expression has also been investigated in CCA. Yoon et al examined thioredoxin expression by immunohistochemistry in 47 patients with CCA of varying grades. Although as a group CCA expressed higher levels of thioredoxin when compared to normal bile ducts, there was no correlation of level of expression with tumour grade (Yoon et al., 2010).

In my study, thioredoxin was overexpressed in cell line KKU M213 (but not in HUCC-T1 or SkChA1), an indication that thioredoxin is over expressed at least in some forms of CCA. This correlates with a study performed by Pak et al where HUCC-T1 cells were exposed to *clonorchis sinensis* excretory secreted products. The study intended to replicate the effect of this liver fluke in vitro. Proteomic analysis by mass spectrometry showed that thioredoxin levels were increased after cell exposure (Pak et al., 2009). With several other thioredoxin inhibiting agents in the development pipeline, this protein and pathway could represent a future target for CCA patient treatment.

4.4.12 Other differentially expressed proteins

Prostaglandin E synthase 3 (PGES-3 also known as p23) is a cytosolic enzyme, part of the biosynthetic pathway for prostaglandin E (a vasodilator). It also acts as a co-chaperone for HSP90 (see section 4.4.7) (McLaughlin et al., 2006). In my study it was found to be overexpressed in cell lines HUCC and SkChA1 which does not correspond to the same cell lines as for HSP90.

Calmodulin (an acronym for calcium modulated protein) is a calcium-binding messenger protein. It is an important intracellular mediator of calcium dependant

signalling. Signals are transduced when calmodulin binds to calcium ions which modify its interactions with a variety of target proteins. These in turn mediate a variety of pathways including proliferation, differentiation, and apoptosis (Pawar et al., 2009). Its differential expression in one of the cell lines (calmodulin was down regulated only in SkChA1) could represent a fall in overall calcium dependent signalling, but this does not necessarily relate to the cell cycle.

5 Conclusions and future directions

Cholangiocarcinoma has been a relatively neglected disease by the scientific community, more apparent when compared to other cancers. It is particularly special in that although its incidence is on the increase, on a global scale, patient numbers remain low, especially in affluent countries. It not so hard to see why the pharmaceutical industry does not consider CCA as an attractive market, having invested in just a handful of phase II/III trials treating patients with this disease.

However, momentum in research is gathering, especially in South East Asian countries and in particular Thailand where the disease incidence is on par with colorectal and lung cancers. Investment and research in CCA has inevitably followed the rapid economic development of the country where the GDP per capita has doubled from approximately US$2,500 to $5,000 in less than a decade.

This study has demonstrated how various chemotherapy combinations can have a diverse effect on cultured CCA cells. This observation reinforces the emerging opinion that CCA is less of a single entity and more of a spectrum of closely related cancers that share an anatomical proximity. It also reinforces the opinion that intrahepatic CCA differs depending on the risk factors the patient was exposed to, grossly separating western and South East Asian variant.

This study has also added another piece to the proteomic jigsaw of cholangiocarcinoma. The results indicate an enigmatic puzzle, with a small subset of the total proteome (10-20%) being altered in the CCA cell lines compared with the cholangiocyte cell line. This subset of differentially-expressed proteins are broadly

speaking the same in all three CCA cell lines, but with each protein in the subset cohort usually being up-regulated in some cell lines but down-regulated in others. However, when viewed in terms of their biological function, it is noteworthy that the differential proteins are principally cellular defence proteins or are calcium-binding and are therefore potentially involved in cell signalling. In all instances, up-regulation was more common than down-regulation amongst the differentially-expressed proteins, suggesting that targeting gene expression is a valid means of controlling CCA. There were no obvious differences between the intra-and extra-hepatic cell lines, with "only" one identified protein being uniquely up-regulated (ATP synthase) and one uniquely downregulated (gamma-synuclein) in the HUCC extra-hepatic cell line compared to the cholangiocyte cell line. The cell line that was most "unique" was SkChA1, with 56 spots upregulated and 98 spots downregulated compared to the cholangiocyte cell line. However, most of these were of low abundance and unfortunately could not be identified. SkChA1 is derived from a Western CCA yet it differs more extensively from the cholangiocyte cell line than the Thai intrahepatic equivalent cell line (M213, where 29 spots were uniquely upregulated and 43 uniquely downregulated compared with the cholangiocyte cell line). This suggests that if the *O. viverrini* parasite does induce CCA as indicated by Sripa et al., (2012) and others, the resultant disease is more differentiated than Western CCA, and therefore possibly more difficult to treat.

Most of the highlighted proteins have been identified in other cancers as well. This observation could be interpreted as a disadvantage as it arguably reduces the chances of finding a diagnostic tool differentiating unique to CCA. However, in the

context of this being a rare disease, it could also be seen as an advantage: any molecular targeted treatments in development for other cancers could potentially translate to CCA.

This study also highlights the role that novel agents might have in the treatment of CCA. The cytotoxic studies demonstrated that a benzodiazepine receptor antagonist enhanced cytotoxic action in an in vitro setting. Agents such as this one are gradually gaining favour at a clinical level in other cancers so the observations in this study show that they may also have a role in the treatment of CCA.

As with other solid cancers, treatment is ultimately shifting towards the tailoring of treatment (including chemotherapy) to the individual patient. The use of a panel of expressed proteins, often referred to as molecular profiling or protein fingerprinting, is thought to be the future of such treatment. Preclinical studies such as this one help provide theoretical guidance for directions to be followed in the clinical setting. Such evidence is all the more important in the investigation of the more rare diseases where patient access is a problem.

It is clear that the findings of this study need to be further validated in real patient samples. As outlined in the introduction to Chapter 4, to date, very few studies have investigated the CCA proteome at patient histology level, and those that have used a very limited number of patients.

Abbreviations

Listed in alphabetical order

2DE	2 Dimensional Electrophoresis
5-FU	5-Fluorouracil
96WP	96 Well Plate
AJCC	American Joint Cancer Committee
ANOVA	Analysis of variance
ASBT	Apical Sodium Dependent Bile Acid Transporter
Bcl	B-cell lymphoma
Bp	Base pairs
Ca 19-9	Carbohydrate antigen 19-9
Cat no	Catalogue Number
CCA	Cholangiocarcinoma
cDNA	Complementary DNA
CEA	Carcinoembryonic antigen
CGH	Comparative genomic hybridisation
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CI	Confidence Interval
CK 19	Cytokeratin 19
CK7	Cytokeratin 7
CN	Copy Number
COX	Cyclooxygenase
DAPI	4',6-diamidino-2-phenylindole
ddH2O	Double distilled water
DIGE	Differential In Gel Electrophoresis
DMEM	Dulbeccos Modified Eagle Medium
DMF	N,N-Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTT	DL-Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
EH	Extra Hepatic
ELISA	Enzyme-Linked Immunosorbent Assay
EMA	Epithelial Membrane Antigen
EtOH	Ethanol
FCS	Foetal Calf Serum
FISH	Fluorescent in situ hybridisation
GB	Gallbladder
GDP	Gross Domestic Product
H&E	Haematoxylin and Eosin
HCC	Hepatocellular carcinoma
HCSG	Human Cancer Studies Group
HPB	Hepatopancreatobiliary
IC_{50}	Half maximal inhibitory concentration

IH	Intra Hepatic
IL	Interleukin
iNOS	inducible Nitric Oxide Synthase
IPG	Immobilised Ph Gradient
LCSGJ	Liver Cancer Study Group of Japan
MALDI	Matrix-assisted laser desorption/ionization
Mb	Megabases
Mcl	Myeloid cell leukaemia
MS	Mass Spectrometry
m/z	Mass / charge
MRA	Magnetic Resonance Angiography
MRCP	Magnetic Resonance Cholangiopancreatography
MRI	Magnetic Resonance Imaging
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCCJ	National Cancer Center of Japan
NCT	National Clinical Trial
NEAA	Non Essential Amino Acids
NNDMF	Diemethyl Formamide
NTCP	Na^+ dependent Taurocholate Co-transporting Polypeptide
OD	Optical Density
OS	Overall Survival
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PFS	Progression Free Survival
PMID	PubMed Identification
PMT	Photomultiplier Tube
PMF	Peptide mass fingerprint
RCT	Randomised Controlled Trial
RNA	ribonucleic acid
rpm	Rotations (revolutions) per minute
RPMI	Roswell Park Memorial Institute
SDS	sodium dodecyl sulfate
SRB	Sulforhodamine B
TACE	Trans- arterial chemoembolisation
TBP	Tributylphosphine
TCA	Trichloroacetic Acid
TEMED	Tetramethylethylenediamine
TGF	Tumour Growth Factor (aka Transforming Growth Factor)
TNF	Tumour Necrosis Factor
TCTP	Translationally controlled tumour protein
TRAIL	Tumour necrosis factor related apoptosis inducing ligand
UICC	Union for International Cancer Control
UK	United Kingdom
US	United States
V	Volts
Vh	Volt hours

Materials, solutions and reagents

Reagent	Company	Catalogue Number
2-D Clean-Up Kit	GE Healthcare	80-6484-51
4-hydroxy-α-cyano-cinnamic acid	Sigma-Aldrich	50149-1G-F
5-Flurouracil	Sigma-Aldrich	F6627
Acetic Acid	VWR	20104.298
Acrylamide	Sigma-Aldrich	01709
Adenine	Sigma-Aldrich	A2786
Albumin	Sigma-Aldrich	A9418
Ammonium persulphate	Sigma-Aldrich	A9164
Ammonium bicarbonate	Sigma-Aldrich	09830
Anti mouse antibody	Jackson ImmunoResearch	715-166-150
Acetonitrile (aqueous)	Sigma-Aldrich	34998
Bio-Safe™ Coomassie Stain	Bio-Rad Laboratories	161-0786EDU
Bromophenol blue	Sigma-Aldrich	B5525
Chamber slide	LabTek	177402
CHAPS	Sigma-Aldrich	C9426
Cisplatin	Enzo Life Sciences	ALX-400-040-M050
CK7	DAKO	IR618/IS618
CK19	DAKO	IR615/IS615
Cy3 DIGE Fluor	GE Healthcare	25-8010-83
Cy5 DIGE Fluor	GE Healthcare	25-8008-62
DAPI	Sigma-Aldrich	D9542
DMEM	Gibco-Invitrogen	21969
DMEM with high glucose	Gibco-Invitrogen	11095098
DMSO	Sigma-Aldrich	D4540
DPBS	Gibco-Invitrogen	14190250
DTT	Sigma-Aldrich	D9779
EMA	DAKO	IR629/IS629
Eosin	Sigma-Aldrich	HT110116
Epidermal Growth Factor	Millipore	01-102
Epinephrine	Sigma-Aldrich	E4250
Etoposide	Sigma-Aldrich	E1383
Foetal calf serum	Sigma-Aldrich	N4637
Gemcitabine (Gemzar)	Lilly pharmaceuticals	7502
Gentamicin	Sigma-Aldrich	G1272
Glycerol	Sigma-Aldrich	G5516
Hematoxylin	Sigma-Aldrich	H3136
Hydrocortisone	Sigma-Aldrich	H0888
Insulin	Sigma-Aldrich	I2643
Iodoacetamide	Sigma-Aldrich	I1149
L-Glutamine	Gibco-Invitrogen	25030081
Lysine	Sigma-Aldrich	L5501
Protease Inhibitor Mix	GE Healthcare	80-6501-23

Methanol	VWR	BDH 101586B AnalR
NaOH	Sigma-Aldrich	71690
NEAA (x100)	Gibco-Invitrogen	11140
NNDMF	Sigma-Aldrich	D4551
P11195	Sigma-Aldrich	C0424
Paraformaldehyde	VWR	28794.295
PBS	Sigma-Aldrich	P4417/P5244
Penicillin/Streptomycin	Gibco-Invitrogen	15140155
Penicillin/Streptomycin	Sigma-Aldrich	15140-122
PK11195	Sigma-Aldrich	C0424 (10mg)
Power Block	Biogenex	HK085-5K
RNeasy Mini Kit	Qiagen	74104
SDS	Sigma-Aldrich	L4390
SRB	Sigma-Aldrich	S9012 (5g)
TCA	Sigma-Aldrich	T6399
TEMED	Sigma-Aldrich	T9281
TGS	BioRad	161-0772
Thiurea	Sigma-Aldrich	T8656
Transferrin	Sigma-Aldrich	T2036
Trizma HCL	Sigma-Aldrich	T3253
Trifluoroacetic acid	Sigma-Aldrich	T6508
Tris Base	Sigma-Aldrich	T1503
Trizma base	Sigma-Aldrich	T1503
Trypan Blue	Sigma-Aldrich	T8154
Trypsin – EDTA solution	Sigma-Aldrich	T4174 (10x)
Trypsin (porcine)	Promega	V511A
Trypsin (proteomics)	Promega	608-274-4330
Urea	Sigma-Aldrich	U6504
β- actin	Applied Biosystems	4310881E
DMEM/F-12	Gibco-Invitrogen	11330057
IPG buffer (pH 3-11)	GE Healthcare	17-6004-40
Ethanol (100%)	VWR	20820.327
3,3 Triiodo-l-thyronine	Sigma-Aldrich	T6397
Pierce 660	Thermo Scientific	22660
RPMI 1640	Sigma-Aldrich	R5886

Kits and Assays

cDNA Reverse Transcription Kit	Invitrogen	4368814
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems	4368814
TaqMan Gene Expression Assay	Applied Biosystems	Hs00236329_m1

Related publications and presentations

Pericleous S; Mathieson W; Middleton N; McKay S; Biswas A; Thomas G; Spalding DR; Hutchins RR. PK11195, a peripheral benzodiazepine receptor ligand, potentiates cytotoxic action on cholangiocarcinoma cell lines. European-African Hepato Pancreatic Biliary Association, 12 Apr 2011 - 16 Apr 2011. (Suppl. 2):1-145.

Pericleous S, SC McKay, W Mathieson, K Unger, DR Spalding, RR Hutchins, G Thomas, G Stamp Array Comparative Hybridisation to Cholangiocarcinoma Cell Lines HuCC-T1 and SkChA-1. Presented at the Pathological Society Scientific Meeting London , 8–9 January 2010 Abstract published in: *The Journal of Pathology*, Volume 220, Issue S1 (p S1-S27) DOI: 10.1002/path.2705

Pericleous S et al. Chemotherapy for cholangiocarcinoma; the effect of dual versus monotherapy on multiple cell lines. The Association for Cancer Surgery Scientific Conference (British Association of Surgical Oncology), (November 2010).

McKay S, Unger K, **Pericleous S**, Spalding D, Hutchins, R, Thomas, G Array comparative genomic hybridization identifies novel potential therapeutic targets for cholangiocarcinoma. - Abstract number: 7614627 Presented at the 9th World Congress of the International Hepato-Pancreato-Biliary Association April 18-22, 2010, Buenos Aires. Abstract published in: *HPB Journal* 12 (Suppl. 1), 1–468. DOI: 10.1111/j.1477-2574.2010.00165.x (Presentation FP-25)

McKay S, Unger K, Sriraksa R, Zeller C, **Pericleous S** et al. Differing copy number alteration profiles identified by array CGH in Thai Intrahepatic Cholangiocarcinoma

related to prognosis . (Abstract 1291) The Liver Meeting 2010 - The American Association for the Study of Liver Diseases 61st Annual Meeting. October 29-November 2, 2010. Hepatology Volume 52, Issue Supplement S1, pages 919A–1020A, October 2010 DOI: 10.1002/hep.23994

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