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The Role of the Cyclooxygenase-2 – Prostaglandin E2 Cascade in Pancreatic Cancer Associated Angiogenesis.

Doctor in Medicine

by Research

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Declaration.

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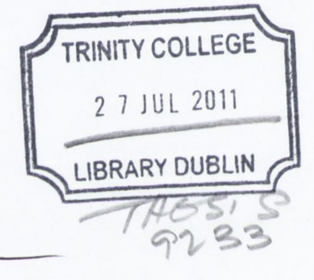
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Dr. Desmond P. Toomey MB, MRCSI

Summary.

Medical therapy has failed to make any significant impact on survival in pancreatic cancer. Non Steroidal Anti-inflammatory Drugs (NSAIDs) have shown promise in several gastrointestinal (GI) cancers. Evidence has suggested a similar effect in pancreatic cancer. Cyclooxygenase-2 (COX-2), a major target of NSAIDs, is upregulated in pancreatic cancer and is associated with worse prognosis. COX-2 upregulation has been shown to correlate with angiogenesis and production of pro-angiogenic growth factors, especially Vascular Endothelial Growth Factor (VEGF), in several GI cancers. Although this relationship between COX-2 and angiogenesis in pancreatic cancer would seem a viable target, clinical trials of COX-2 or VEGF inhibitors have demonstrated no survival benefit.

This study developed an *in vitro* co-culture model of pancreatic cancer angiogenesis. A COX-2 positive and negative pancreatic cancer cell line were identified and validated. This model enabled investigation of the role of COX-2 in angiogenesis and its relationship with VEGF. The study also went on to investigate the COX-2 independent effects of NSAIDs and the therapeutic potential of a Celecoxib derivative with no COX-2 inhibitory activity.

VEGF was found to be independent of COX-2. Furthermore, VEGF and not COX-2 stimulated endothelial cell proliferation/survival in this

model of the tumour microenvironment. However, in line with the findings of other authors, there was evidence suggesting that COX-2 does play a role in other steps of angiogenesis – migration and cord formation. NSAIDs had differing effects at high concentrations including upregulation of VEGF by Celecoxib. The Celecoxib derivative, OSU-03012, had marked cytotoxic ability at physiological concentrations without inhibiting COX-2.

The finding that VEGF is independent of COX-2 and that they are responsible for different steps in the angiogenic process suggests that combination therapy may improve clinical effect. Furthermore, although COX-2 remains an important target, new agents that exploit the COX-2 independent effects of NSAIDs have exciting promise in pancreatic cancer.

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Acknowledgments.

I would like to thank several people, without whom I could not have completed this project.

Professor Kevin Conlon for providing me with the opportunity to undertake this research fellowship. Your motivation, supervision and guidance were crucial for completion of this project.

Dr. Joseph Murphy, your constant direction kept me focussed on the project in hand and your knowledge and guidance were invaluable.

Ms. Ellen Manahan, Dr. Annamarie Rogers and Dr. Ciara McKeown for everything from teaching me to pipette to listening to me complain. You were always willing and able to help me out. Your assistance was invaluable and your comradeship indispensable.

Dr. Michael Geary, his team and the midwives on the labour ward in the Rotunda Hospital who obtained consent from the mothers and gathered the umbilical cords. This research could not have been undertaken without your assistance. I hope that this fruitful relationship will continue into the future to ensure continued progression in this field.

The University of Dublin, Trinity College and The Royal College of Surgeons in Ireland who provided vital funding for this project. Your assistance is very much appreciated.

All the staff in the Trinity Centre for Health Sciences in AMNCH, Tallaght for your help, advice, support and friendship over the last two years.

And finally my wife Sarah, who always supports me in everything I do.

Thank you everybody,

Des.

Publications.

1. DP Toomey, JF Murphy, KC Conlon. **"COX-2, VEGF and Tumour Angiogenesis".** The Surgeon 2009;7(3): 174-181.

2. DP Toomey, E Manahan, C McKeown, A, Rogers, H McMillan, M Geary, KC Conlon, JF Murphy. **"Vascular Endothelial Growth Factor and not Cyclooxygenase-2 Stimulates Endothelial Cell Viability in the Pancreatic Tumour Micro-environment".** Pancreas 2009. In print.

Presentations.

Oral Presentations. *(Presenter underlined)*

1. DP Toomey, E Manahan, C McKeown, A Rogers, H McMillan, M Geary, KC Conlon, J Murphy. **"VEGF and not COX-2 promotes endothelial cell viability in the pancreatic tumour micro-environment".** RAMI / St Lukes Young Investigator of the Year Award, January 2009. (Shortlisted)

2. DP Toomey, E Manahan, C McKeown, A Rogers, H McMillan, S Thow, M Geary, KC Conlon, J Murphy. **"Inhibition of VEGF and Not COX-2 Prevents Endothelial Cell Survival and Proliferation in Pancreatic Cancer."** Sylvester O' Halloran Meeting, Limerick, March 2008.

3. DP Toomey, E Manahan, C McKeown, A Rogers, KC Conlon, J Murphy. **"A Celecoxib Derivative, OSU-03012, has Potential in Pancreatic Cancer Treatment."** Sylvester O' Halloran Meeting, Limerick, March 2008.

4. DP Toomey, J Murphy, E Manahan, C McKeown, A Rogers, T El Hantati, S Sheehan, M Geary, KC Conlon. **"COX-2: A Target for Combating Tumour Angiogenesis in Pancreatic Cancer?"** Sir Peter Freyer Meeting, Galway, September 2007.

5. DP Toomey, J Murphy, E Manahan, A Rogers, KC Conlon. **"Upregulation of Cyclooxygenase-2 and the Expression of Pro-angiogenic Growth Factors By Pancreatic Cancer Cells in Response to Two Organic Compounds."** RAMI Biomedical Sciences Section Winter Meeting, Dublin, January 2007.

6. J Murphy, DP Toomey, A Rogers, E Manahan, K Conlon. **"Prostaglandin E2 and VEGF Production by Pancreatic Cancer Cells".** Irish Society of Gastroenterology Winter Meeting, Dublin, November 2006.*

*First prize – oral presentation section.

Poster Presentations.

1. DP Toomey, J Murphy, E Manahan, C McKeown, A Rogers, S. Thow, H. McMillan, M Geary, KC Conlon. **"Is there a link between COX-2, VEGF and Tumour Angiogenesis in Pancreatic Cancer?"** Irish Society of Gastroenterology Winter Meeting, Dublin, November 2007.

2. DP Toomey, J Murphy, E Manahan, C McKeown, A Rogers, S. Thow, H. McMillan, M Geary, KC Conlon. **"Is COX-2 linked with Tumour Angiogenesis in Pancreatic Carcinoma?"** Pancreatic Society of GB and Ireland, UK, November 2007.

3. D.P. Toomey, J. Murphy, A. Rogers, C. McKeown, E. Manahan, K.C. Conlon. **"Classification of pancreatic cancer cell lines, by level of COX-2 expression, in order to determine the role of COX-2 in angiogenesis."** European Pancreatic Club, UK, July 2007.

4. DP Toomey, J Murphy, E Manahan, C McKeown, A Rogers, KC Conlon. **"Development of a model to investigate the role of COX-2 in pancreatic cancer associated angiogenesis."** Irish Society of Gastroenterology Spring Meeting, Kilarney, April 2007.

Abbreviations.

APC	Adenomatous Polyposis Coli
APC^{min}	APC Gene knockout mice
Akt	Also known as Protein Kinase B
Camp	Camptothecin
CO_2	Carbon Dioxide
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
dH_2O	Deionised water
DMSO	Dimethyl Sulphoxide
EC	Endothelial Cell
EDTA	Ethylenediaminetetraacetic Acid
FAP	Familial Adenomatous Polyposis Coli
FCS	Foetal Calf Serum
FDA	Food And Drug Administration (USA)
FGF	Fibroblast Growth Factor
GF	Growth Factor
GI	Gastrointestinal
h	Hours
HIF	Hypoxia Inducible Factor
HIF-1α	Hypoxia Inducible Factor 1α
HIF-1β	Hypoxia Inducible Factor 1β
HUVEC	Human Umbilical Vein Endothelial Cells

IκB	Inhibitor κB
LC_{50}	Lethal Concentration 50
min	Minutes
mRNA	Messenger Ribonucleic Acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyyltetrazolium bromide
MVD	Micro Vessel Density
NF-κB	Nuclear Factor κB
NSAID	Non Steroidal Anti-inflammatory Drug
PBS	Phosphate Buffered Saline
PDK-1	Phosphoinisotide Dependent Kinase-1
PG Synthases	Prostaglandin Synthases
PGD2	Prostaglandin D2
PGE2	Prostaglandin E2
PGF2	Prostaglandin F2
PGH2	Prostaglandin H2
PGI2	Prostacyclin
PLA2	Phospholipase A2
PlGF	Placental Growth Factor
PMA	Phorbol 12-Myrisitate-13-Acetate
Pos Control	Positive Control
PPARγ	Peroxisome Proliferator Activated Receptor γ
rpm	revolutions per minute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SEM	Standard Error of the Mean
TXA2	Thromboxane A2

v/v	Concentration volume / volume
VEGF	Vascular Endothelial Growth Factor
VEGF Neut Ab	Vascular Endothelial Growth Factor Neutralising Antibody
VEGF-A	Vascular Endothelial Growth Factor A
VEGF-B	Vascular Endothelial Growth Factor B
VEGF-C	Vascular Endothelial Growth Factor C
VEGF-D	Vascular Endothelial Growth Factor D
VEGF-E	Vascular Endothelial Growth Factor E
VEGFR1	Vascular Endothelial Growth Factor Receptor 1
VEGFR2	Vascular Endothelial Growth Factor Receptor 2
VEGFR3	Vascular Endothelial Growth Factor Receptor 3
WST	Water Soluble Tetrazolium

For Sarah.

Chapter 1: Background & Literature Review.

1.1 Introduction.

Despite the positive impact of medical and surgical advances on the prognosis of many different tumours, 7870 Irish patients died of cancer in 2004.[1] Although the incidence of pancreatic cancer remains relatively low, at approximately 400 cases per annum, its dismal survival rate ensures that it is the fifth leading cause of cancer death.[1-3] The search continues for agents with minimal toxicity which are effective in both the chemoprevention and adjuvant therapy fields.

Over the past three decades, epidemiological evidence has shown a protective effect of regular Non Steroidal Anti-inflammatory Drugs (NSAID) use against developing many different types of gastrointestinal cancer. Pancreatic cancer may be included in this group.[4-7] These findings prompted the investigation of cyclooxygenase, the enzyme inhibited by NSAIDs, as a possible therapeutic target in pancreatic cancer. However more recently cyclooxygenase independent effects of these agents have also come to light.[8]

1.2 Cyclooxygenase-2.

Cyclooxygenase is an enzyme that catalyses the conversion of arachadonic acid to prostaglandin H2 (PGH2). Specific synthases rapidly convert PGH2 into one of several prostaglandins, depending on

the type and function of the cell involved (Fig. 1.1). These prostaglandins are both autocrine and paracrine intercellular messengers.[9]

Two isoforms of cyclooxygenase have been well described. Cyclooxygenase-1 (COX-1) is a constitutive enzyme present in fairly constant amounts in most cells of the body. Its functions include production of gastric mucous and mediating platelet aggregation.[10, 11, 12] In contrast Cyclooxygenase-2 (COX-2) is generally not present in most cells under normal conditions[13] but can be rapidly induced by various intercellular messengers including growth factors, inflammatory mediators and tumour promoters.[9, 14]

Early NSAIDs, such as Aspirin, reduce production of pro-inflammatory prostaglandins by non specific inhibition of COX. This can cause side effects, especially upper gastrointestinal ulceration and bleeding. Hence, the discovery of the inducible isoform (COX-2) and delineation of its role in inflammation, prompted development of specific COX-2 inhibitors.[14] Meanwhile, epidemiological evidence linked NSAIDs to a reduced risk of developing gastrointestinal tumours, including colorectal,[5, 6, 15-17] gastric,[5, 17] oesophageal[4, 17] and pancreatic cancer.[7] As COX-2 is upregulated in the majority of these tumours, its role and the potential of specific COX-2 inhibitors as chemoprotective or therapeutic agents is under investigation.[15, 18]

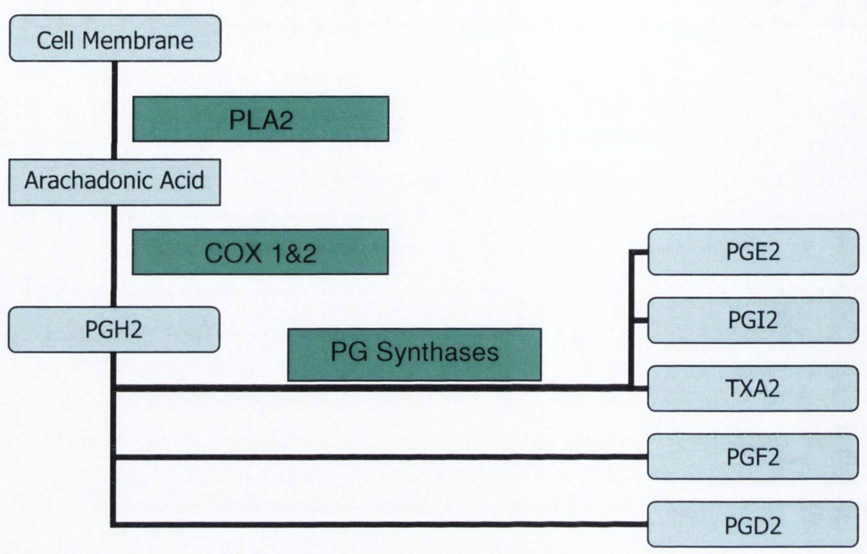


Figure 1.1 Cyclooxygenase Cascade.

Arachadonic acid is released from the cell membrane by Phospholipase A2 and is converted to PGH2 by cyclooxygenase. PGH2 is then rapidly converted into downstream prostaglandins by specific prostaglandin synthases depending on the cell type and function.

PLA2 – Phospholipase A2; COX – Cyclooxygenase; PG Synthases – Specific Prostaglandin Synthases; PGH2 – Prostaglandin H2; PGE2 – Prostaglandin E2; PGI2 – Prostacyclin; TXA2 – Thromboxane A2; PGF2 – Prostaglandin F2; PGD2 – Prostaglandin D2.

1.3 Cyclooxygenase-2 in Cancer.

Increased levels of COX-2 have been demonstrated in the majority of colon,[13, 19-21] prostate,[19] lung,[19, 22] breast,[19] oesophageal,[12] pancreas,[23-26] head and neck[27] and gastric tumours.[28, 29] In clinical studies, high COX-2 levels in tumours are associated with increased clinical and pathological stage,[12, 13, 21, 22, 29] higher histological grade[21, 22, 29] and worse prognosis.[13, 22, 29]

The tumourigenic properties of COX-2 have been clearly demonstrated in *in vivo* mouse studies. Liu *et al.* (2001) conducted a study on mice transfected with human COX-2 in their mammary epithelial tissue and found that post lactation, mammary involution was delayed due to decreased apoptosis. Furthermore >85% of multi-parous females developed mammary tumours (a spectrum from hyperplasia through to metastatic carcinoma).[30] Similarly, lung cancer xenografts in COX-2(-/-) mice resulted in smaller tumours than those in COX-1(-/-) or controls. Treatment with Celecoxib, a specific COX-2 inhibitor, resulted in smaller tumours however it had a less marked effect than COX-2 knockout.[31] Oshima *et al.* (1996) demonstrated that COX-2 is only present in polyps and not in normal mucosa in APC (Adenomatous Polyposis Coli Gene) knockout mice. COX-2 knockout in these mice produced significantly smaller polyps and also reduced polyp numbers by 66% and 86% for one and two allele knockouts, respectively. Furthermore they developed no colonic polyps.[32]

Clinical trials have demonstrated that Celecoxib reduces the polyp burden in Familial Adenomatous Polyposis Coli (FAP) by 28% and this agent is licensed by the FDA for chemoprevention in this condition.[33] Several NSAIDs have been shown to inhibit tumour proliferation in laboratory models[26, 32], however it is debatable whether this is entirely via inhibition of the COX-2 – prostaglandin cascade. Other mechanisms, including inhibition of Akt phosphorylation, blocking of transcription factor binding and nuclear membrane receptor activation, may also play a significant role.[34-37]

Prostaglandin E2 (PGE2) is generally considered to be the dominant downstream mediator of COX-2 activity in tumours.[18, 38-41] Pugh *et al.* (1994) found that colonoscopic biopsies of carcinomas and adenomas produced more PGE2 than normal mucosa and that "normal" mucosa from cancer patients had increased PGE2 levels.[42] A small study on patients with genotypic but not phenotypic FAP treated with Sulindac (NSAID) found that patients on Sulindac who did not later develop polyps had lower PGE2 levels than those who did.[43] *In vitro* colon cancer studies have shown that upregulated COX-2 and PGE2 decrease apoptosis[44] and that PGE2 can upregulate COX-2 in a positive feedback manner.[45] Furthermore, Backlund *et al.* (2005) found that 15 Prostaglandin Dehydrogenase, an enzyme which metabolises PGE2, can be down-regulated in many tumour types including

colorectal cancer cell lines, colorectal tumours (85%) and in polyps from APC^{min} mice.[16]

Several tumourigenic processes are directly linked with increased COX-2 expression. These include increased proliferation and invasion and decreased apoptosis and host immune response. However, one exciting target that has emerged is the role of COX-2 in tumour angiogenesis.[18]

1.4 Angiogenesis.

Angiogenesis is the growth of new capillary vessels from existing vasculature resulting in the formation of a new vascular network.[46, 47] This process is part of normal growth and development and is important in inflammation and wound repair. It is largely quiescent in healthy adults apart from a role in the female reproductive cycle.[46, 48] Angiogenesis, under normal physiological conditions, is tightly controlled by a balanced symphony of angiogenic and angiostatic growth factors. The “angiogenic switch” refers to alteration of growth factor expression to form a pro-angiogenic environment during growth, tissue hypoxia or inflammation.[47, 48] In solid neoplasms, cells can initially survive by diffusion of oxygen and metabolites from local vessels. However, once the tumour diameter exceeds 2-3 mm angiogenesis must construct a neo-vasculature to supply the rapidly growing cancer with oxygen and nutrients. In theory, agents that

reverse the angiogenic switch would have significant therapeutic benefit.[49]

Sprouting is the dominant form of angiogenesis in tumours (Fig. 1.2).[47] Following the angiogenic switch, the action of Angiopoeitin-1, which maintains vessels in the quiescent state, is blocked by Angiopoeitin-2. The endothelial cells, which line the vascular compartment, are activated by pro-angiogenic growth factors, lose some of their cell to cell adhesions and proliferate. Vascular support cells (smooth muscle cells, pericytes, etc.) dissociate from the destabilised vessel. The basement membrane and extracellular matrix are degraded by matrix metalloproteinases. This enables endothelial cells to migrate into the tumour where they align themselves in cords and form tubules which ultimately join up into a vascular network. Endothelial cells are prone to cell death while separated from the basement membrane but growth factors, especially Vascular Endothelial Growth Factor (VEGF), stimulate survival until support cells lay down a new basement membrane and stabilise the maturing vasculature.[46, 47, 50]

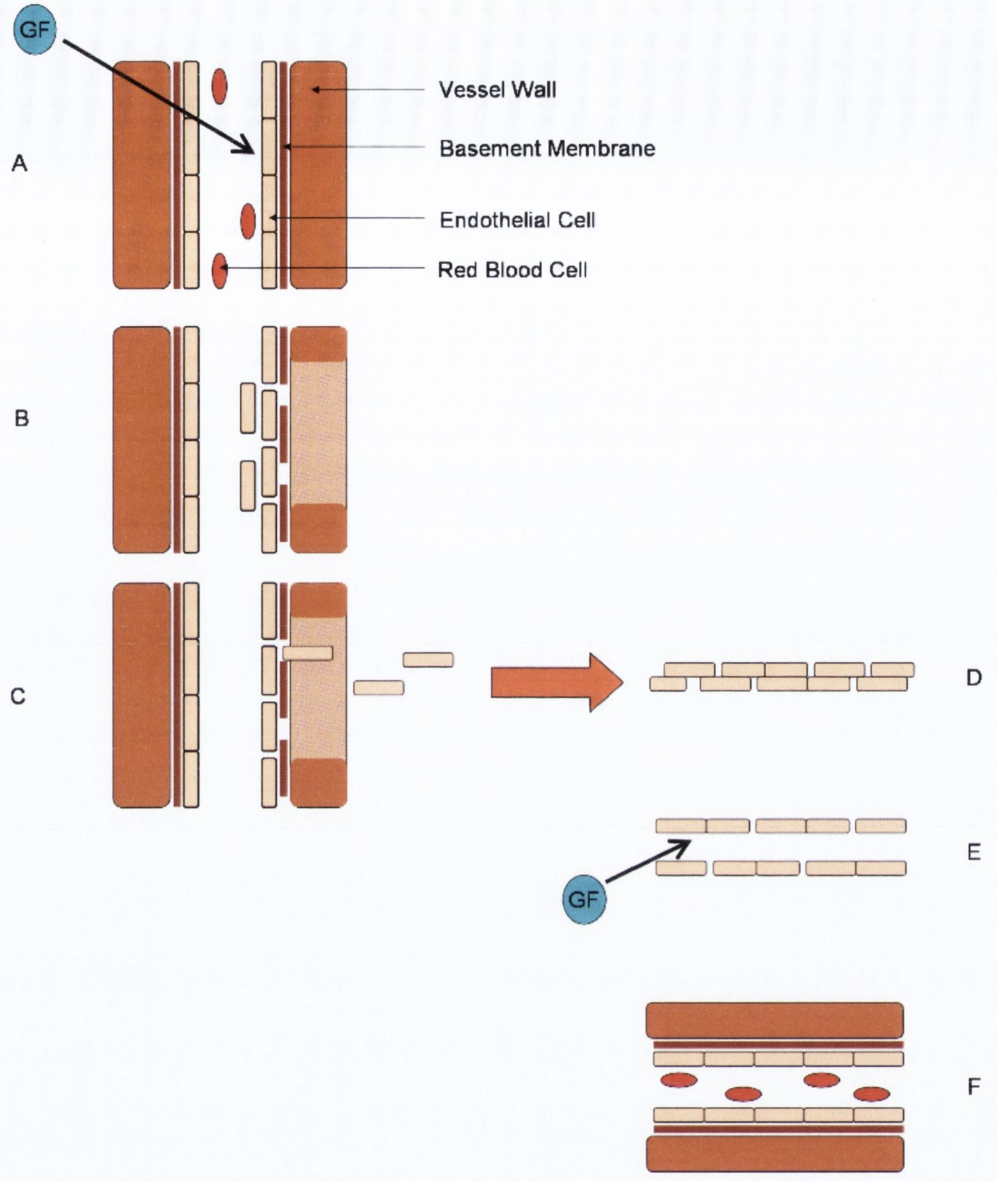


Figure 1.2 Sprouting Angiogenesis.

A. *Pro-angiogenic growth factors (GF) activate endothelial cells (EC) in the quiescent vessel.* ***B.*** *EC proliferate and cell to cell adhesions loosen. Proteases degrade the basement membrane and Extracellular Matrix.* ***C***. *EC migrate out of the vessel into the tumour.* ***D***. *EC align themselves in cords.* ***E***. *Cords of EC tubulise. Growth factors stimulate EC survival.* ***F***. *Vascular support cells migrate to the neo-vessel, lay down a basement membrane and vessel stabilises and matures.*

VEGF is the best described angiogenic growth factor. There are six members of the VEGF family – VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and Placenta Growth Factor (PlGF).[47] VEGF-A (VEGF) is a potent stimulator of angiogenesis which has been shown to specifically stimulate endothelial cell proliferation and survival.[10, 47] Animal studies suggest an involvement of VEGF-B and VEGF-C in coronary and lymphatic development, respectively. Roles for VEGF-D, VEGF-E and PlGF remain elusive.

There are 3 VEGF receptors (VEGFR1, VEGFR2 and VEGFR3).[47] VEGFR2 knockout mice develop minimal vasculature and endothelial cells. This suggests an important role for this receptor in vascular development. In contrast, VEGFR1 knockout mice have an excess of endothelial cells arranged in chaotic tubular structures, suggesting that this is a decoy receptor. VEGFR3 seems to be important in lymphatic development.[47, 48, 50]

As a tumour grows and the distance from local vessels increases, nutrients including oxygen become depleted. In hypoxic cells, Hypoxia Inducible Factor 1α (HIF-1α), which is rapidly degraded under normoxic conditions, stabilises and translocates to the nucleus where it binds with HIF-1β forming HIF. This transcription factor stimulates production of VEGF resulting in angiogenesis and growth of new vessels into the tumour.[47, 48, 51, 52] Similarly, other growth factors, including Epidermal Growth Factor, Fibroblast Growth Factor (FGF)

and Chemokines, are produced by hypoxic tumours by less well understood mechanisms.[47, 53, 54]

Tumour angiogenesis is not the finely tuned process seen under normal physiological conditions. An imbalance in growth factor concentration and timing results in an excess of vessels developing which are often fragile, incomplete, chaotic and leaky.[55] A major reason for this is that oncogenes can up-regulate growth factors in an uncontrolled manner with no regulation. For example, VEGF can be upregulated by mutant k-ras,[56] or mutant raf,[47] and by deactivation of P53[10, 47] or von Hippel-Lindau tumour suppressor genes.[57] It has been hypothesised that this disordered vasculature facilitates haematogenous metastases as tumour cells are in direct contact with blood flow (vascular mimicry).[46, 53, 55] It is probable that tumour angiogenesis is driven by a combination of hypoxia and oncogene induced growth factors.

Angiogenesis is essential for tumour growth, invasion and metastases.[58] As pancreatic cancer mostly affects older people in whom physiological angiogenesis is largely quiescent, there is exciting potential for tumour specific therapies with minimal side effects.[46, 48]

1.5 Cyclooxygenase-2 and Angiogenesis.

Tumour cell COX-2 is thought to stimulate angiogenesis and so promote the malignant phenotype.[59] Tumours with high levels of COX-2 have been associated with increased micro vessel density (MVD) in colon, lung, head and neck and gastric cancer specimens[20-22, 27, 29] and in various laboratory models.[31, 60, 61] Furthermore, *in vivo* studies have linked prostaglandin production, in particular Thromboxane A2 (TXA2) and PGE2 with increased angiogenic activity.[20, 28, 31, 60, 62-65]

Several studies on clinical specimens in different tumour types have suggested a relationship between increased COX-2 and increased pro-angiogenic growth factors, in particular VEGF. Expression of VEGF correlates with COX-2 levels in oesophageal,[12] colon,[12, 20] gastric,[28] head and neck,[27] and lung[22] cancer specimens and Williams *et al.* (2000) showed that COX-2 and VEGF had a similar distribution in lung cancer xenografts.[31]

Transfection studies have also suggested a causative relationship between COX-2 and VEGF. A COX-2 negative colon cancer cell line transfected with COX-2 upregulated VEGF production that was blocked by NS398 (a specific COX-2 inhibitor).[60] A similar link between COX-2, PGE2 and VEGF production, possibly via HIF-1α, was shown using a gastric cancer cell line transfected with COX-2.[28] Furthermore

rat intestinal epithelial cells transfected with oncogenic Ras showed upregulated COX-2, Prostacyclin (PGI2) and VEGF.[66]

In *in vitro* studies, NSAIDs have been shown to reduce angiogenesis and production of angiogenic growth factors. Rofecoxib, SC560 and diclofenac (COX-2, COX-1 and COX inhibitors, respectively) reduced, in the short term, VEGF mRNA production by oesophageal carcinoma *in vitro* but the effect was not sustained.[12] NS398 reduced VEGF and inhibited endothelial cell migration and tubule formation in *in vitro* models of colon cancer[60] and gastric cancer[28] transfected with COX-2. Similar inhibitory effects have been demonstrated in COX-2 positive pancreatic cancer cell lines although high concentrations of NSAID were used or cyclooxygenase activity was amplified by addition of exogenous arachadonic acid.[61, 67]

In vivo models generally support a role for COX-2 in tumour angiogenesis. Systemic COX-2 inhibitors, NS398 and Celecoxib, markedly inhibited FGF induced angiogenesis in corneal micropocket models.[50, 63, 68] Sulindac and Celecoxib also reduced MVD, FGF and VEGF in gastric cancer xenografts.[69] In pancreatic cancer the evidence is less clear. Celecoxib decreased MVD in one *in vivo* pancreatic cancer cell line study[70] and decreased tumour size, metastases, VEGF and MVD in another.[71] However, in resected human tumours xenographed into mice, it had no impact on tumour size, angiogenesis or VEGF levels.[72] The impact on angiogenesis and

growth factor production would seem to differ significantly between NSAIDs and tumours.

COX-2 in the endothelial and stromal cells would also seem to orchestrate angiogenesis. Immunohistochemistry demonstrates COX-2 in angiogenic cells in the tumour vasculature but not in matched normal vessels.[19, 21, 68] In Williams' study, VEGF was reduced in fibroblasts from COX-2(-/-) mice and a specific COX-2 inhibitor decreased VEGF production from wild type fibroblasts (92%).[31]

Murphy *et al.* (2001) showed, *in vitro,* that exogenous VEGF, acting through VEGFR2 receptor, upregulated COX-2 and PGI2 in endothelial cells. There was a corresponding increase in endothelial cell proliferation and tubule formation[11, 73] This group later showed upregulation of COX-2 in response to $\alpha V\beta 3$ clustering, a major endothelial cell integrin in angiogenesis.[74] Other similar studies using various types of endothelial cells also found that VEGF upregulated COX-2 and PGI2 in the endothelial cell.[50, 75, 76]

The evidence is clear that COX-2 is upregulated in many cancers and that it portends a poorer prognosis. In immunohistochemistry studies COX-2 is strongly associated with increased angiogenesis, a process essential for tumour growth and progression. However, while it remains very likely that there is a causative relationship between COX-2 and tumour angiogenesis the exact link and the mechanisms

involved remain obscured. Upregulated COX-2 in tumours often correlates with VEGF activity however this does not prove a causative relationship and they may in fact occur in parallel or independently of each other. One issue with studies that suggest a causative link between COX-2 – PGE-2, VEGF and angiogenesis is that they demonstrate possible pathways using COX-2 transfection that are then inhibited by NSAIDs. In the natural environment it is probable that more than one pathway is involved in growth factor production and so single enzyme inhibition would be insufficient. A second issue surrounds the use of single reagents at supraphysiological concentrations over short periods of time. High concentration or out of proportion growth factors and messengers may have effects in experiments that do not occur in physiological situations and caution is required when drawing conclusions from such data. Thirdly, as malignant angiogenesis is an imbalanced and disordered process, results from studies on normal angiogenesis may not be completely transferable.

1.6 Cyclooxygenase-2 Independent Effects of NSAIDs.

It is increasingly being accepted that inhibition of COX-2 cannot completely explain the therapeutic effects of NSAIDs.[8] In many of the above studies there is great disparity between the concentrations of NSAIDs used and these are often wildly in excess of that required to inhibit COX-2. Celecoxib, at high concentrations, can induce tumour

cell apoptosis, regardless of cellular COX-2 levels.[35, 77, 78] In contrast, other effective COX-2 inhibitors, such as NS398, do not display such marked concentration-dependent effects on proliferation or apoptosis.[61] Ibuprofen and several other high concentration NSAIDs reduced HIF-1α levels and subsequent VEGF production in prostate cancer cells regardless of COX-2 expression suggesting COX-2 independent effects.[79]

Two recent studies, which support this theory, raise significant concerns about using these agents without understanding the mechanism involved. Eibl *et al.* (2005), in a pancreatic cancer study, showed that high dose Nimesulide induces VEGF from a COX-2 negative cell line through PPARγ activation and *in vivo* it stimulated both increased tumour angiogenesis and growth.[80] This correlates with the clinical findings of Ueno *et al.* (2006) which showed that breast cancer patients taking Celecoxib as part of their chemotherapy regime have significantly increased circulating VEGF levels.[81]

It is thought that this COX-2 independent activity may, at least in part, be mediated by inhibition of Phosphoinositide-dependant kinase 1 (PDK-1) phosphorylation of Akt (Fig. 1.3). When phosphorylated, Akt migrates from the plasma membrane into the cytoplasm activating cascades involved in migration, cell cycle control, proliferation, apoptosis and response to hypoxia.[82, 83] Ras, a major oncogene which is mutated in 90% of pancreatic cancers, is an important upstream

stimulator of this pathway and 80% of pancreatic carcinoma cell lines have constitutively activated Akt.[84] Furthermore, NF-κB, a transcription factor upregulated in many tumours, is a known downstream target of Akt and can be inhibited by Aspirin, Sulindac (colorectal cancer) or Celecoxib (pancreatic cancer).[35, 85]

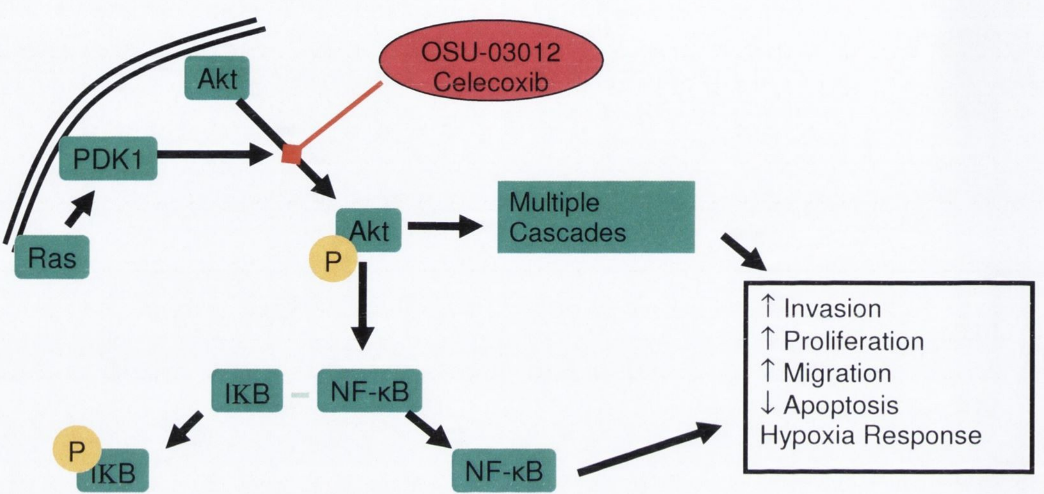


Figure 1.3 OSU-03012 and Celecoxib inhibit phosphorylation of Akt by PDK-1.

Celecoxib and its derivatives can inhibit phosphorylation of Akt by PDK-1. When phosphorylated, Akt moves away from the cell membrane into the cytoplasm where it activates multiple cascades (including NF-κB cascade) involved in the regulation of invasion, proliferation, migration, apoptosis and response to hypoxia.

PDK-1 – Phosphoinisotide Dependent Kinase -1; P – PO_4^{2-}.

The concentration required to achieve COX-2 independent effects *in vitro* is unachievable *in vivo* without severe adverse events. This, combined with concerns regarding cardiovascular side effects of NSAIDs, has led to the development of Celecoxib derivates.[8] These derivatives are designed to have improved efficacy against non COX-2 targets thus enhancing their anticancer effects at physiological concentrations. In addition, reduced COX-2 inhibition should minimise gastrointestinal and cardiovascular complications. *In vitro* studies with these derivatives in several cancers, including prostate, breast, colon, pancreas and leukaemia, have shown decreased cellular proliferation and stimulation of apoptosis.[86]

One particular compound that shows considerable promise is the Celecoxib derivative OSU-03012. Marked reduction in cell proliferation following treatment with OSU-03012, at physiological concentrations has been shown in multiple cancer types.[82, 84, 87, 88] Celecoxib requires higher concentrations (5 – 10 fold) to achieve a similar effect.[35] OSU-03012 can decrease Akt phosphorylation by competitively inhibiting the ATP binding site on PDK-1 and so preventing phosphorylation of Akt at Threonine 308.[86, 88] In thyroid cancer it has also been shown to directly inhibit activation of p21 activated kinases independent of upstream PDK-1 inhibition.[82] In malignant B cells and in B cells from COX-2 deficient mice, OSU-03012 decreased intracellular glutathione, an important cofactor in cellular processes including apoptosis.[89] Both the intrinsic and extrinsic apoptosis pathways are implicated in OSU-

03012 and Celecoxib induced apoptosis.[83, 87] OSU-03012 is reported to be well tolerated in animal models with plasma concentrations exceeding those required for *in vitro* apoptosis.[87]

Celecoxib derivatives have been shown to result in cell cycle arrest at various stages in different tumours.[77, 82, 83, 85, 90] However, the intracellular mechanisms by which OSU-03012, NSAIDs and other similar compounds reduce proliferation and stimulate apoptosis seem to vary from tumour to tumour and are not fully understood. In his review article, Kashfi (2005) describes over ten pro-cancerous targets, including Akt and NF-κB, that have been linked to NSAIDs in various studies.[85]

Although showing promise, the use of NSAIDs in clinical practice has been somewhat disappointing. In the preventative setting, they reduced colorectal cancer by 40-50% and in FAP Celecoxib reduced polyps by 28%.[6, 33] Conversely, this means they did not prevent 50-60% of cancers and 72% of FAP polyps. Their benefit in pancreatic cancer would seem to be less marked despite similar expression levels of COX-2. Furthermore, clinical trials with many of these agents are suspended because of adverse cardiovascular events. Therefore, delineation of the pathways involved and development of derivatives, which uncouple COX-2 inhibition from other beneficial effects, is needed in pancreatic cancer to facilitate design of multi-agent therapies with specific, synergistic targets.

1.7 Summary.

Like most cancers, COX-2 is upregulated in pancreatic cancer and NSAIDs, which inhibit COX-2, have significant therapeutic potential. As angiogenesis is essential for tumour growth and metastases, it is an exciting target that is strongly linked to COX-2 and NSAID activity. However, positive results from experimental models have not translated into a clinical effect in pancreatic cancer clinical trials.[91, 92, 95] A likely explanation is the failure of models to accurately reflect the physiological tumour environment and to identify all the mechanisms involved.

It remains unclear whether or not COX-2, VEGF and other growth factors function in series or parallel and the exact role of NSAIDs in combating (or even stimulating) pancreatic tumour growth and angiogenesis in patients remains unclear. Pancreatic adenocarcinoma is an unusually aggressive and resistant cancer and so caution is required when drawing conclusions on its pharmacodynamics based on results from other cancers. Furthermore, it is probable that NSAIDs in pancreatic cancer have a duel effect at both COX-2 and non COX-2 targets thus knowledge of the pathways involved is required to design synergistic therapies. The development of NSAID derivates, which uncouple the inhibition of COX-2 from the other beneficial effects, is a development that may help solve this enigma.

1.8 Project Overview.

This project verified that two pancreatic cancer cell lines were distinctly COX-2 positive and negative. These two models were then used to investigate the relationship between tumour cell COX-2 and PGE2 to VEGF production. Using an *in vitro* co-culture model of endothelial cell survival– crucial in angiogenesis – the significance of each messenger was determined. Furthermore, the ability of NSAIDs to affect tumour cells independently of COX-2 inhibition was investigated and compared with OSU-03012, a Celecoxib derivative with reported, minimal COX-2 inhibitory activity.

Chapter 2: Methods.

2.1 Cell Culture and Reagents.

All reagents were supplied by Sigma-Aldrich (St. Louis, MI, USA) unless otherwise specified. AsPC-1, BxPC-3 and MIA PaCa-2 permanent pancreatic carcinoma cell lines were purchased from The American Tissue Culture Collection (Manassas, VA, USA). AsPC-1 and BxPC-3 were grown in RPMI-1640 supplemented with 2mM L-glutamine, 10mM HEPES (Invitrogen, NY, USA), 1mM sodium pyruvate, 4.5g/L glucose, 1.5g/L sodium bicarbonate and 10% foetal calf serum (FCS) (Invitrogen). MIA PaCa2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 4mM L-glutamine, 4.5g/L glucose, 1.5g/L sodium bicarbonate, 10% foetal calf serum and 2.5% horse serum (Invitrogen). Cells were propagated in T75 flasks in a humidified atmosphere at 37°C and 5% CO_2. At 80 – 100% confluency, cells were trypsinised (0.05% EDTA) (Invitrogen), centrifuged (2000 rpm for 10 min) and divided into four T75 flasks for further propogation or frozen in freezing medium/DMSO (Invitrogen) in liquid nitrogen. All experiments were performed using passages 5 to 12.

Phorbol-12-myrisitate-13-acetate (PMA) and Camptothecin (Biovision, Mountain View, CA, USA) were reconstituted in Dimethyl Sulphoxide (DMSO) for a stock solution of 1mM which was stored in aliquots at -20°C. Aspirin was dissolved fresh each day in Phosphate Buffered Saline (PBS) (Invitrogen) to form a 200μM solution. NS398, SC560,

OSU-03012 (Caymen Chemical Co., Ann Arbor, MI, USA) and Celecoxib (LKT Laboratories, St Paul, MN, USA) were reconstituted in DMSO for a 10mM or 100mM stock solution which was aliquoted and stored at -20°C. Arachadonic acid was reconstituted in PBS for a stock solution of 15mM and stored in aliquots at -20°C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyyltetrazolium bromide (MTT) was dissolved in PBS at a concentration of 5mg/ml and frozen in aliquots at -20°C. Unless otherwise stated a working solution of each reagent was made by dilution of stock solution in RPMI supplemented with 2.5% FCS.

2.2 Human Umbilical Vein Endothelial Cell Isolation.

Umbilical cords were collected in collaboration with Dr. Michael Geary, Rotunda Hospital, Dublin 1. Ethical approval for harvesting of HUVECs and their use in this study was provided by The Rotunda Hospital Ethics Committee and informed consent was obtained from the mother in each case (Appendix A). The cannulated umbilical vein was flushed twice with PBS and collagenase injected. After 15 minutes (min) incubation at 37°C the vein was flushed with PBS and the discharge collected and centrifuged (1500 rpm for 10 min). The resultant pellet was resuspended in M199 (Invitrogen) supplemented with 25,000 units heparin ammonium (porcine), 15mg endothelial growth factor, 2mM L-glutamine, 1mM sodium pyruvate and 20% heat inactivated FCS. Cells were propagated in T75 flasks coated with 1% gelatine in a humidified

atmosphere at 37°C and 5% CO_2. At 80-100% confluency, cells were split into 3 by trypsinisation and centrifugation. All experiments were performed at passages 2 or 3.

2.3 Western Blot.

Confluent cells in T25 flasks were serum starved overnight in RPMI supplemented with 2.5% FCS. They were then washed twice with PBS and treated either PMA [1μM] or Camptothecin [2μM] for 4 hours (h) or 24h. The supernatant was discarded and the cells were harvested using trypsinisation and centrifugation. Cells were washed twice in PBS and resuspended in 10:1 RIPA buffer : Protease Inhibitor Cocktail solution. After 30 min shaking on ice, the solution was sonicated for 5 min and then centrifuged at 13,000g for 5 minutes. The supernatant was then removed and frozen at -80°C until further analysis.

The concentration of protein in each sample was quantified using the Bio-Rad Protein Assay according to the manufacturer's instructions (Bio-Rad, Munich, Germany). Samples containing a fixed amount of protein (30μg) were fractionated by SDS-PAGE gel (10%) using a Mini-PROTEAN 3® Cell and transferred to nitrocellulose membrane with a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad). Ponceau S staining confirmed protein transfer. Membranes were blocked with a BSA (10% in 0.1% Tween in PBS) and then incubated with appropriate primary antibody overnight. Bound proteins were then

detected using the amplified 4-Opti-CN (Bio-Rad) detection system according to the manufacturer's instructions.

2.4 RT-PCR.

Total RNA was isolated from pancreatic cancer cells using an RNeasy 96 Kit (Qiagen), including a DNase digestion step. Concentration and integrity of the RNA samples was measured by capillary electrophoresis using an RNA 6000 Nano Chip Kit on the Agilent 2100 Bio-analyser system. Only samples with a peak area ratio > 2.0 of 28S to 18S rRNA and a RNA integrity number of 8-9 were used.

RNA was reverse transcribed into cDNA using a High Capacity cDNA Archive Kit (Applied Biosystems). Pre-designed and pre-optimised Taqman Gene Expression assays, for COX-2 and a housekeeping gene (18S), were purchased from Applied Biosystems. Real-time RT-PCR was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) with template cDNA and Taqman Gene Expression Assay Mix (Applied Biosystems). Measurements were carried out in triplicate and repeated using 3 passages. Data from gene expression analysis, using quantitative real-time RT-PCR, was analysed using the $2^{-\Delta\Delta CT}$ method.

2.5 PMA / Camptothecin Assay.

Confluent cells were washed twice with PBS and serum starved overnight in RPMI supplemented with 2.5% FCS. Cells were washed twice with PBS between each of the following treatments. Aspirin [200μM] in PBS for 45 min then PMA [1μM] or Camptothecin [2μM] or DMSO [0.1% v/v] in RPMI supplemented with 2.5% FCS for 4h or 24h. Selected wells were treated with NS398 [1μM] in PBS for 45 min. Arachadonic acid [150μM] in PBS was added to each well for 10 min and then harvested and stored at -20°C until further analysis by ELISA. Cell viability of similarly treated cells was determined by MTT assay instead of addition of arachadonic acid.

2.6 Endothelial Cell Proliferation / Survival Assay.

Confluent AsPC-1 or BxPC-3 cells were washed twice with PBS and the appropriate treatment added in RPMI supplemented with 2.5% FCS. HUVECs at passage 2 or 3 were seeded at 2.5 x 10^4 in M199 supplemented with FCS (20%) in inserts with 0.4μm porous membrane coated with 0.1% gelatin and allowed to adhere overnight. Supernatant was replaced with M199 supplemented with FCS (2.5%) and the inserts combined with the companion plate (Fig. 2.1). The coculture was incubated for 3-5 days with a change of medium (including treatments) on days 2 and 4. Supernatant from both upper and lower chambers was harvested (timpoint determined by HUVEC

viability on Giemsa Staining) and frozen at -20°C until further analysis. HUVEC viability was quantified by WST assay and Giemsa staining. Tumour cell viability was confirmed by MTT assay.

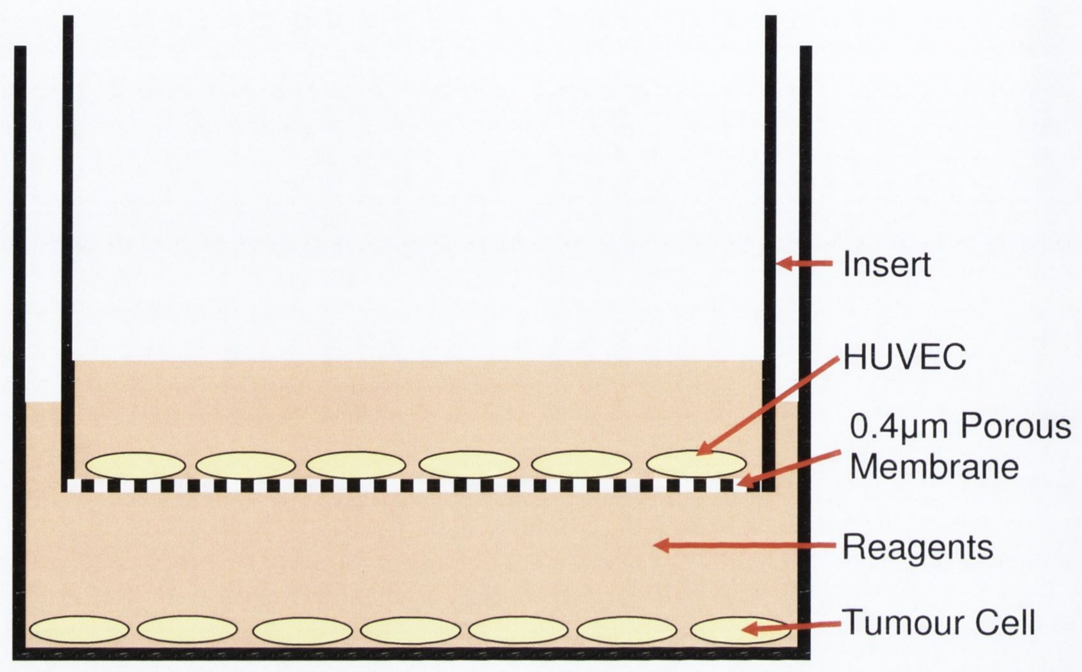


Figure 2.1 Coculture Assay.

AsPC-1 or BxPC-3 cells were seeded in the lower chamber and HUVECs in the upper chamber. COX-2 or VEGF inhibitors were added to the lower chamber. After 3-5 days the medium was harvested for PGE2 or VEGF ELISA, tumour cell viability was determined by MTT assay and HUVEC viability was quantified by WST assay.

HUVEC – Human Umbilical Vein Endothelial Cells; Tumour Cell – AsPC-1 or BxPC-3; Reagents – NS398, VEGF Neutralising Antibody or DMSO.

2.7 NSAID / OSU-03012 Assay.

AsPC-1 and BxPC-3 cells at 60-75% confluency were used to ensure proliferation. Cells were serum starved overnight in RPMI supplemented with FCS (2.5%). Cells were washed twice with PBS and treated with NS398, Celecoxib or OSU-03012, in RPMI supplemented with FCS (2.5%), at various concentrations and time points. Supernatant was harvested and stored at -20°C until further analysis by ELISA. Cell viability was determined by MTT assay.

2.8 PGE2 / VEGF ELISA.

Harvested supernatant was stored at -20°C until analysis by ELISA. PGE2 was quantified by PGE2 ELISA kit (Cambridge Bioscience, Cambridge, UK) and VEGF by VEGF Duokit Sandwich ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.9 MTT Assay.

Following treatment, MTT [0.5mg/ml] was added to each well and they were incubated at 37°C in the dark for 1 h. During this time MTT was cleaved into purple formazan crystals by viable cells. Supernatant was discarded and cells and crystals dissolved in DMSO (250µL). Homogenicity was ensured by gentle swirling. Absorption of light at

570nm was measured using a Tecan Infitinite F200 spectrophotometer (Eppendorf) and cell viability was calculated as a percentage of control.

2.10 WST-1 "Quick Cell Proliferation Assay" Kit.

All medium was removed from inserts and stored at -20°C for further analysis. WST solution (1/10 in PBS, Cambridge Bioscience) was added to each insert and incubated in the dark at 37°C for 1h. During this time viable cells cleaved the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases. The supernatant was transferred to a 96 well plate and the absorbance at 450nm was measured using a Tecan Infitinite F200 spectrophotometer. The optical density for each sample was divided by that of the control to calculate the cell viability.

2.11 Giemsa Staining.

The supernatant was removed from inserts and stored at -20°C for further analysis. Cells were fixed in methanol (99%) for 15 min and then stained for 13 min with Giemsa stain (dissolved 1/6 in deionised water (dH_20) and filtered). After copious washing with dH_20 membranes were allowed to dry and were visualised using a Leica DMIL inversion microscope. Images were captured using a Sony Cybershot 6.0 megapixel digital camera.

2.12 Statistical Analysis.

Two or more duplicates of each experiment were performed. Results were expressed as the mean of the replicates ± standard error of the mean (SEM). Significance was calculated using single sample or independent sample t test, as appropriate (SPSS Version 14.0, SPSS Inc. USA). Equal variances were not assumed.

Due to the narrow range of the PGE2 ELISA kit, several results were reported as "<min" or ">max". These were either disregarded and the experiment repeated or, if appropriate, the minimum or maximum value applied as the most conservative possible concentration (Table 2.1). This increased the p value of some significance calculations as marked in the text (*).

Table 2.1. Examples of Conservative Estimates of PGE2 Concentrations.

Variable	Reading (Range: 39 – 2000 pg/ml)	Outcome
Control Inhibitor	> Max 367 pg/ml	2000 pg/ml 367 pg/ml
Control Inhibitor	> Max > Max	Disregard and Repeat
Control Inhibitor	< Min < Min	Disregard and Repeat
Control Inhibitor	1589 pg/ml < Min	1589 pg/ml 39 pg/ml

Chapter 3: Results.

3.1. Validation of COX-2 Positive and COX-2 Negative Permanent Pancreatic Cancer Cell Lines.

3.1.1 Potential Cell Lines.

In order to choose two permanent pancreatic cancer cell lines that were consistently and reliably COX-2 positive and COX-2 negative, the literature was reviewed, three cell lines were selected and preliminary experiments performed. The characteristics of the three cell lines are detailed in Table 3.1.1.

In preliminary optimisation experiments, the three cell lines were treated in a time and concentration-dependent manner with different combinations of NSAIDs – Aspirin (COX inhibitor) and NS398 (COX-2 inhibitor), the pro inflammatory agent, PMA, and the pro-apoptotic agent, Camptothecin. Response was measured by quantifying VEGF production and conversion of arachadonic acid to PGE2 by ELISA.

During these preliminary, optimisation experiments it emerged that the MIA PaCa2 cells were very intolerant of Aspirin with near complete loss of cells at the initial stages of each experiment. Patterns also emerged suggesting that BxPC-3 are COX-2 positive and AsPC-1, COX-2 negative. Furthermore, both AsPC-1 and BxPC-3 cells were grown in RPMI medium but Mia-PaCa2 were grown in DMEM. Therefore AsPC-1 and BxPC-3 were selected as COX-2 negative and COX-2 positive cell lines, respectively, for the remainder of the experiments.

Table 3.1.1. Candidate cell lines and their characteristics.

Name	AsPC-1	MIA PaCa2	BxPC-3
Type	Pancreatic adenocarcinoma	Pancreatic adenocarcinoma	Pancreatic adenocarcinoma
Origin	Ascitic metastasis	Primary tumour	Primary tumour
COX-2 status (literature)	Weak	Weak – moderate	Very strong
Morphology			

3.1.2 Concentration and Efficacy of Compounds.

In order to verify the COX-2 status of the two cell lines as "positive" and "negative" they were exposed to two extremes – PMA, a tumour / inflammation promoter and Camptothecin, a pro-apoptotic agent.

PMA.

Following preliminary concentration-timepoint-response curves and based on institutional experience and manufacturer's guidelines, cell viability assays (MTT) were performed. PMA was cytotoxic to the cells (Fig. 3.1.1). In BxPC-3 cells there was a decrease in viability of 54% following treatment with 1μM PMA at 4h and 68% at 24h, respectively. In contrast AsPC-1 cells were relatively resistant displaying decreases of 28% at 4h and 37% at 24h. These changes were all significant relative to untreated controls ($P<0.01$). The vehicle, DMSO had negligible effect on cell viability (Figure 3.3.2).

In AsPC-1, the proposed COX-2 negative cell line, maximum stimulation was sought to ensure no inducible COX-2. As the concentration of 1μM only resulted in a cytotoxicity of approximately 35% in AsPC-1, the cell line to be stress tested for COX-2 negativity, this concentration was used in all future experiments to ensure a high degree of stimulation and thus a robust "COX-2 negative" conclusion.

Camptothecin.

The concentration of Camptothecin recommended by the manufacturer was 2µM. Cell viability was measured for each cell line treated with Camptothecin [2µM] for 4h and 24h (Fig. 3.1.2).

The recommended concentration was non-toxic to the cells with cell viability of 100% and 97% in AsPC-1 cells at 4h and 24h. BxPC-3 were more susceptible to cytotoxic effects, with a decrease of 13% at 4h and 40% after 24h. The latter effect approached significance relative to untreated control ($p=0.057$). This concentration was used in all future experiments.

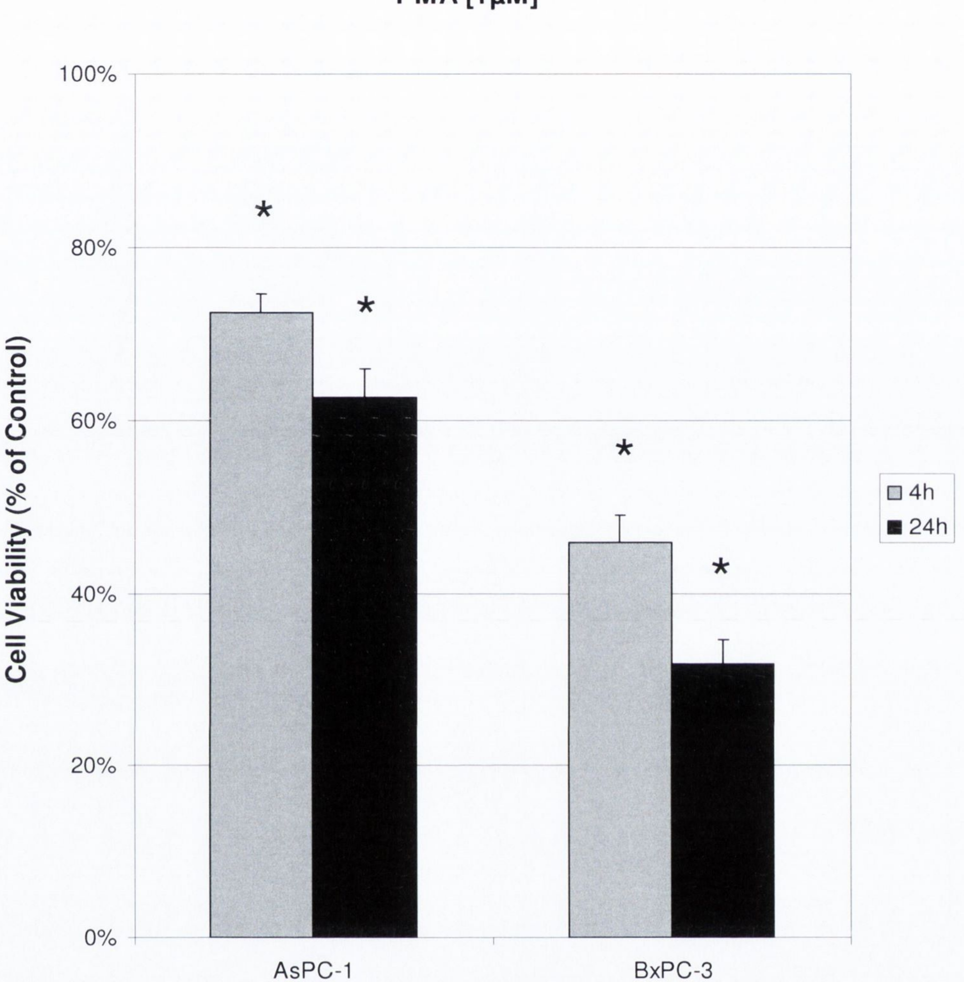


Figure 3.1.1 PMA is cytotoxic to cells.

*Serum starved, confluent AsPC-1 and BXPC-3 cells were treated with PMA at a final concentration of 1μM for 4h or 24h. MTT assay quantified cell viability relative to untreated control wells. The vehicle DMSO [0.1% v/v] had negligible effect on cell viability (data not shown, see Figure 3.3.2). Columns are means of 3 replicates ± SEM. *p<0.05 relative to control.*

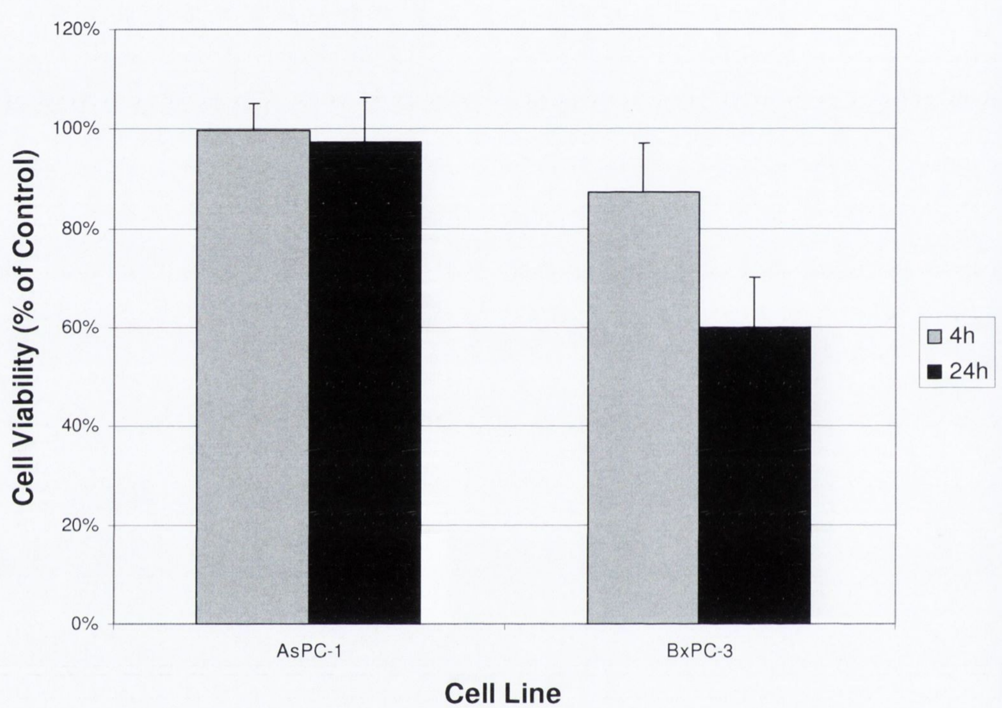


Figure 3.1.2 Camptothecin [2μM] is cytotoxic to BxPC-3 cells.

Serum starved, confluent AsPC-1 and BxPC-3 cells were treated with Camptothecin, final concentration 2μM, for 4h or 24h. MTT assay quantified cell viability relative to untreated control. The vehicle, DMSO [0.1% v/v] had negligible effect on cell viability, (data not shown). Columns are means of at least 3 replicates ± SEM.

Non steroidal Anti-inflammatory Drugs.

Two NSAIDs were chosen as COX inhibitors. Aspirin is probably the oldest and best known, non specific COX inhibitor while NS398 was one of the first specific COX-2 inhibitors discovered and has been extensively used in many studies.

Aspirin.

As a result of previous optimization studies, a concentration of 200μM Aspirin was used.

Cell viability was measured after a 45 minute treatment with Aspirin followed by 4h or 24h incubation in RPMI supplemented with 2.5% FCS. This NSAID had a significant cytotoxic effect on AsPC-1 cells with a 22% and 12% reduction in cell viability at 4h and 24h respectively ($p<0.05$). In contrast Aspirin had no effect on BxPC-3 viability (Fig. 3.1.3). Of note, treatment with Aspirin for longer than 45 min resulted in marked cell death, therefore this concentration was used for all future experiments for a maximum treatment time of 45 min.

NS398.

NS398 [1μM] was used to specifically inhibit COX-2. This concentration is lower than that used by other authors but successfully inhibited PGE2 production by COX-2 with no cytotoxic effect (Section 3.1.5). This NSAID, at higher concentrations, did have other effects such as cytotoxicity (Chapter 3.3).

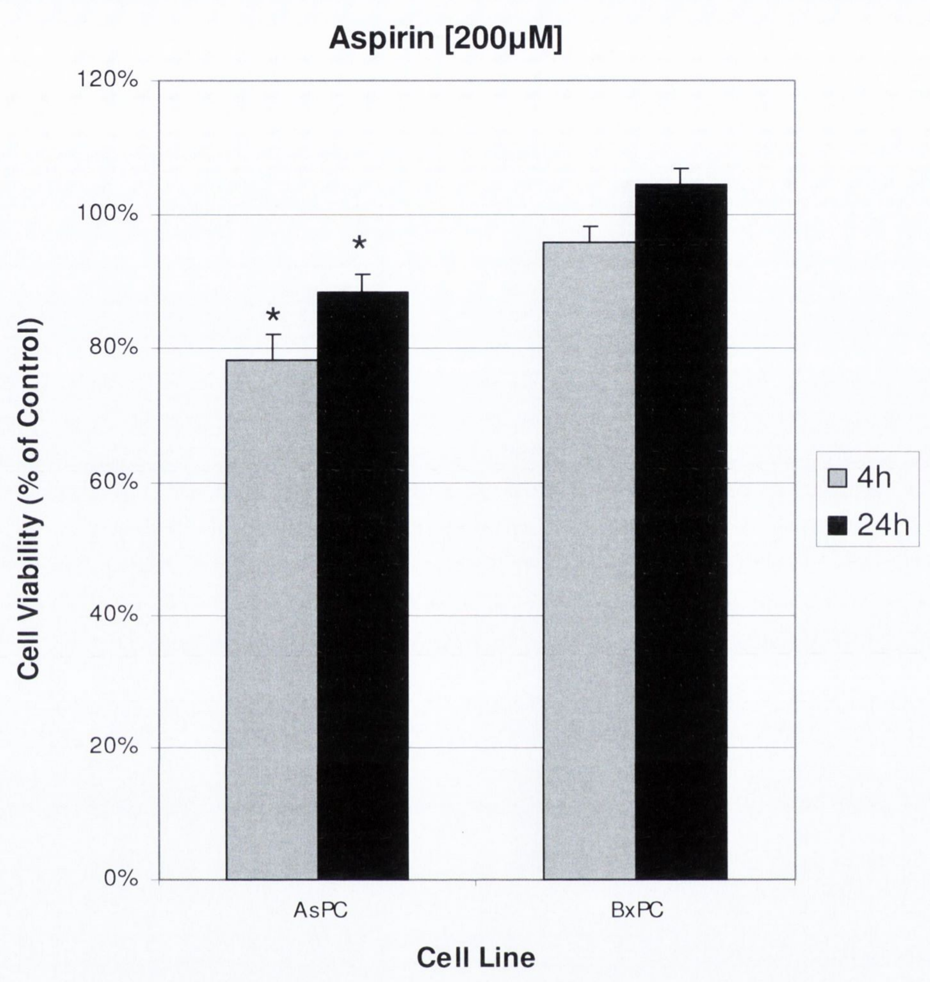


Figure 3.1.3 Minimal cytotoxicity with Aspirin [200μM] treatment.

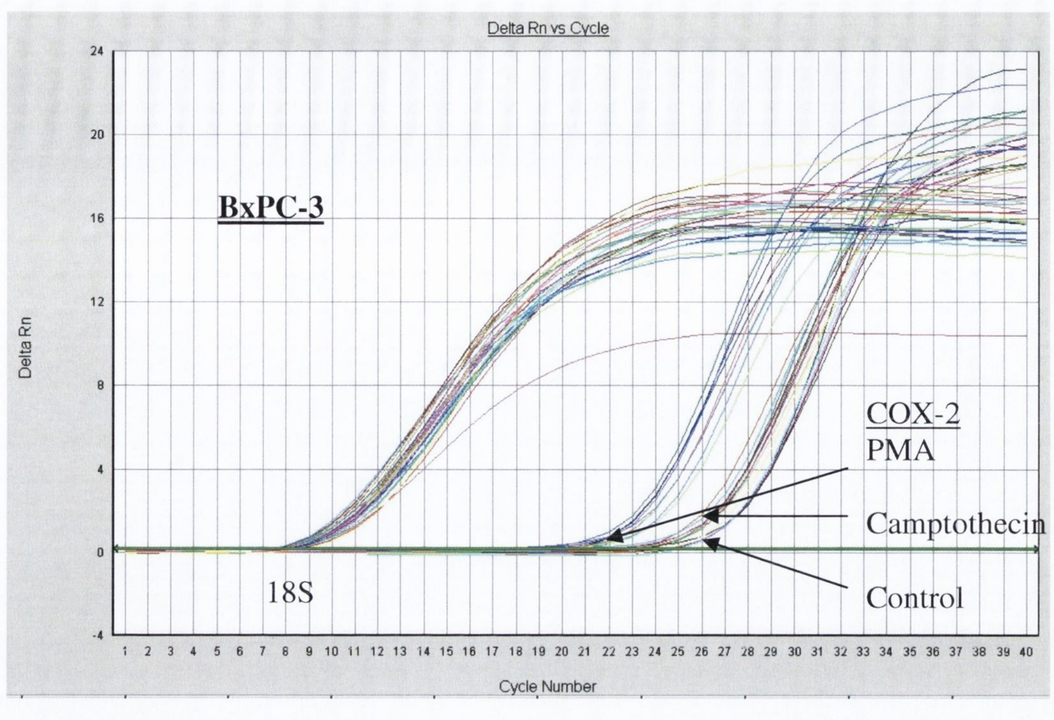
*Serum starved, confluent AsPC-1 and BxPC-3 cells were treated with Aspirin [200μM] in PBS for 45 minutes. Aspirin was removed and then RPMI supplemented with 2.5% FCS added for 4h or 24h. MTT assay then quantified cell viability relative to untreated control. Columns are means of at least 3 replicates ± SEM. *p<0.05 relative to control.*

3.1.3 COX-2 mRNA Expression Levels.

PMA and Camptothecin treated BxPC-3 and AsPC-1 cells (4h) were further analysed in a parallel project carried out by our group. COX-2 mRNA levels were measured by real-time quantative RT-PCR.

COX-2 mRNA was quantified under each treatment condition in the two cell lines at 4h. 18S was used as an endogenous control and was detected at cycle 7 in BxPC-3 and at cycle 8 in AsPC-1. In the BxPC-3 cells COX-2 mRNA was evident at cycle 25 in control cells, cycle 23 in Camptothecin treated cells and cycle 20 in PMA treated cells. This demonstrates a high baseline COX-2 mRNA levels in this cell line that is augmented by PMA and, to a lesser extent, by Camptothecin. COX-2 mRNA was essentially undetectable (>cycle 37) in the AsPC-1 cells regardless of treatment.

Calculation of quantative results by the $2^{-\Delta\Delta CT}$ method shows a 16.4 and a 2.2 fold upregulation in PMA and Camptothecin treated BxPC-3 cells, respectively, relative to control (Fig. 3.1.5). As COX-2 mRNA was undetectable in most AsPC-1 samples it was not possible to calculate fold changes.



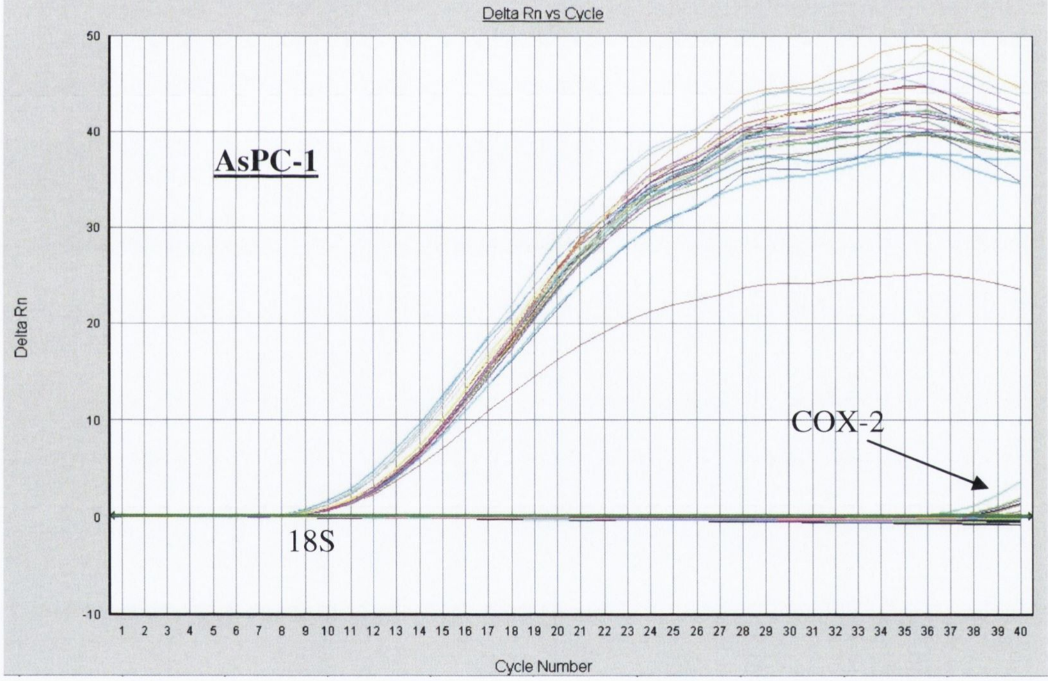


Figure 3.1.4 COX-2 mRNA is undetectable in AsPC-1 cells.

Confluent AsPC-1 and BxPC-3 cells were treated with PMA [1μM] or Camptothecin [2μM] for 4h. Cells were lysed and total RNA harvested. Quantative RT-PCR was performed for COX-2 and 18S (Control) mRNA.

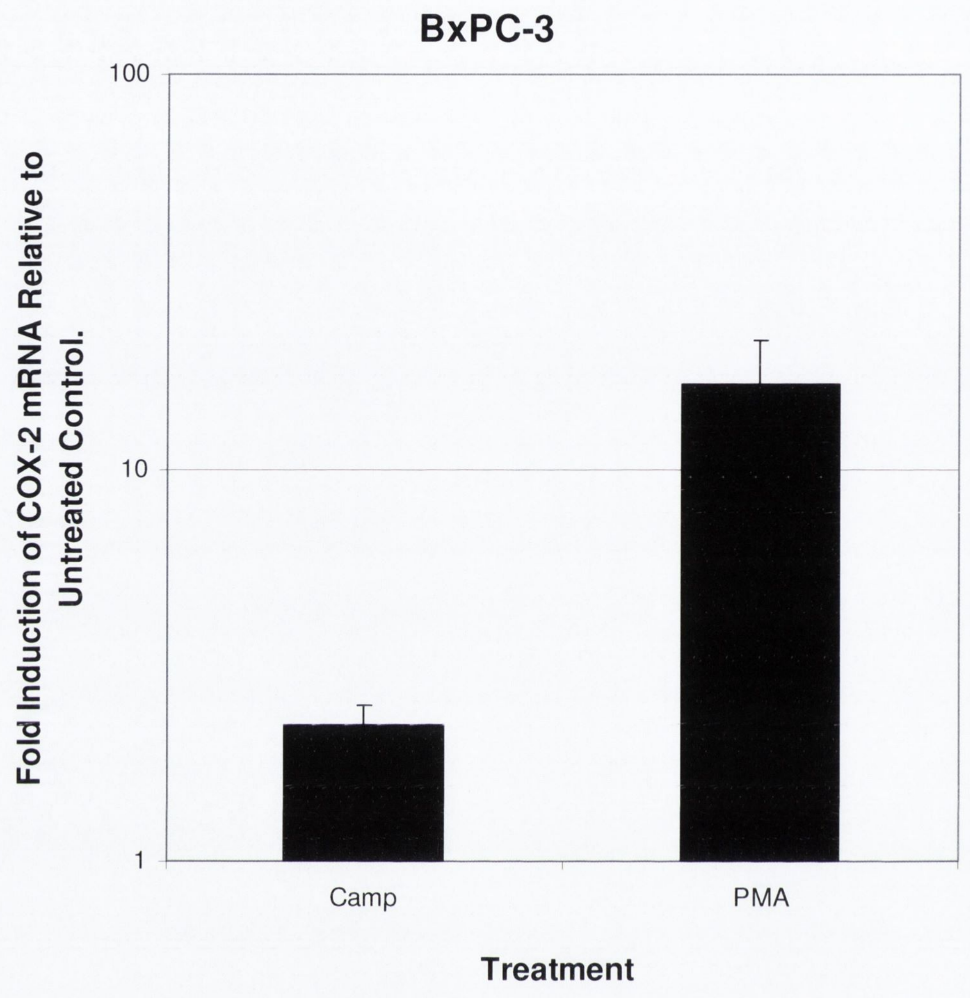


Figure 3.1.5 COX-2 is upregulated in BxPC-3 cells

Confluent AsPC-1 and BxPC-3 cells were treated with PMA [1μM] or Camptothecin [2μM] for 4h. Cells were lysed and total RNA harvested. Quantative RT-PCR was performed for COX-2 and 18S (Control) mRNA. As COX-2 mRNA was undetectable in AsPC-1 cells, fold changes could not be calculated. Columns are means of 3 replicates ± SEM.

3.1.4 COX-2 Protein Levels.

Western blots were used to quantify COX-2 protein levels both in controls and following treatment with PMA and Camptothecin at 4h and 24h (Fig 3.1.6). In BxPC-3 cells, COX-2 was evident at baseline and was augmented by PMA and to a lesser extent by Camptothecin at each timepoint. There was no increase in COX-1 levels.

In AsPC-1 cells COX-2 was not detected at either timepoint despite using an amplification system (Opti-4CN amplification system) sensitive to 2 pg/ml.

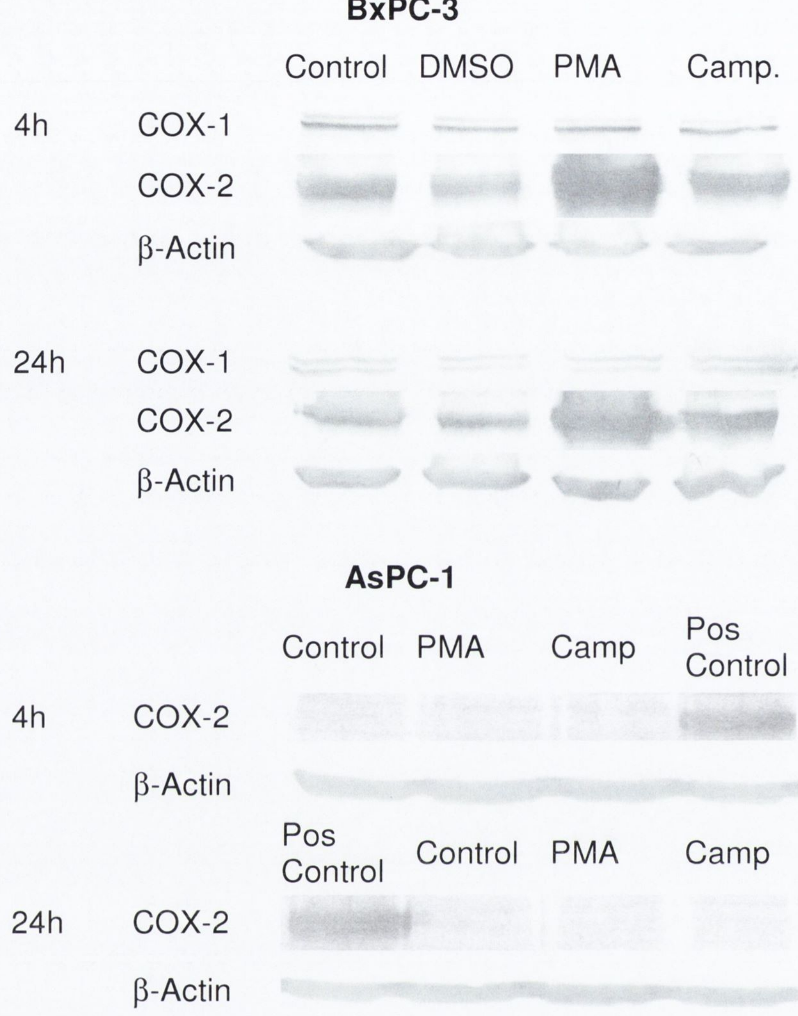


Figure 3.1.6 COX-2 Protein is present in BxPC-3 but not AsPC-1 cells.

Confluent AsPC-1 and BxPC-3 cells were treated with PMA or Camptothecin in RPMI supplemented with 2.5% FCS for 4h or 24h. Cells were lysed in RIPA buffer. Lysate (30µg of protein) was fractionated on 10% SDS-PAGE and transferred to Nitrocellulose Membrane. Protein was detected using appropriate antibody and the Opti-4CN kit with amplification. β-Actin ensured equal loading. Images are representative of 3 replicates.

3.1.5 COX-2 Activity.

To confirm maximal COX-2 activity, cells were pre-treated with Aspirin to inhibit any baseline COX activity. Cells were then treated with PMA or Camptothecin for 4h or 24h before arachadonic acid, the substrate for COX, was added and PGE2 quantified by ELISA. Treatment with NS398 prior to arachadonic acid differentiated COX-1 from COX-2 activity.

The mean PGE2 produced in the AsPC-1 control wells was 634 pg/ml in the 4h group and 545 pg/ml in the 24h. The concentration of PGE2 in BxPC-3 control wells was 13 fold greater than this (7824 pg/ml and 7062 pg/ml, respectively) ($p<0.05$). NS398 [1μM] reduced this by 71% at 4h ($p<0.05$) and 47% at 24h ($p<0.05$) in the BxPC-3 cells but had no effect on AsPC-1 cells (Fig. 3.1.7 and 3.1.8).

PMA treatment increased PGE2 production by the BxPC-3 cells 7.5 ($p=0.089$)* and 8.4 fold ($p=0.288$)* after 4h and 24h treatment. This was reduced 77% at 4h ($p=0.075$)* and 61% ($p=0.231$)* at 24h by NS398 [1μM]. There was no change in PGE2 concentration in the AsPC-1 cells with either treatment.

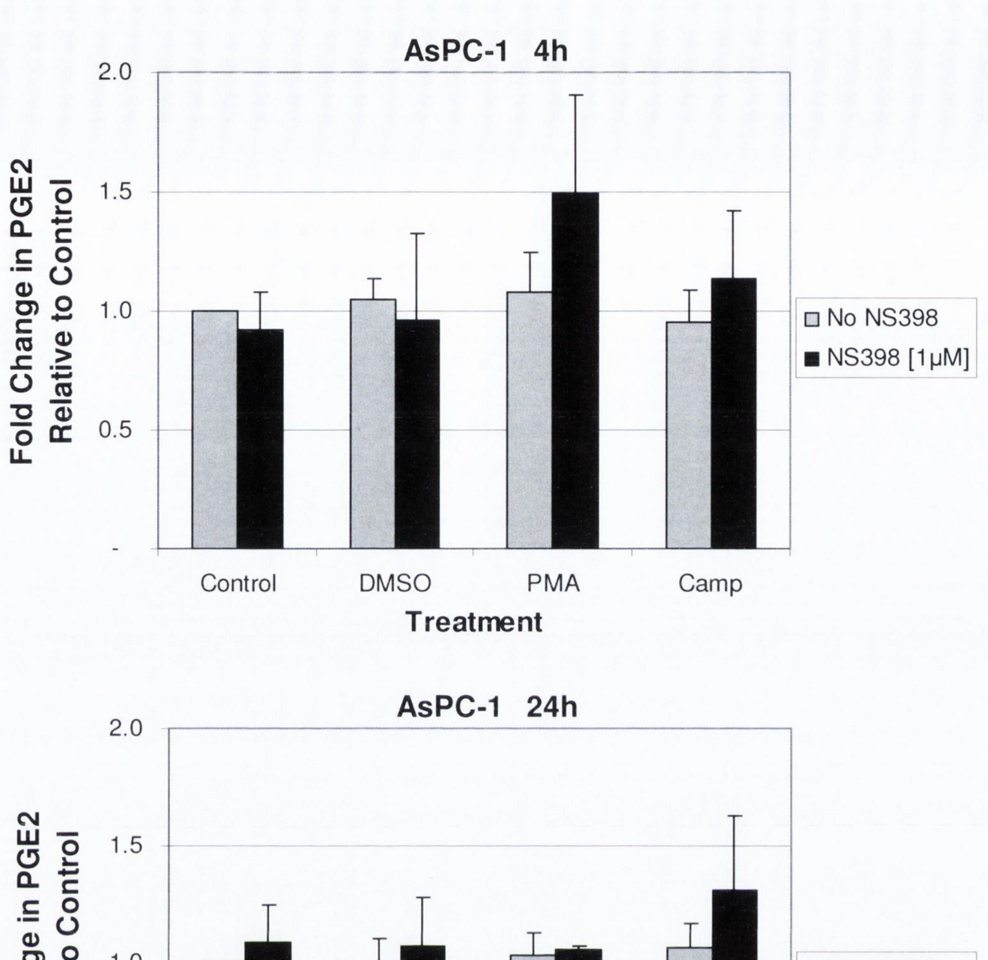
BxPC-3 cells treated with Camptothecin displayed a similar, although less marked, pattern of PGE2 production. After a 4h treatment PGE2 was increased by 3.6 fold ($p=0.068$)* and this was reduced 84% by

NS398 [1μM] (p=0.114)*. Following 24h treatment, PGE2 was increased 5.5 fold (p=0.057)* and this was reduced 48% by [1μM] NS398 (p=0.228)*. Neither treatment had any effect in AsPC-1 cells.

Investigations using MTT assay showed that there was no difference between BxPC-3 cells treated with NS398 and those that were not. This confirms that the reduction in PGE2 was due to enzyme inhibition and not cytotoxicity (Fig. 3.1.9).

PGE2 ELISA on medium from cells in the serum starved state confirmed that this difference in COX-2 activity / PGE2 production persisted in cells not exposed to exogenous arachadonic acid. This would be used in future models of apoptosis and angiogenesis (Fig. 3.1.10). PGE2 in supernatant from BxPC-3 cells (395.3 pg/ml) serum starved for 24h was 5.9 times higher than in AsPC-1 samples (67.4 pg/ml) ($p<0.05$).

* See Chapter 2.12



AsPC-1 24h
2.0
1.5
1.0
0.5
-
Fold Change in PGE2 Relative to Control
No NS398
NS398 [1µM]
Control
DMSO
PMA
Camp
Treatment

Figure 3.1.7 No increase in COX-2 activity in AsPC-1 cells.

Serum starved confluent AsPC-1 cells were treated with Aspirin [200µM] for 45 min and then PMA [1µM] or Camptothecin [2µM] for 4h or 24h. Selected cells were then treated with NS398 [1µM] for 45 min. NS398 was removed and Arachadonic acid [150µM] added for 15 min and the supernatant harvested for PGE2 ELISA. Columns are means of at least 3 replicates ± SEM.

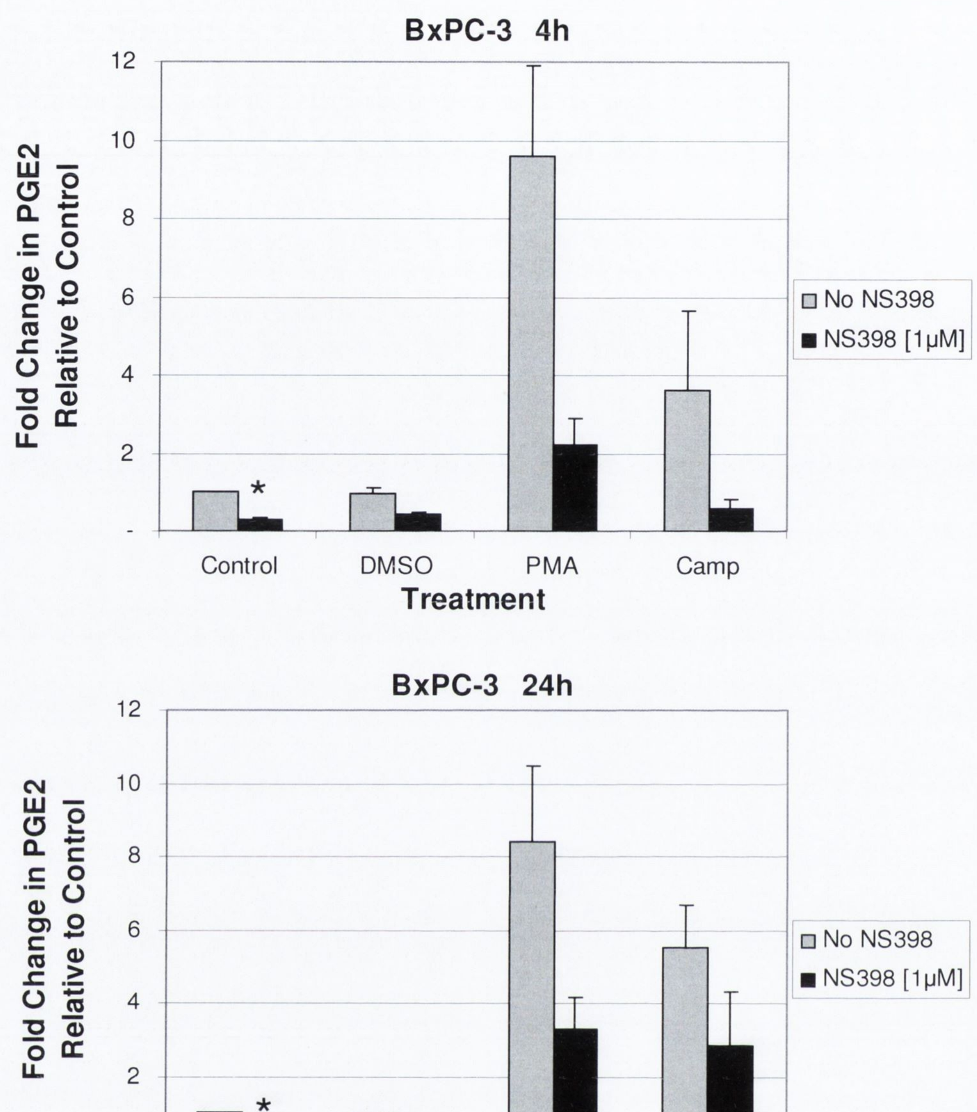


Figure 3.1.8 High COX-2 activity in BxPC-3 cells.

*Serum starved confluent BxPC-3 cells were treated with Aspirin [200μM] for 45 min and then PMA [1μM] or Camptothecin [2μM] for 4h or 24h. Selected cells were then treated with NS398 [1μM] for 45 min. NS398 was removed and Arachadonic acid [150μM] added for 15 min and the supernatant harvested for PGE2 ELISA. Columns are means of at least 3 replicates ± SEM. *p<0.05 relative to control.*

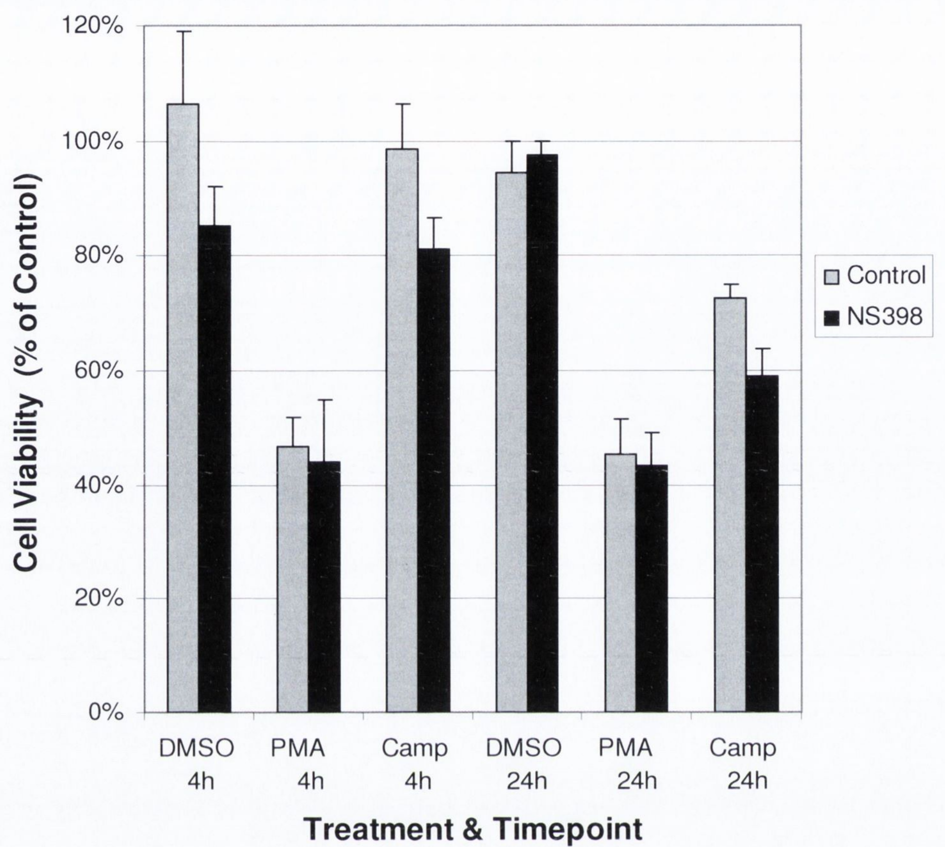


Figure 3.1.9 NS398 inhibition of PGE2 production is not due to cytotoxicity.

Serum starved, confluent BxPC-3 cells were treated with Aspirin [200μM] then either PMA [1μM] or Camptothecin [2μM] for 4h or 24h. Selected cells were then treated with NS398 [1μM] in PBS for 45 min. MTT assay quantified cell viability relative to control wells. Columns are means of at least 3 replicates ± SEM.

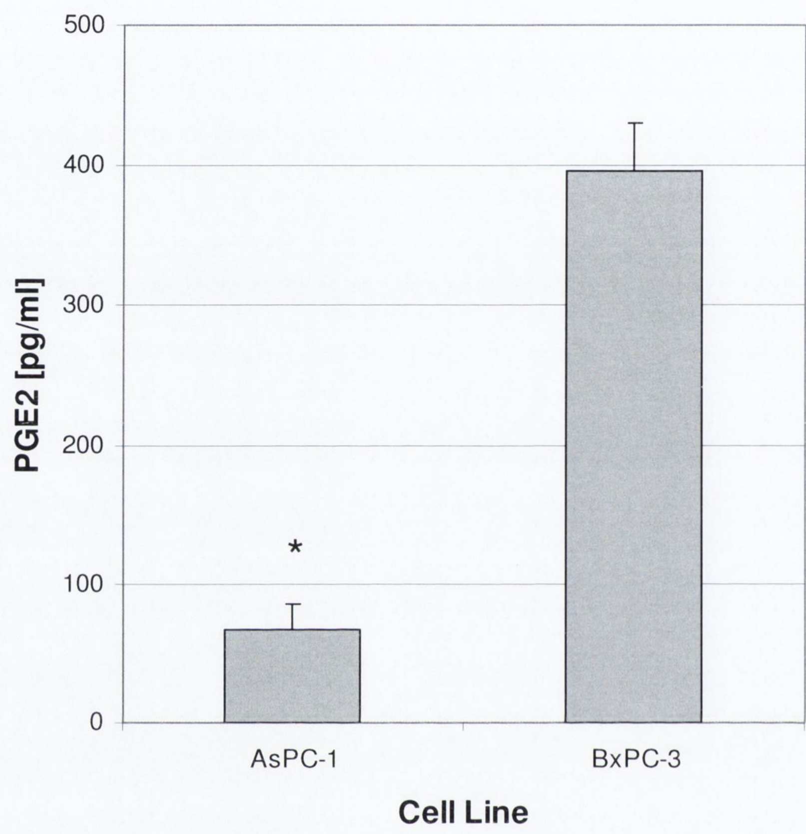


Figure 3.1.10 BxPC-3 cells have high baseline COX-2 activity.

*Confluent AsPC-1 and BxPC-3 cells were serum starved for 24hr and the concentration of PGE2 in the supernatant measured by ELISA. Columns are means of at least 3 replicates ± SEM. *p<0.05 relative to BxPC-3 control.*

3.1.6 VEGF Production.

To confirm stimulation of AsPC-1 cells by PMA and Camptothecin, VEGF concentration was measured by ELISA. Supernatants were harvested immediately before treatment with NS398 in the COX-2 activity model.

Mean VEGF produced by AsPC-1 cells was 164 pg/ml and 468 pg/ml at 4h and 24h. In BxPC-3 cells, baseline VEGF production was higher at 740 pg/ml at 4h ($p<0.05$) and 1755 pg/ml at 24h ($p<0.05$).

PMA increased VEGF production by 1.7 fold in AsPC-1 cells at both 4h and 24h and by 1.3 and 7.1 fold in BxPC-3 cells at 4h and 24h respectively (Fig. 3.1.11). These changes were significant relative to untreated control ($P<0.05$).

In BxPC-3 cells, Camptothecin increased VEGF production by 31% at 4h ($p<0.05$) and 18% ($p=0.186$) at 24h relative to untreated controls (Fig. 3.1.12) but was also cytotoxic at 24h (Fig. 3.1.9). Conversely, Camptothecin decreased VEGF by 16% at 4h and 38% at 24h in AsPC-1 cells with no cytotoxic effect. (Fig. 3.1.12)

To eliminate possible vehicle effect, DMSO [0.1%v/v] was investigated. DMSO reduced VEGF production by 11% in AsPC-1 cells and 15% in BxPC-3 cells at 24h (Fig. 3.1.13). When compared to

DMSO, the decrease in VEGF in AsPC-1 cells in response to Camptothecin approached significance at 24h ($p=0.058$).

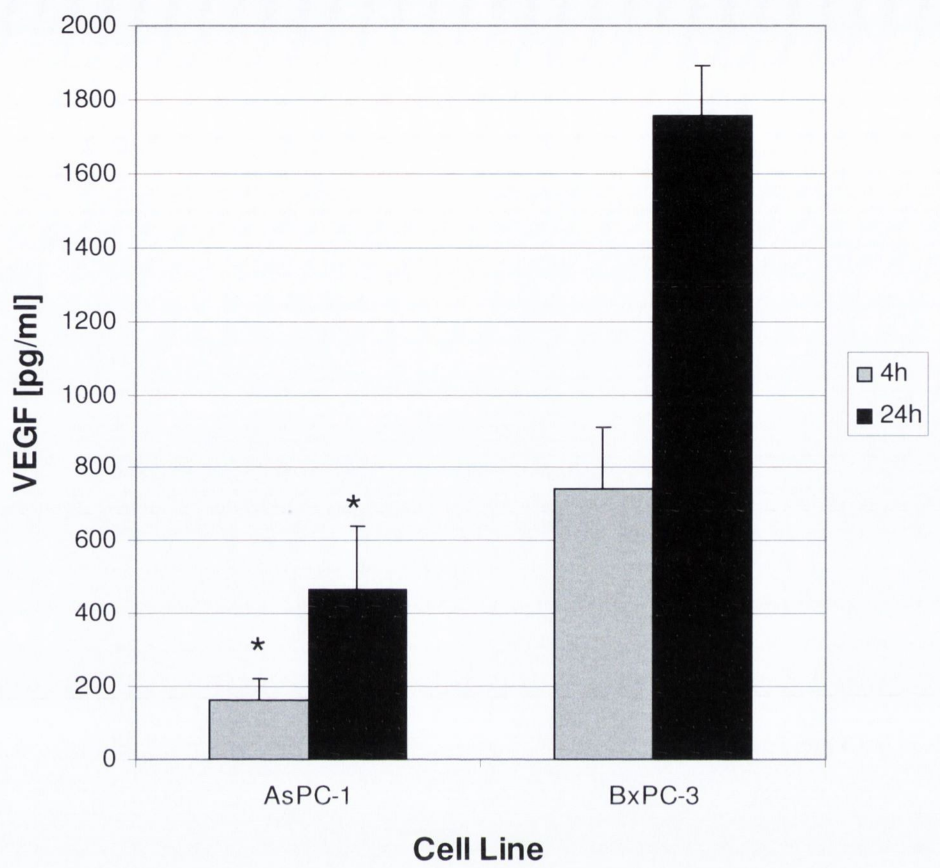
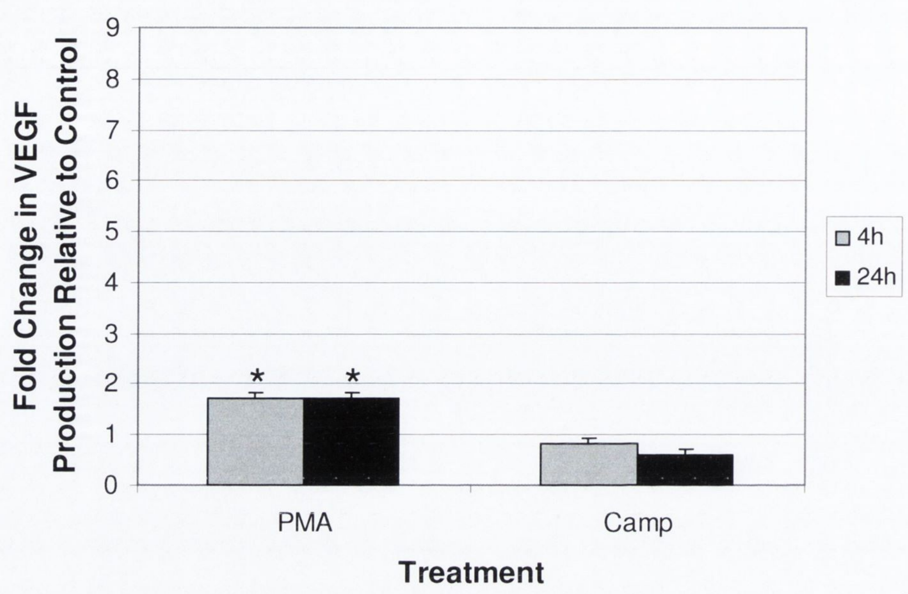


Figure 3.1.11 Baseline VEGF levels in BxPC-3 and AsPC-1 cells.

*Serum starved, confluent AsPC-1 and BxPC-3 cells were treated with Aspirin [200μM] for 45 min and then RPMI supplemented with 2.5% FCS for 4h or 24h. Supernatant was harvested for VEGF ELISA. Columns are means of at least 3 replicates ± SEM. *p<0.05 relative to BxPC-3 control.*

AsPC-1



BxPC-3

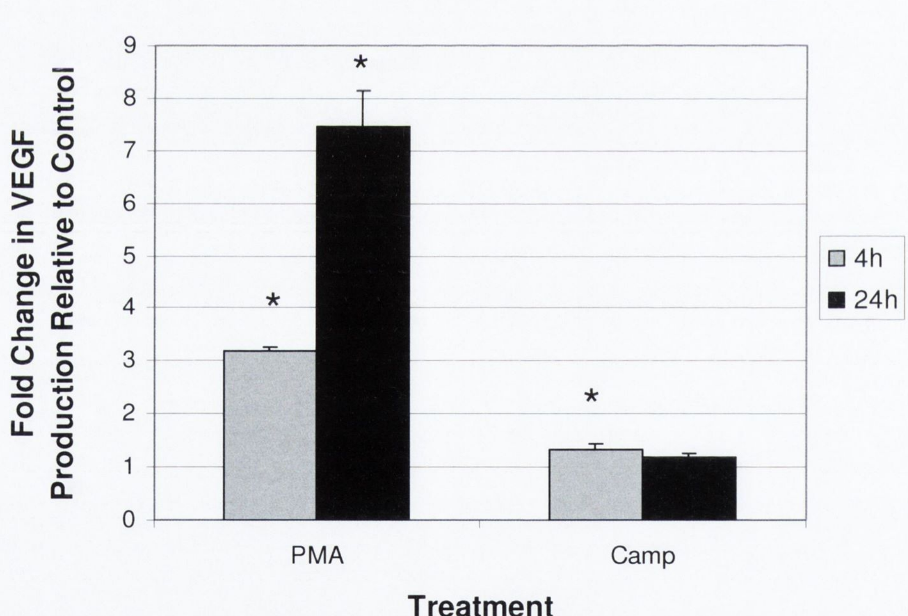


Figure 3.1.12 PMA and Camptothecin alter VEGF production.

*Serum starved, confluent AsPC-1 and BxPC-3 cells were treated with Aspirin [200μM] for 45 min and then PMA [1μM] or Camptothecin [2μM] for 4h or 24h. Supernatant was analysed for VEGF. Columns are means of at least 3 replicates ± SEM. *p<0.05 relative to control.*

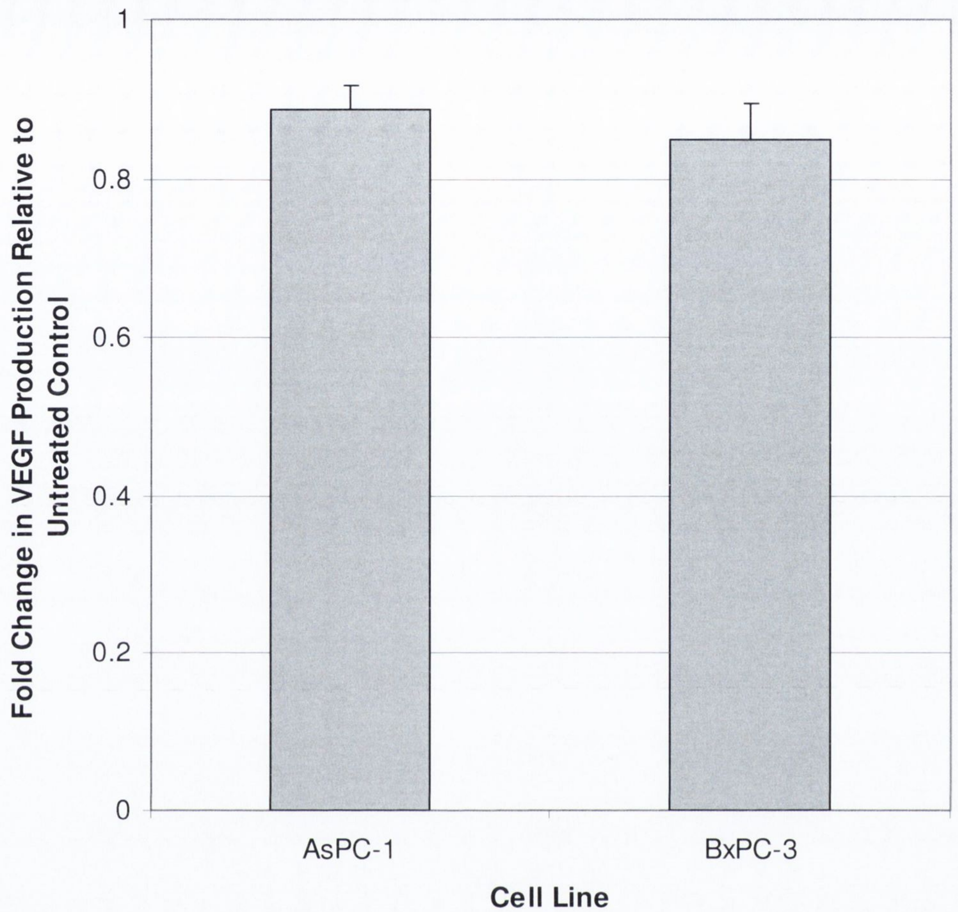


Figure 3.1.13 Changes in VEGF are not due to vehicle effect.

Serum starved, confluent AsPC-1 and BxPC-3 cells were treated with DMSO at a final concentration of [0.1% v/v] in RPMI supplemented with 2.5% FCS. The concentration of VEGF in the supernatant was then quantified by ELISA. Columns are means of at least 3 replicates ± SEM.

3.2. Endothelial Cell Proliferation / Survival Assay.

3.2.1 Proliferation / Survival Assay.

An *in vitro* assay of endothelial cell proliferation / survival using a co-culture system was developed. This mimics a crucial step in tumour angiogenesis where the endothelial cell must proliferate and survive in the tumour microenvironment until a new basement membrane is laid down (Fig. 3.2.1). This assay enabled investigation of the role of COX-2 in this process.

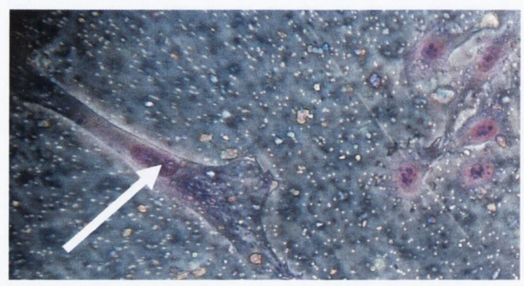


Figure 3.2.1 HUVEC Proliferation.

HUVECs co-cultured with BxPC-3 cells and stained with Giemsa as in experiment depicted in Fig. 3.2.4. Ongoing HUVEC proliferation is evident (arrow).

3.2.2 COX-2.

Mean PGE2 was 2.4 times higher in the BxPC-3 wells (286 pg/ml) than in the AsPC-1 wells (120 pg/ml) ($p=0.059$)*. These levels are similar to those in controls without HUVECs (Fig. 3.1.10). NS398 reduced PGE2 to undetectable or barely detectable levels in each cell line ($p=0.054$)* (Fig. 3.2.2). This was not due to tumour cell death (Fig. 3.2.3). HUVEC controls (no tumour cells in lower chamber) had unexpectedly high levels of PGE2 that were comparable to those in BxPC-3 controls. These were significantly higher than the AsPC-1 controls ($p<0.05$).

Giemsa staining of HUVECs after 3 to 5 days of co-culture demonstrated that there were more viable HUVECs in COX-2 positive, BxPC-3 co-cultures than in COX-2 negative, AsPC-1 wells. However, COX-2 inhibition by NS398 did not affect HUVEC cell numbers (Fig 3.2.4). However, Giemsa staining did suggest that HUVECs co-cultured with BxPC-3 cells tended to behave in an angiogenic manner by migrating to form clusters and cords and NS398 treatment possibly reduced this behaviour (Fig. 3.2.5).

WST cell viability assay confirmed that there were twice as many viable HUVECs in BxPC-3 wells, when compared to AsPC-1 wells, after 3 to 5 days of co-culture ($p<0.05$). Furthermore, NS398 [1μM] did not alter HUVEC viability (Fig. 3.2.6).

3.2.3 Vascular Endothelial Growth Factor.

VEGF was also quantified in the supernatant from the lower chamber. Mean VEGF in the AsPC-1 wells was 910 pg/ml and in the BxPC-3 wells was 2370 pg/ml ($p<0.05$). Treatment with NS398 [1μM] did not change VEGF production in either cell line.

Treatment with VEGF Neutralising Antibody resulted in VEGF being generally undetectable in both AsPC-1 ($p=0.069$)* and BxPC-3 ($p<0.05$) co-cultures (Fig. 3.2.7). HUVEC controls (no tumour cells in bottom chamber) had no detectable VEGF. The VEGF neutralising antibody did not affect tumour cell viability (Fig. 3.2.3) nor PGE2 levels in the co-cultures (Fig. 3.2.8).

Giemsa staining showed a marked reduction in HUVEC's in BxPC-3 co-cultures treated with VEGF Neutralising Antibody. Cells also seemed less clustered together (Fig. 3.2.4). WST confirmed that BxPC-3 co-cultures treated with VEGF Neutralising Antibody had 48% fewer viable HUVECs than controls ($p<0.05$). This HUVEC viability level was comparable to that of AsPC-1 co-culture controls. This was not due to a direct cytotoxic effect on HUVECs (Fig. 3.2.9).

*See Chapter 2.12.

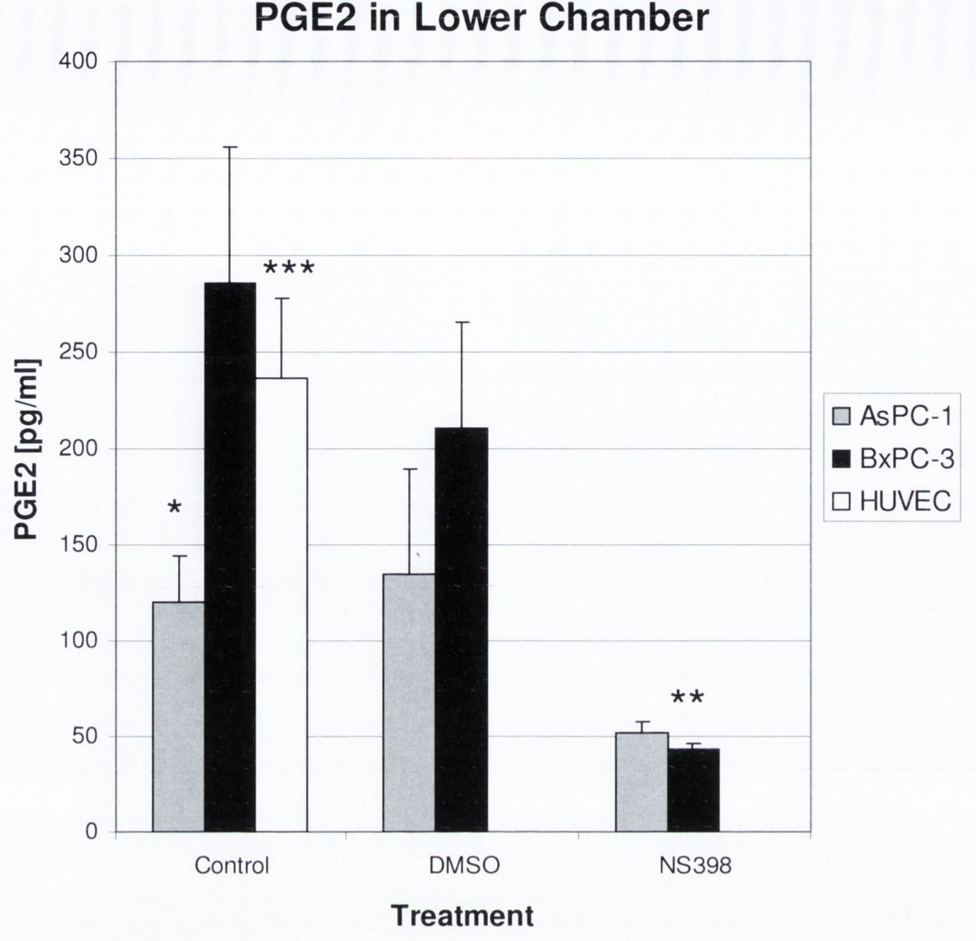


Figure 3.2.2 NS398 Inhibits PGE2.

*Confluent BxPC-3 and AsPC-1 cells, seeded in the lower chamber on the companion plate, were co-cultured (0.4μm porous membrane) with HUVECs (2.5 x 10^4 cells per insert). No tumour cells were seeded in HUVEC controls. DMSO [0.01% v/v] or NS398 [1μM] was added to the lower chamber. Supernatant from the lower chamber was harvested between day 3 and 5, depending on HUVEC viability, and PGE2 quantified by ELISA. Bars represent means of at least 3 replicates ± SEM. *p=0.059 or **p=0.054 relative to BxPC-3 control. ***p<0.05 relative to AsPC-1 control.*

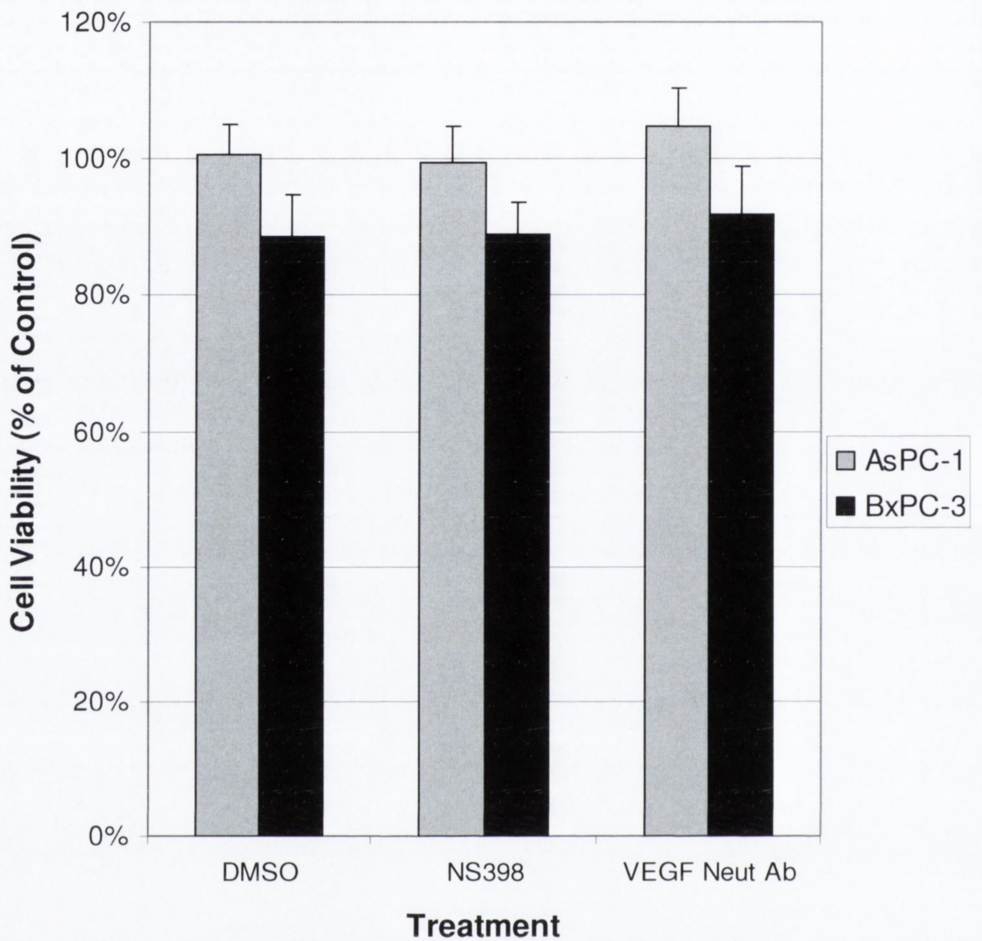


Figure 3.2.3 Decrease in intracellular messengers not due to tumour cell death.

Confluent BxPC-3 and AsPC-1 cells, seeded in the lower chamber on the companion plate, were co-cultured (0.4μm porous membrane) with HUVECs (2.5 x 10^4 cells per insert). DMSO [0.01% v/v], NS398 [1μM] or VEGF Neutralising Antibody [0.5 μg/ml] were added to the lower chamber. Supernatant from the lower chamber was harvested between day 3 and 5, depending on HUVEC viability, and tumour cell viability quantified by MTT assay and expressed as percentage of control. Bars represent means of at least 3 replicates ± SEM.

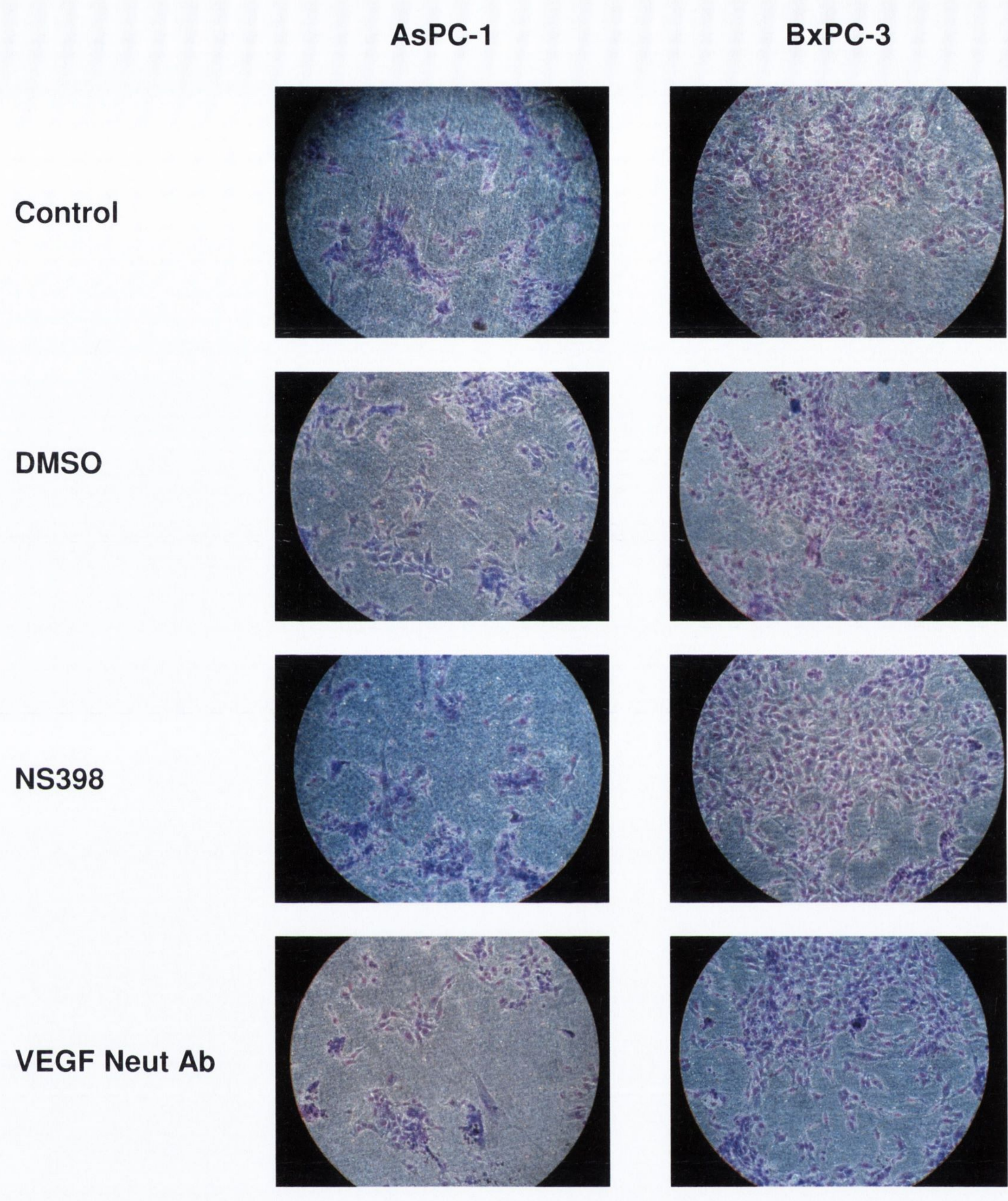
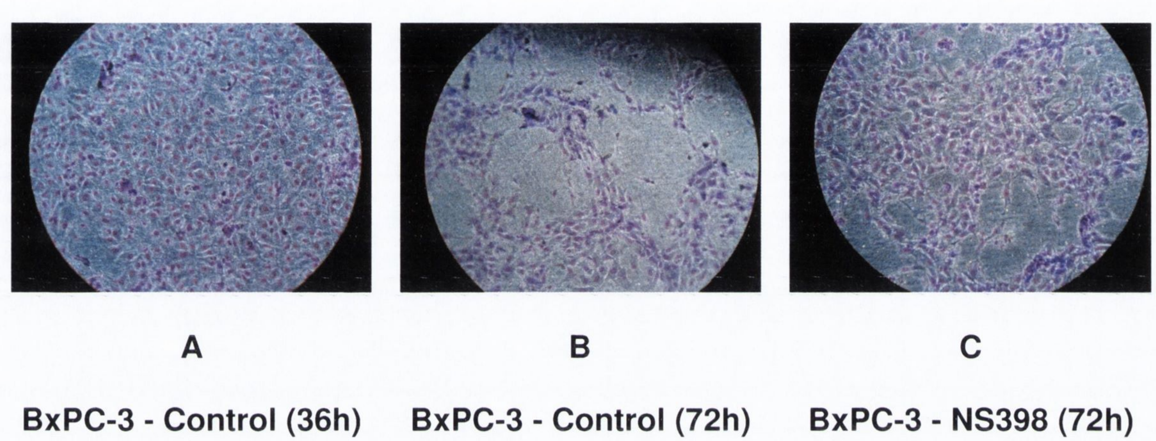


Figure 3.2.4 HUVEC proliferation and morphology.

Figure 3.2.4 HUVEC proliferation and morphology.

Confluent BxPC-3 and AsPC-1 cells, seeded in the lower chamber on the companion plate, were co-cultured (0.4μm porous membrane) with HUVECs (2.5 x 10^4 cells per insert). DMSO [0.01% v/v], NS398 [1μM] or VEGF Neutralising Antibody [0.5 μg/ml] was added to the lower chamber in RPMI supplemented with 2.5% FCS. Medium was removed at 72h, HUVECs fixed in methanol, stained with Giemsa and photographed at 200X. Images are from a single experiment and representative of 3 replicates.



BxPC-3 - Control (36h) **BxPC-3 - Control (72h)** **BxPC-3 - NS398 (72h)**

Figure 3.2.5 Angiogenic activity by HUVECs.

HUVECs co-cultured with BxPC-3 cells and stained with Giemsa from experiment depicted in Fig. 3.2.4. Initially HUVECs form a homogenous sheet of cells at 36h (A) but then redistribute into cord like structures at 72h (B). NS398 [1μM] reduces angiogenic behaviour (C).

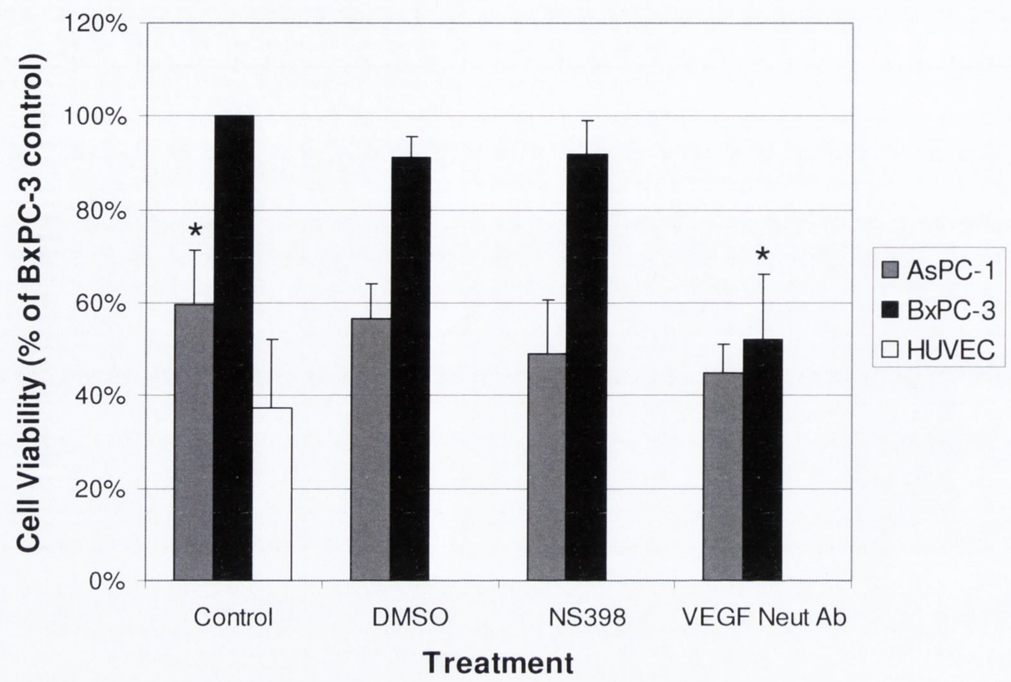


Figure 3.2.6 NS398 did not affect HUVEC viability.

*Confluent BxPC-3 and AsPC-1 cells, seeded in the lower chamber on the companion plate in RPMI supplemented with 2.5% FCS, were co-cultured (0.4μm porous membrane) with HUVECs (2.5 x 10^4 cells per insert) in M199 supplemented with 2.5% FCS. DMSO [0.01% v/v], NS398 [1μM] or VEGF Neutralising Antibody [0.5 μg/ml] was added to the lower chamber. Insert was removed at 72h – 120h and supernatant harvested. Cell viability was quantified by WST assay and expressed as a percentage of BxPC-3 control. Bars represent means of 3 replicates ± SEM. *p<0.05 relative to BxPC-3 control.*

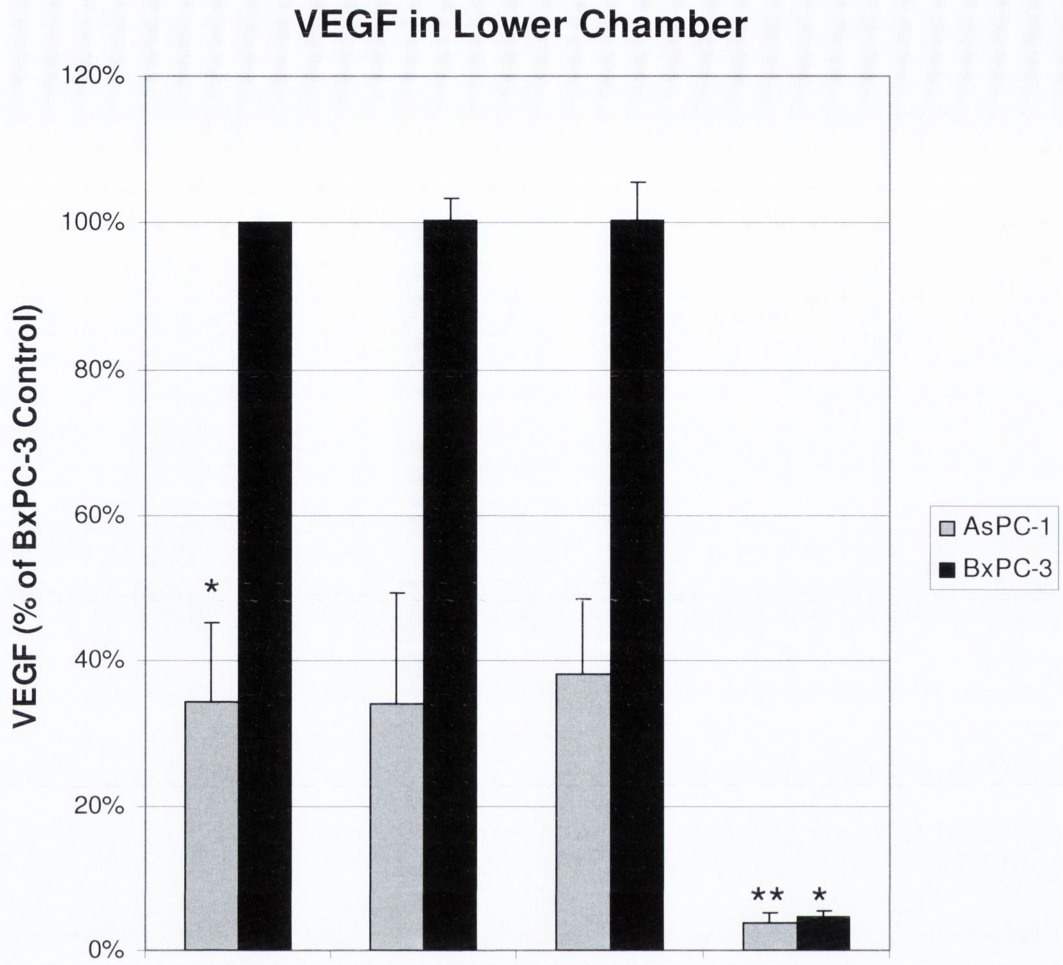


Figure 3.2.7 COX-2 inhibition does not reduce VEGF.

*Confluent BxPC-3 and AsPC-1 cells, seeded in the lower chamber on the companion plate, were co-cultured (0.4μm porous membrane) with HUVECs (2.5 x 10^4 cells per insert). Supernatant from the lower chamber was harvested between day 3 and 5, depending on HUVEC viability, and VEGF quantified by ELISA. Bars represent means of at least 3 replicates ± SEM. *$p<0.05$ relative to BxPC-3 control. **$p=0.069$ relative to AsPC-1 control.*

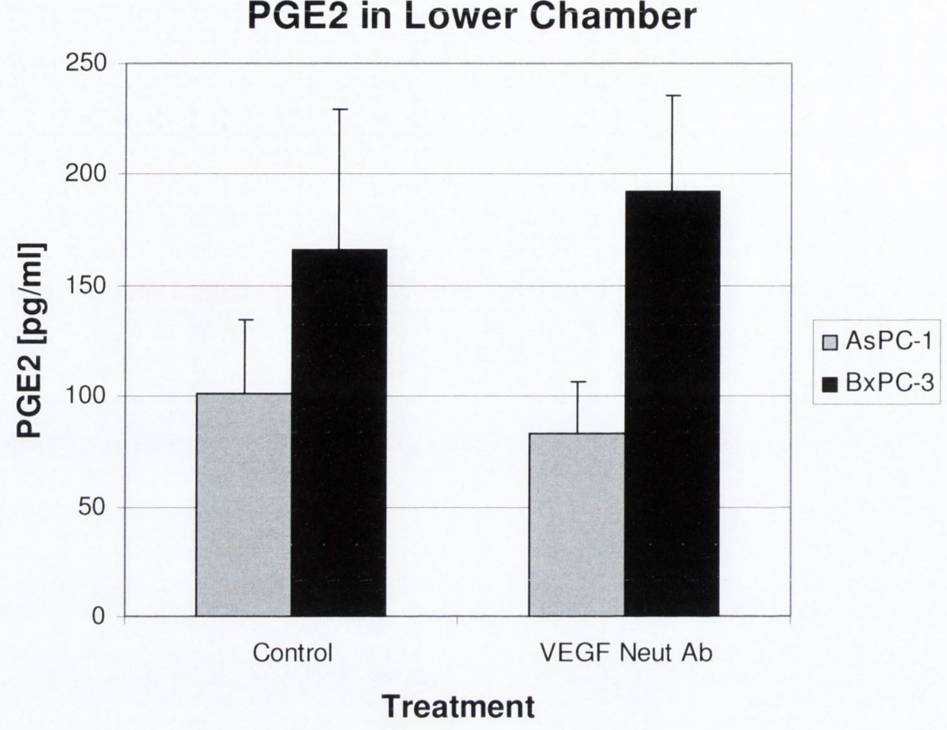
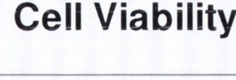


Figure 3.2.8 VEGF Neutralising Antibody did not reduce PGE2.

Confluent BxPC-3 and AsPC-1 cells, seeded in the lower chamber on the companion plate, were co-cultured (0.4μm porous membrane) with HUVECs (2.5 x 10^4 cells per insert). VEGF Neutralising Antibody [0.5μg/ml] was added to the lower chamber. Supernatant from the lower chamber was harvested between day 3 and 5, depending on HUVEC viability, and PGE2 quantified by ELISA. Bars represent means of 2 replicates ± SEM.



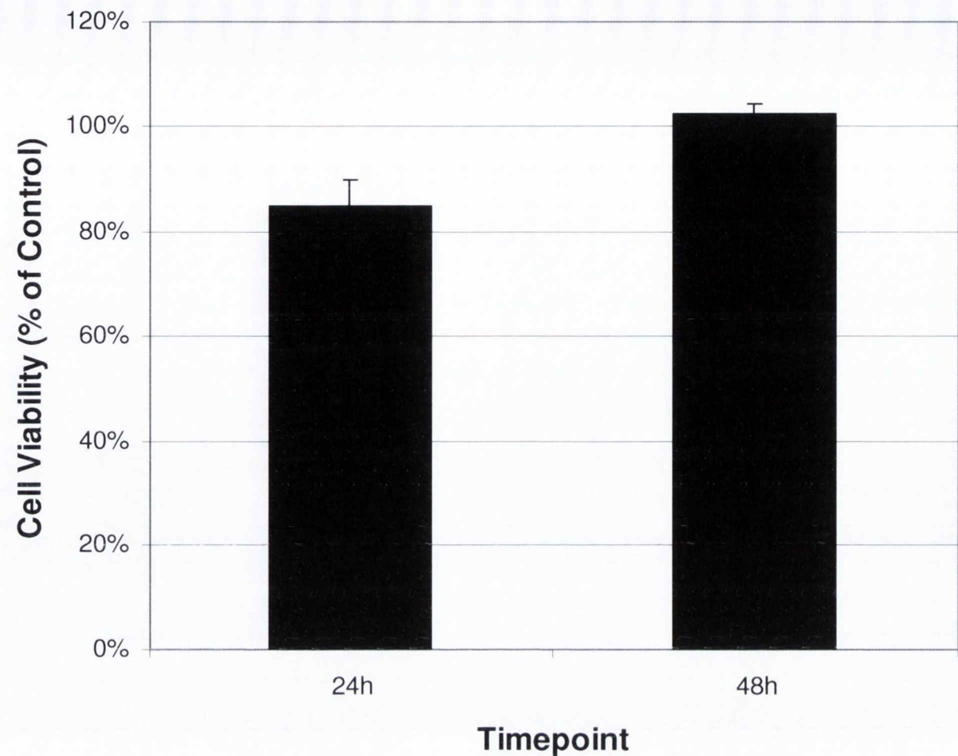


Figure 3.2.9 VEGF Neutralising Antibody is not directly toxic to HUVECs.

HUVECs, plated in 24 well plates, were treated with VEGF Neutralising Antibody [0.5μg/ml] in M199 supplemented with 2.5% FCS. Cell viability was measured at 24h and 48h by WST assay. Columns are means of 2 replicates ± SEM.

3.3. COX-2 Independent Effects of NSAIDs.

3.3.1 Introduction.

In Chapter 3.2 it was clearly evident that effective inhibition of COX-2, as demonstrated by reduction in PGE2 following treatment with NS398 [1μM], did not impact on VEGF levels. As other authors have demonstrated inhibition of VEGF production by high concentration NSAIDs, the COX-2 independent effects of two specific COX-2 inhibitors (NS398, Celecoxib) were investigated. A third reagent, OSU-03012, a Celecoxib derivative with reduced COX-2 inhibitory activity was also examined.

3.3.2 Cell Viability.

The vehicle, DMSO, had a consistent effect on cell viability at concentrations between 0.01% and 1% [v/v]. In sub-confluent, proliferating cells, viability was approximately 80% to 90% after 24h. DMSO had negligible effect on viability of confluent cells at 24h (Fig. 3.3.2).

In proliferating cells, Celecoxib had a concentration-dependent effect on cell viability in each cell line at 24h. BxPC-3 cells tended to be more sensitive than AsPC-1 cells although this did not reach significance at any of these concentrations. Celecoxib [50μM] resulted in cell viability of 66% in AsPC-1 cells ($p<0.05$) and 60% in BxPC-3 cells ($p<0.05$) at 24h. There was almost complete cell death following treatment with

Celecoxib [100μM] for 24h in each cell line ($p<0.05$). The LC_{50} for Celecoxib in each cell line was therefore between 50μM – 100μM (Fig. 3.3.3).

NS398 was also cytotoxic to proliferating cells at higher concentrations however the effect was not as marked as that seen with Celecoxib treatment. BxPC-3 cells treated with NS398 [100μM] had a cell viability of 51% at 24h ($p<0.05$). Similarly treated AsPC-1 cells were more resistant to the cytotoxic effects and had a cell viability of 70% ($p<0.05$). When compared, the difference in viability between these two cell lines following 24h treatment with NS398 [100μM] was significant ($p<0.05$). Therefore BxPC-3 cells are more sensitive than AsPC-1 and the LC_{50} for NS398 was >100μM in each cell line (Fig. 3.3.3).

OSU-03012 was 3-5 times more cytotoxic than Celecoxib. In AsPC-1 cells, OSU-03012 [10μM] had a significant effect on cell death with a cell viability of 62% at 24h ($p<0.05$). When AsPC-1 cells were treated with OSU-03012 [20μM] there was marked cell death and viability was reduced to 30% ($p<0.05$). BxPC-3 cells were more resistant at concentrations up to 15μM however the effect of treatment with OSU-03012 [20μM] was similar to that seen in AsPC-1 cells with a cell viability of 33% ($p<0.05$). Therefore the LC_{50} for OSU-03012 was between 15μM and 20μM in both AsPC-1 and BxPC-3 cells (Fig. 3.3.4).

To investigate the mechanism of OSU-03012 induced cell death and its potential to target tumour cells, its effects on proliferating and non proliferating BxPC-3 cells were compared. This cell line was chosen as it quickly reaches a confluent monolayer and stops proliferating (Fig. 3.3.1).

Proliferating BxPC-3 cells were more susceptible to the cytotoxic effects of OSU-03012 than non proliferating cells (Fig. 3.3.5). As proliferating cells were also more susceptible to vehicle effects (Fig 3.3.2), cell viability following treatment with OSU-03012 [20μM] was normalised to the appropriate DMSO control for comparison. Normalised cell viability of proliferating cells was 46% but viability of non proliferating cells was significantly higher at 95% ($p<0.05$).

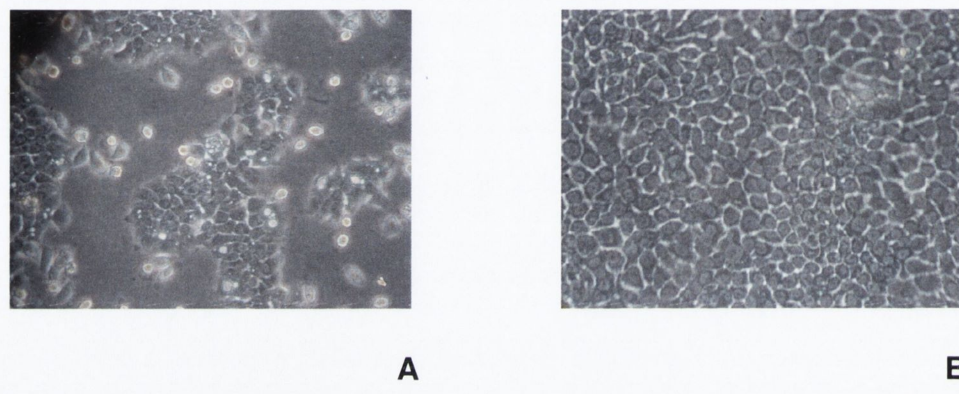
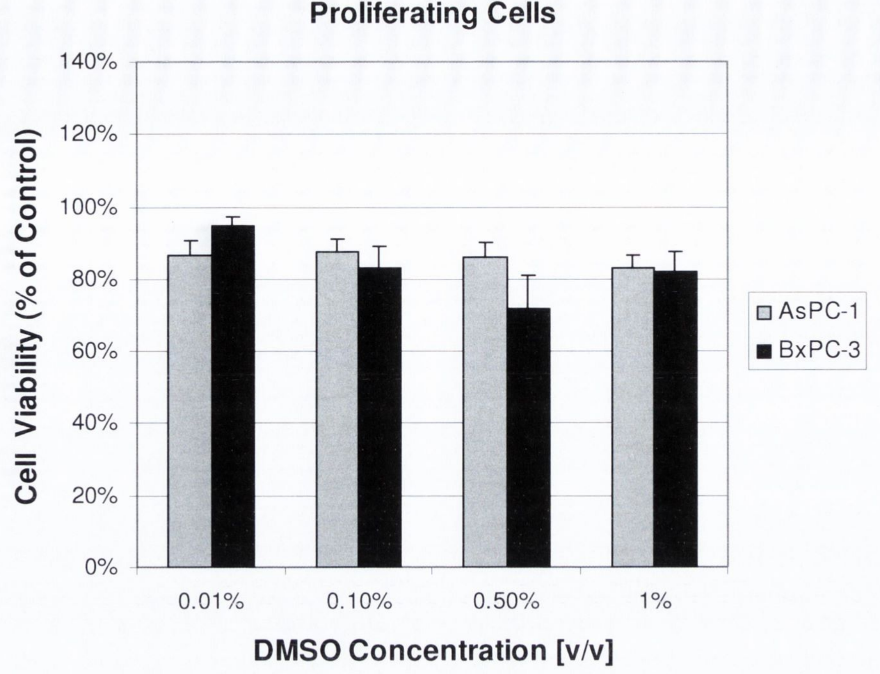


Figure 3.3.1 Proliferating and Non Proliferating BxPC-3 Cells.

BxPC-3 cells were counted by haemocytometer and plated in 24 well plates at 1.5 x 10^5 cells per well (A) to ensure they were sub-confluent and proliferating. To ensure they were confluent and non-proliferating, BxPC-3 cells were plated in excess of 5 x 10^5 per well (B).



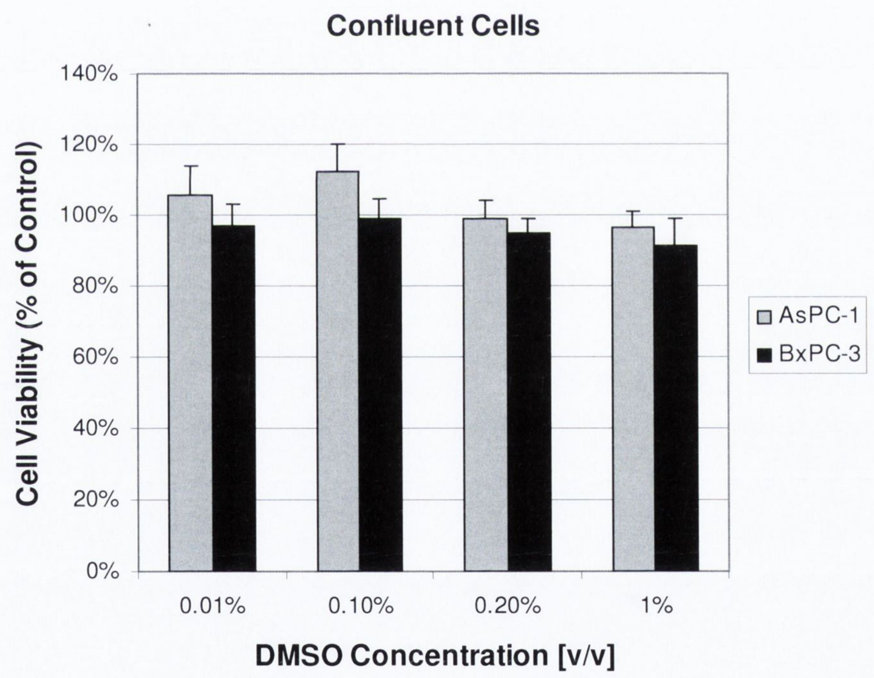
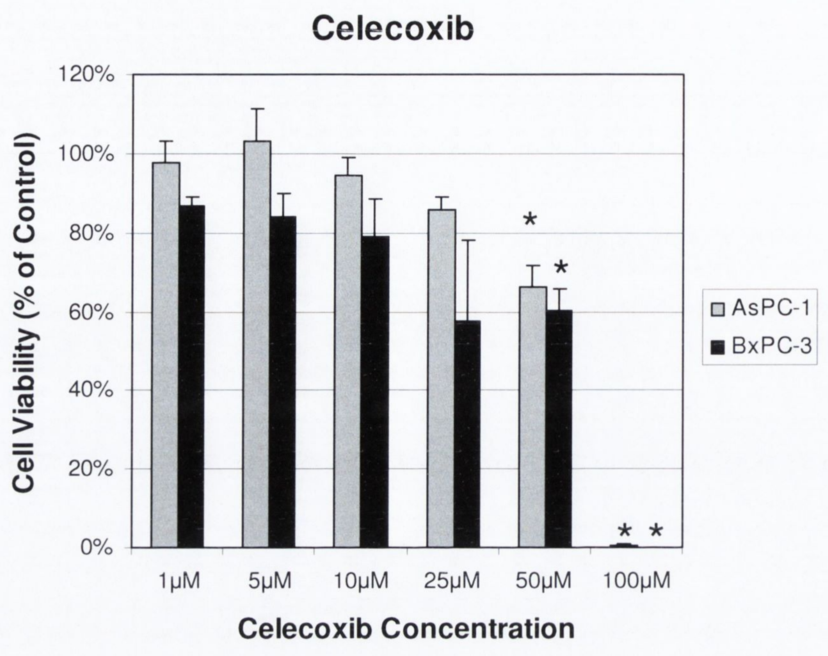


Figure 3.3.2 No concentration effect of DMSO on cell viability.

Serum starved, proliferating (top) or confluent (bottom) AsPC-1 and BxPC-3 cells were treated with increasing concentrations of DMSO for 24h. Cell viability was determined by MTT assay. Columns are means of at least 3 replicates ± SEM.



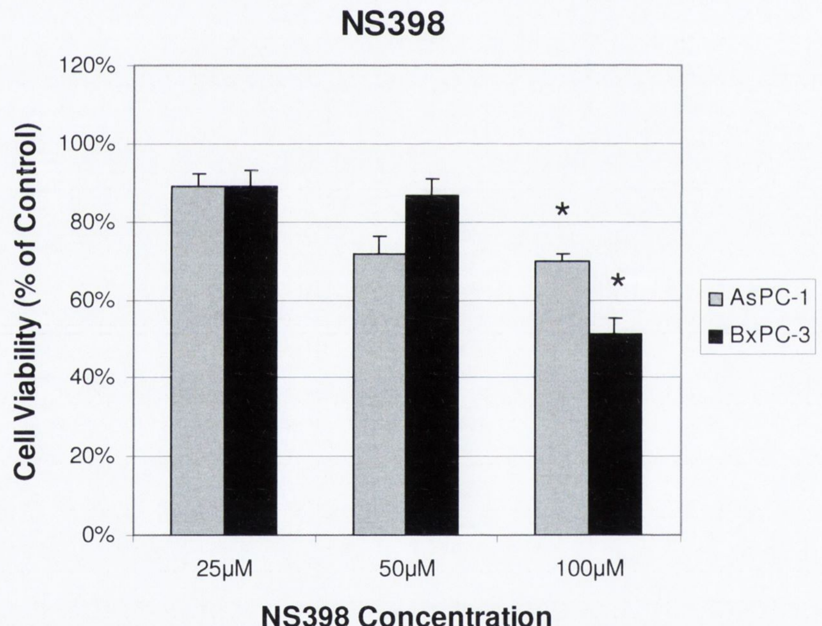
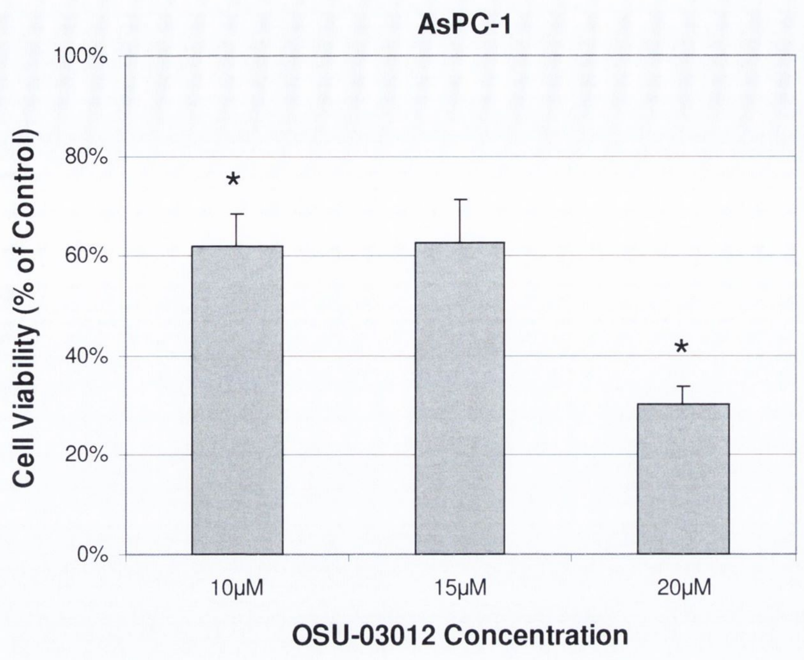


Figure 3.3.3 Concentration dependent cytotoxicity of Celecoxib and NS398.

*Serum starved, proliferating AsPC-1 and BxPC-3 cells were treated with increasing concentrations of Celecoxib (top) or NS398 (bottom) for 24h. Cell viability was determined by MTT assay. Columns are means of at least 3 replicates ± SEM. *p<0.05 relative to control.*



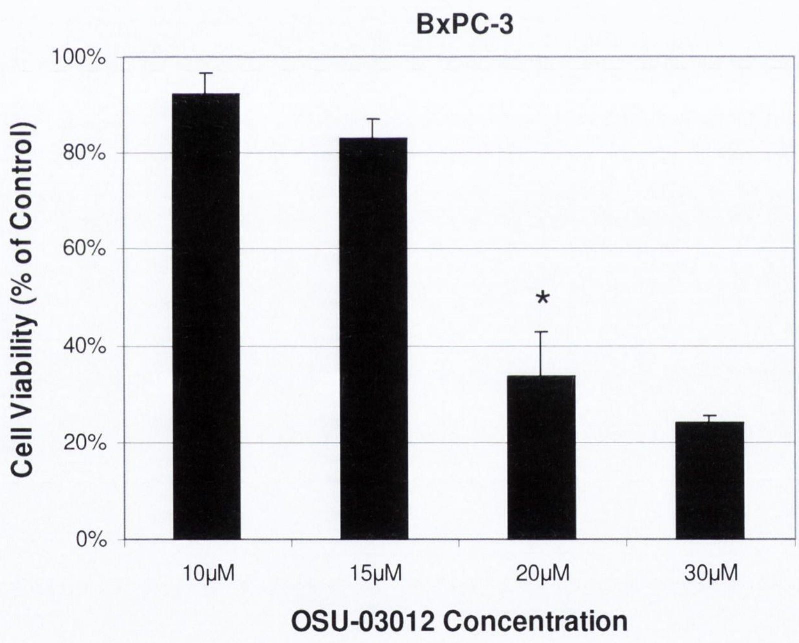
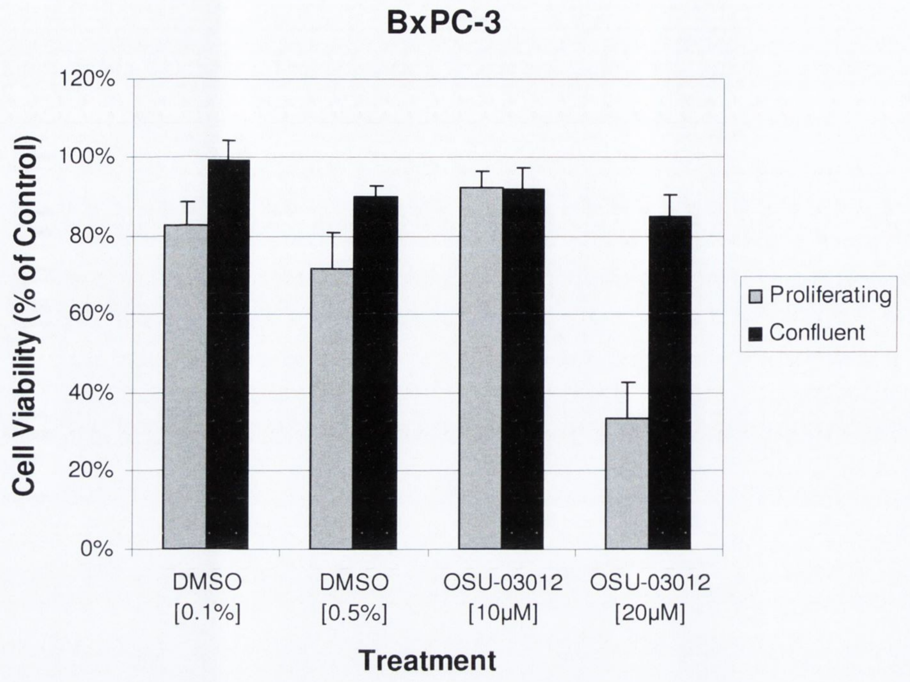


Figure 3.3.4 OSU-03012 is cytotoxic.

*Serum starved, proliferating AsPC-1 (top) or BxPC-3 (Bottom) cells were treated with increasing concentrations of OSU-03012 for 24h. Cell viability was determined by MTT assay. Columns are means of at least 3 replicates ± SEM. *p<0.05 relative to control.*



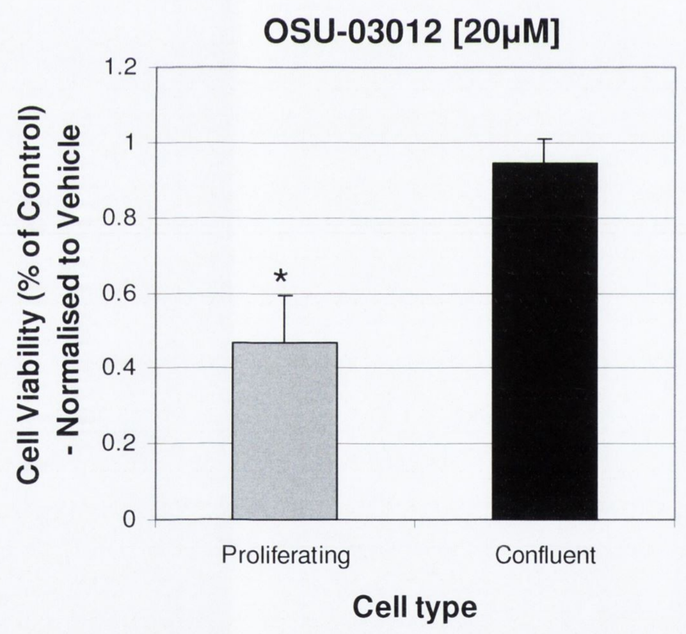


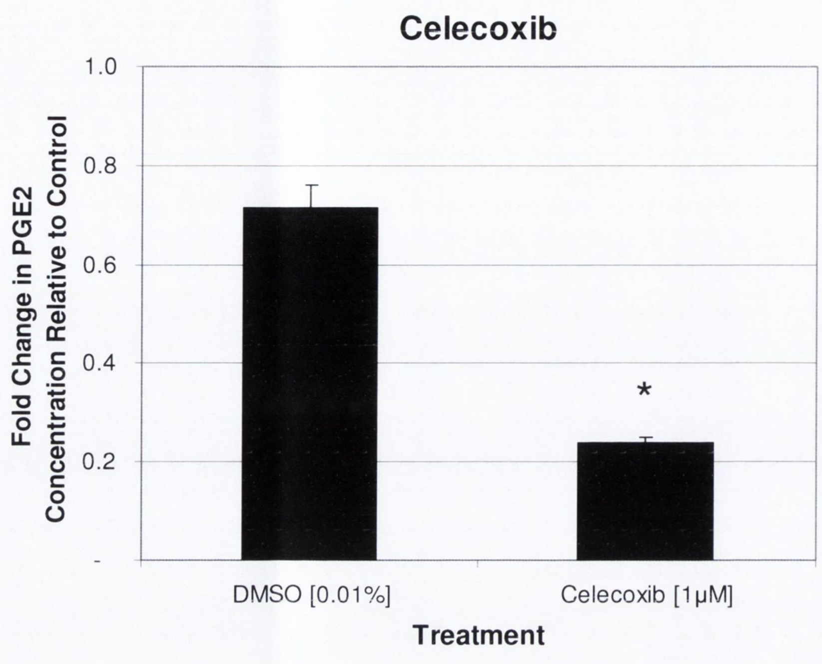
Fig 3.3.5 Non proliferating cells are resistant to cytotoxicity.

*Serum starved, confluent BxPC-3 cells were treated with increasing concentrations of OSU-03012 (top). Cell viability was determined by MTT assay and normalized to vehicle control (Bottom). Columns are mean of at least 2 replicates ± SEM. *p<0.05 relative to confluent cells.*

3.3.3 COX-2 Inhibition.

As shown in Chapters 3.1.5 and 3.2.2, NS398 [1μM] effectively inhibited COX-2 and significantly reduced PGE2 production. The ability of Celecoxib and OSU-03012 to inhibit COX-2 was investigated by quantifying PGE2 in supernatant from BxPC-3 cells (COX-2 positive) treated with each reagent.

Celecoxib [1μM] reduced PGE2 concentration by 64% relative to vehicle control ($p<0.05$). In contrast, despite increasing the concentration to 10μM, OSU-03012 only resulted in a 21% decrease that was not significant (Fig. 3.3.6).



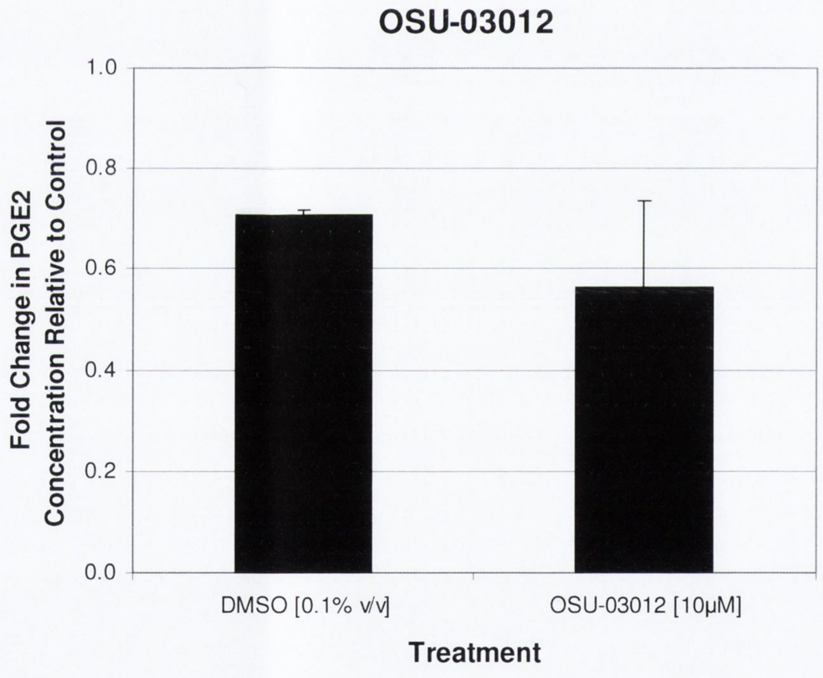


Figure 3.3.6 Celecoxib inhibits COX-2 but OSU-03012 does not.

*Serum starved, confluent AsPC-1 and BxPC-3 cells were treated with Celecoxib [1μM] or OSU-03012 [10μM] for 24h. PGE2 concentration in the supernatant was quantified by ELISA. Columns are means of at least 3 replicates ± SEM. *p<0.05 relative to control.*

3.3.4 VEGF Production.

The effect of each of the three reagents on VEGF concentration after 24hr was investigated. Mean VEGF levels in 24h controls were approximately 4 fold higher in BxPC-3 than in AsPC-1 cells (Chapter 3.1.6). However similar patterns of concentration-dependent effects were seen in the two cell lines. Results were not normalised to cell viability and so any increases in VEGF may be more profound.

Celecoxib increased VEGF levels in a concentration dependent manner in both AsPC-1 and BxPC-3 cells. In AsPC-1 cells Celecoxib [50μM] resulted in a 2 fold increase VEGF ($p<0.05$). In BxPC-3 cells a 1.3 fold increase was seen in response to Celecoxib [10μM], 1.6 fold following 25μM and 3.4 fold increase in cells treated with 50μM for 24h ($p<0.05$). (Fig. 3.3.7)

NS398, to a maximum concentration of 100μM, did not alter VEGF levels from either cell line. (Fig. 3.3.8).

OSU-03012 [20μM] had no significant effect on VEGF production in either cell line (Fig. 3.3.9). Higher concentrations of OSU-03012 could not be investigated due to marked cell death (Fig. 3.3.4).

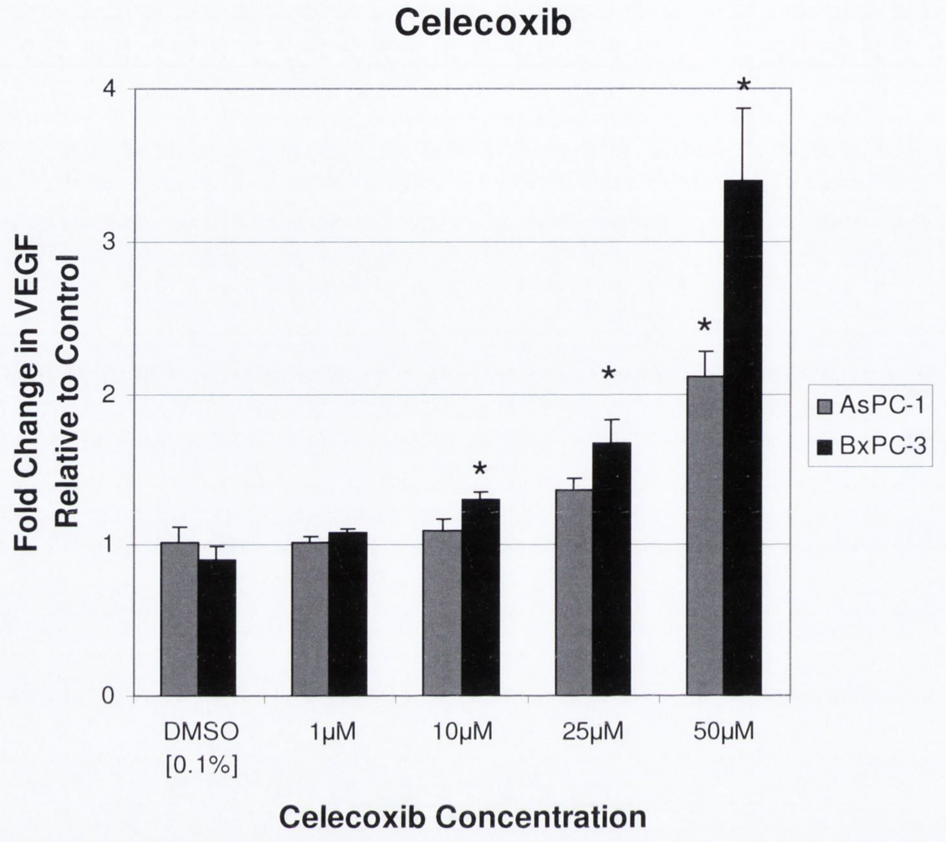


Figure 3.3.7 Celecoxib stimulates VEGF production.

*Serum starved, proliferating AsPC-1 and BxPC-3 cells were treated with increasing concentrations of Celecoxib for 24h. VEGF concentration in the supernatant was quantified by ELISA. Columns are means of at least 3 replicates ± SEM. *p<0.05 relative to control.*

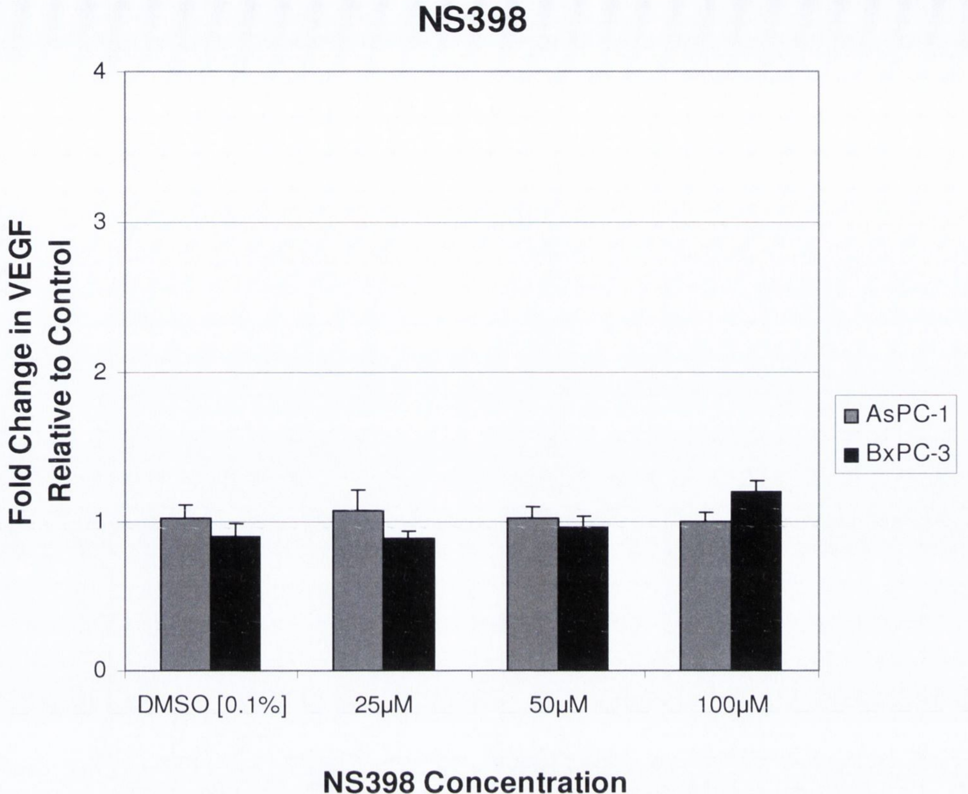


Figure 3.3.8 NS398 does not stimulate VEGF production.

Serum starved, proliferating AsPC-1 and BxPC-3 cells were treated with increasing concentrations of NS398 for 24h. VEGF concentration in the supernatant was quantified by ELISA. Columns are means of at least 3 replicates ± SEM.

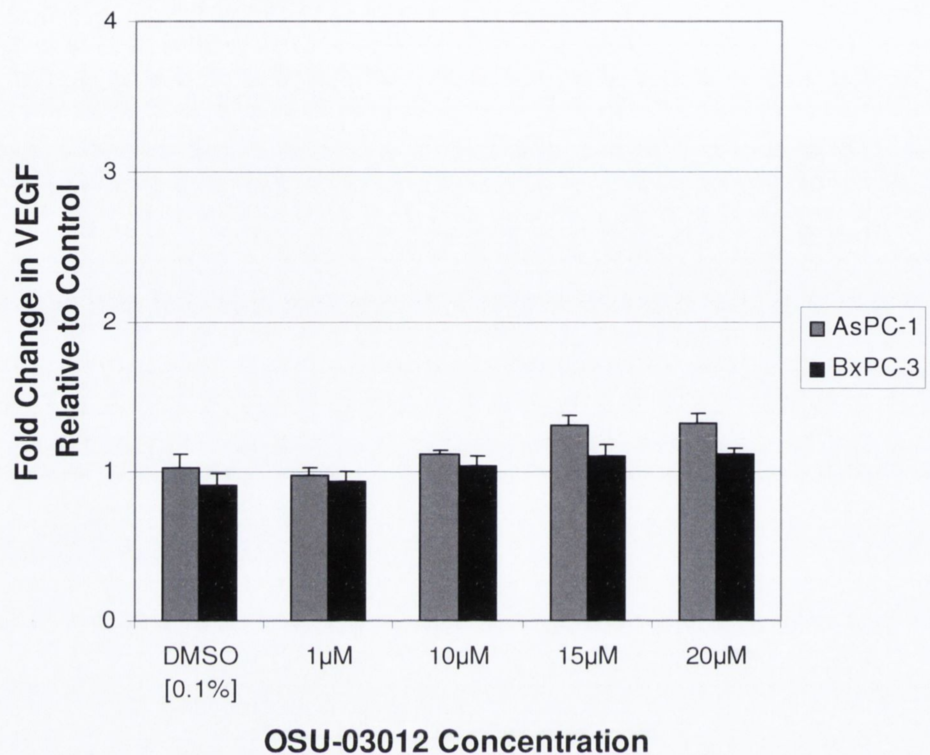


Figure 3.3.9 OSU-03012 does not stimulate increase in VEGF production.

Serum starved, proliferating AsPC-1 and BxPC-3 cells were treated with OSU-03012 for 24h. VEGF concentration in the supernatant was quantified by ELISA. Columns are means of at least 3 replicates ± SEM.

Chapter 4: Discussion.

COX-2, which is upregulated in pancreatic cancer, is strongly associated with worse prognosis.[23-26] In cancer, COX-2 has been definitively associated with decreased apoptosis and immune response and increased proliferation, invasion and angiogenesis.[18, 39, 41] Analysis of a prospective database of over 28,000 women found a significant trend towards decreased pancreatic cancer in regular NSAID users and estimated that 43% of pancreatic cancers in non NSAID users are potentially preventable.[7] Despite this, clinical trials of NSAIDs in pancreatic cancer have been very disappointing.[91, 92]

It is well accepted that angiogenesis is a viable target in cancer and as such it has therapeutic potential in pancreatic cancer.[49, 51] Although anti-angiogenic effects have been demonstrated *in vitro*, to date, NSAIDs have failed to impact on prognosis in pancreatic cancer.[91, 92] VEGF is a major, pro-angiogenic growth factor that has been linked to COX-2 in many cancers. VEGF and VEGFR2 are upregulated in pancreatic cancer and are associated with increased metastases and worse prognosis.[93, 94] Despite promising pre-clinical results, Bevacizumab, a VEGF inhibitor, failed to show any benefit in a major clinical pancreatic cancer trial.[95] This highlights the need to accurately delineate the biochemical mechanisms that stimulate tumour angiogenesis so that effective, targeted therapies can be developed. Our project developed an *in vitro* model to investigate the role of COX-2 in pancreatic cancer angiogenesis and in particular its regulation of VEGF. Furthermore, having found that NSAIDs could dysregulate

VEGF independent of COX-2, this project examined the COX-2 independent effects of NSAIDs and the therapeutic potential of a novel Celecoxib derivative in pancreatic cancer.

Two permanent pancreatic adenocarcinoma cell lines were selected. Their COX-2 status was verified by exposing them to pro-inflammatory and pro-apoptotic stimuli. High concentrations of each reagent were chosen to ensure that AsPC-1 cells were intensely stimulated. BxPC-3 cells had high levels of COX-2 mRNA and this was augmented by both PMA and Camptothecin. A similar pattern was demonstrated in COX-2 protein expression ruling out any significant post-transcriptional regulation. In contrast, AsPC-1 cells had virtually undetectable COX-2 mRNA and no detectable COX-2 protein under either condition.

COX-2 enzyme activity was also determined in each cell line. Cells were saturated with arachadonic acid, the substrate for COX, and its conversion into PGE2 assayed. Treatment with NS398 prior to arachadonic acid separated COX-1 activity from that of COX-2. The results correlated with mRNA expression and protein levels. AsPC-1 cells showed no COX-2 activity whereas BxPC-3 cells had markedly higher baseline PGE2 levels that were augmented by PMA and Camptothecin. NS398 inhibition clearly delineated this as COX-2 activity.

In control cells these results were statistically significant. In treated BxPC-3 cells, the differences in PGE2 concentration failed to reach significance. However, the narrow range of the PGE2 ELISA kit frequently resulted in readings that were ">max". In this situation the maximum value of the ELISA range was applied to that sample as a conservative estimation of the true value. Furthermore, the narrow range necessitated different dilutions of control and treated samples thus disproportionately magnifying some errors. Despite this, a definite pattern was evident that correlated with both the PCR and Western Blot data. This allowed the conclusion that BxPC-3 are strongly COX-2 positive and AsPC-1 are COX-2 negative. Several other studies have drawn the same conclusion.[61, 67, 70]

AsPC-1 and BxPC-3 cells were then used in the co-culture model of angiogenesis. Initially high concentrations of FCS were used in the media. Although this did not alter the effect of reagents on growth factor production, it stimulated the endothelial cells (already activated when separated from their basement membrane) to proliferate to confluency. This masked the effect of altering growth factor concentrations on endothelial cell viability. To prevent this, the assay was performed using medium supplemented with 2.5% FCS. As endothelial cells apoptose in a serum depleted environment,[48] this resulted in HUVEC cell numbers decreasing during the course of the experiment despite a degree of ongoing proliferation evident on Giemsa staining. Hence, the assay measured the tumour cells'

influence on the endothelial cell proliferation / apoptosis balance. This mirrored the physiological environment. Once activated *in vivo*, the proliferating endothelial cells separate from their basement membrane and migrate into the tumour. Here growth factors must stimulate their ongoing survival until a new basement membrane is laid down and vascular support cells migrate to the maturing vessel.[46, 47, 50]

Several authors have demonstrated the pro-angiogenic role of COX-2 and prostaglandins in endothelial cell migration and tubule formation.[60, 61, 63, 64] Similarly, in our model, Giemsa staining, although not quantative, did support these findings that COX-2 inhibition can reduce endothelial cell clustering and cord formation. In contrast, the function of tumour cell COX-2 in endothelial cell proliferation and survival has been under investigated although Tsujii *et al.* (1998) does refer to tumour cell COX-2 having no effect on endothelial cell proliferation.[60, 61] We have shown that, despite HUVEC viability in the COX-2 positive co-cultures (BxPC-3) being twice that of the COX-2 negative co-cultures (AsPC-1), COX-2 inhibition had no impact on endothelial cell viability.

VEGF is thought to be the primary messenger responsible for activating endothelial cells and promoting their proliferation and survival.[48, 50] BxPC-3, the COX-2 positive cell line, produced markedly more VEGF than AsPC-1. As COX-2 inhibition did not reduce endothelial cell viability, its effect on VEGF concentrations in the co-

culture was investigated. COX-2 inhibition did not affect VEGF production. This explains why NS398 did not reduce endothelial cell viability (Fig. 4.1). To confirm this, a VEGF neutralising antibody was added to the lower chamber. This inactivated all VEGF in the assay and dramatically reduced endothelial cell viability. A subsequent assay on endothelial cells showed no direct cytotoxic effect of this antibody confirming that the response in the co-culture was due to inhibition of tumour – endothelial interaction.

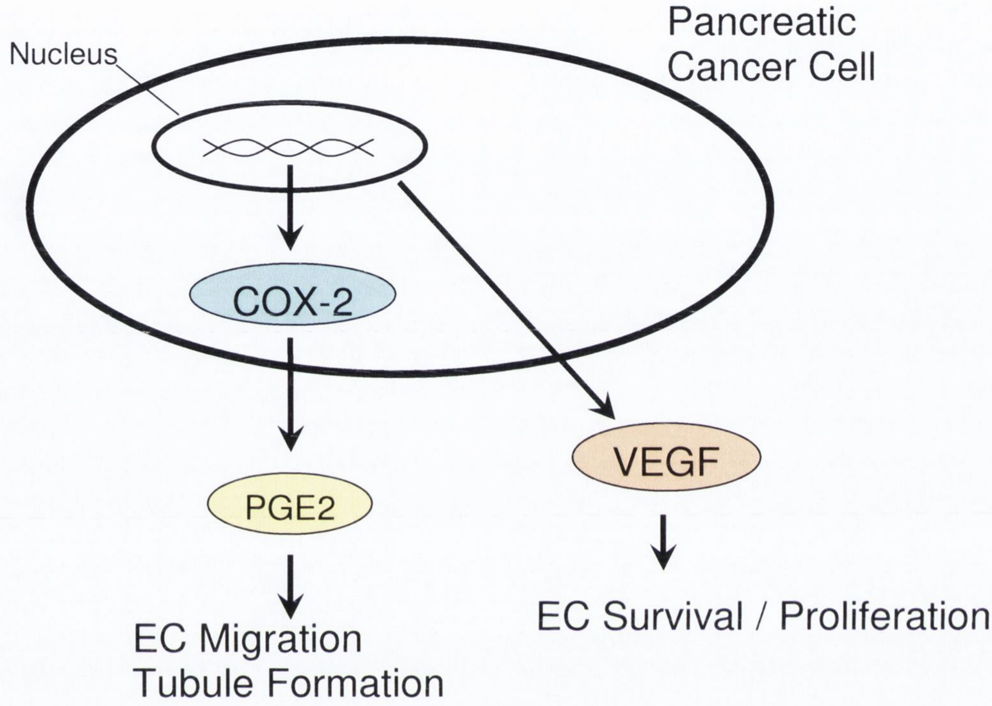


Figure 4.1 VEGF is not downstream of COX-2.

Upregulation of COX-2 and downstream PGE2 are not responsible for upregulation of VEGF in pancreatic cancer cells. VEGF and not COX-2 stimulates EC viability in the tumour micro-environment.

COX-2 – Cyclooxygenase-2; PGE2 – Prostaglandin E2; VEGF – Vascular Endothelial Growth Factor; EC – Endothelial Cell; NSAIDs – Non Steroidal Anti-inflammatory Drugs.

In immunohistochemical studies on clinical specimens[12, 20, 22, 27, 28], correlation and co-localisation of COX-2 and VEGF does not indicate a causative relationship as a common upstream regulator or two parallel, independent pathways would produce the same results. Of note, these studies do not refer to pancreatic cancer. Studies in which COX-2 negative cancer cells are transfected with COX-2 have definitively shown that upregulated COX-2 can upregulate VEGF however this does not necessarily mean that it does so in nature.[28, 60] Multiple pathways are likely. Our findings that PMA upregulates VEGF independent of COX-2 (in AsPC-1) and that COX-2 inhibition does not downregulate VEGF (in BxPC-3) clearly demonstrate that COX-2 and VEGF are parallel or independent of each other in these two cell lines.

In this study, inhibition of VEGF, but not COX-2, tipped the endothelial cell proliferation / survival balance and Giemsa stains supported the hypothesis that, as found by other authors,[60, 61] COX-2 is important in migration and tubule formation. This potentially, at least in part, explains the failure of Bevacizumab and NSAIDs in clinical pancreatic cancer. In this aggressive disease, if they each target different steps of the angiogenic cascade, in isolation they may not reach the angiostatic threshold required to see a clinical benefit. We were unable to find any clinical cancer studies that combined treatment of VEGF and COX-2 inhibitors. Furthermore, trials of Bevacizumab are performed in advanced pancreatic cancer which would already have established tumour vasculature whose basement membranes facilitate endothelial

cell survival and negate the need for VEGF. It may therefore have a more significant role in early disease or the adjuvant setting.

The relatively high PGE2 in HUVEC controls relative to AsPC-1 controls remains an enigma. It is possible that this cell line has acquired a mutation that upregulates an angiostatic factor (perhaps angiopoietin-2 or a similar substance). This would counteract the inherent activation of HUVECs that are separated from their basement membrane. Whatever the cause, this finding indicates a role for stromal/endothelial COX-2 in angiogenesis and highlights the importance of models that accurately reflect all elements of the physiological environment – both tumour and host.

The conclusion that COX-2 does not regulate VEGF production is contrary to that of many other studies in various cancers. Our findings do not explain how several authors have shown down regulation of VEGF by NSAIDs. Closer examination of the methodology of these studies reveals that they used high concentrations of NSAIDs markedly in excess of that required to inhibit COX-2.[57, 61, 71, 79, 96] The majority of these studies did not investigate pancreatic cancer. This leads to the hypothesis that NSAIDs also act at COX-2 independent targets.

To investigate this further, two specific COX-2 inhibitors were selected. NS398 is a laboratory based COX-2 inhibitor used in the previous

experiments and Celecoxib is a clinical COX-2 inhibitor with preventative properties in gastrointestinal cancers, especially colorectal cancer and FAP.[18, 33] A third agent selected was OSU-03012. This is a derivative of Celecoxib that has an enhanced pro-apoptotic profile in several tumour types and minimal COX-2 inhibitory activity.[86]

Each of the cell lines was treated with increasing concentrations of the three reagents and their cytotoxicity measured. NS398 was the least cytotoxic (LC_{50} >100μM). Celecoxib, in line with previous clinical experience,[33] had a more cytotoxic profile (LC_{50} 50μM – 100μM). Celecoxib completely killed both tumour cell lines at 100μM. OSU-03012 was markedly more cytotoxic in each cell line (LC_{50} 15μM – 20μM). This degree of cytotoxicity is less than that reported by other authors.[84, 86, 87] Our study used relatively high FCS levels (2.5%) and serum is thought to interfere with the efficacy of these drugs.[88] In clinical practice, serum and interstitial proteins may reduce the bioavailability of drugs and so this model more closely represents the tumour microenvironment than serum free media does. The relatively sustained efficacy of OSU-03012 in this assay therefore holds significant promise for clinical trials.

As discussed previously, OSU-03012 is thought to act on several biochemical pathways including Akt phosphorylation.[77, 78, 84, 87, 88, 90] At lower concentrations it was significantly more cytotoxic to the AsPC-1

cells than BxPC-3 cells. AsPC-1 cells have been reported to upregulate Akt in an oncogenic manner.[84] Conversely Celecoxib trended towards increased apoptotic activity in BxPC-3 cells relative to AsPC-1 cells, perhaps suggesting that it synergistically inhibited COX-2 and other targets. This implies that the role of COX-2 should not be disregarded and future therapies should include it in their target list.

OSU-03012 was also investigated on proliferating and non proliferating BxPC-3 cells. Its cytotoxicity was significantly higher in proliferating cells. This result is promising for future clinical studies as it indicates a tendency for the drug to target the rapidly proliferating tumour cells seen in this aggressive disease.

The COX-2 inhibitory activity of the three reagents was determined by measuring PGE2. Both NS398 and Celecoxib inhibited COX-2 at 1μM however, OSU-03012 showed no significant inhibition at 10μM (higher concentrations of OSU-03012 were cytotoxic to the BxPC-3 cell line). These results reinforce the theory that, at high concentrations, these reagents are acting in a COX-2 independent manner.

The effect of high concentration NSAIDs on VEGF production is controversial. It is generally accepted that they reduce VEGF.[18, 67] However, while Chu *et al.* (2003) showed that NS398 [50μM] decreased VEGF by 20% in BxPC-3, Raut *et al.* (2004) and Eibl *et al.* (2005) showed an increase in VEGF from BxPC-3 with NS398

[100μM] and Nimesulide [100μM], respectively.[61, 70, 80] More worryingly an *in vivo* study in pancreatic cancer demonstrated stimulation of angiogenesis and tumour growth[80], and a clinical study in breast cancer showed increased serum VEGF, in response to systemic NSAIDs.[81] Our project found that while NS398 had no effect on VEGF, Celecoxib increased its production in both cell lines. The concentration at which this was seen was less than that required for apoptosis. The mechanism for how this occurs is not understood but Eibl *et al.* (2005) showed that Nimesulide can stimulate PPARγ and hence downstream VEGF production.[80] Another possibility is that the tumour cell reacts to the blockade of the Akt response to hypoxia by upregulating other pathways that result in growth factor production. These discrepancies demonstrate the urgent need to understand the biochemical mechanisms involved in pancreatic cancer angiogenesis and how the response to NSAIDs is likely to differ significantly between tumours. OSU-03012 stimulated less VEGF production than Celecoxib at similar concentrations but had markedly more cytotoxic ability. We could find no evidence that this has been investigated before. This pro-angiogenic response to NSAIDs at concentrations below that needed for apoptosis could help explain their failure in the clinical treatment of this disease.

This project has shown that multiple pathways, both COX-2 dependent and independent, are involved in regulation of pancreatic tumour angiogenesis. Stark differences between our findings and those in

other GI malignancies[97] highlight the danger of assuming that mechanisms from other cancers transfer to pancreatic cancer. Clear delineation of pancreatic mechanisms is required to develop targeted agents against both angiogenesis and tumour proliferation. Further research to model, *in vitro,* other elements of angiogenesis and to incorporate stromal cells (macrophages, neutrophils, etc) will improve understanding of the tumour – host interplay and the messengers involved. Our group is currently undertaking such a project that utilises the co-culture model. Use of gene expression arrays and, in the future, proteomic arrays will both validate the existing targets and identify others to aid the development of synergistic therapies. An obvious limitation of our study is that it uses two commercially available permanent cell lines however the results show definite interactions and patterns that will enable design of further studies. Animal models, using xenographed, resected human tumours, will be crucial to confirming biochemical pathways and determining the clinical potential of new agents and combination therapies in advance of clinical trials. This aggressive cancer is often advanced at presentation and so a determined effort is required to recruit patients with early stage disease who may be more responsive to treatment.

NSAID derivatives, and especially OSU-03012, are an exciting development. With their cytotoxic efficacy they should be effective at physiological concentrations without the detrimental effects of growth factor upregulation. Furthermore, by decoupling these effects from

COX-2 inhibition they may offer a solution to the cardiovascular side effects that have plagued the use of NSAIDs in other cancers. Our data shows that COX-2 is important in pancreatic cancer angiogenesis but does not regulate VEGF and that NSAIDs have viable COX-2 independent targets. This suggests that triple therapy, targeting VEGF, COX-2 (low dose COX-2 specific NSAIDs) and COX-2 independent targets (NSAID derivatives), in combination with existing or developing anti-neoplastic therapies, may impact on tumour growth and angiogenesis and thus improve survival in pancreatic cancer (Fig. 4.2).

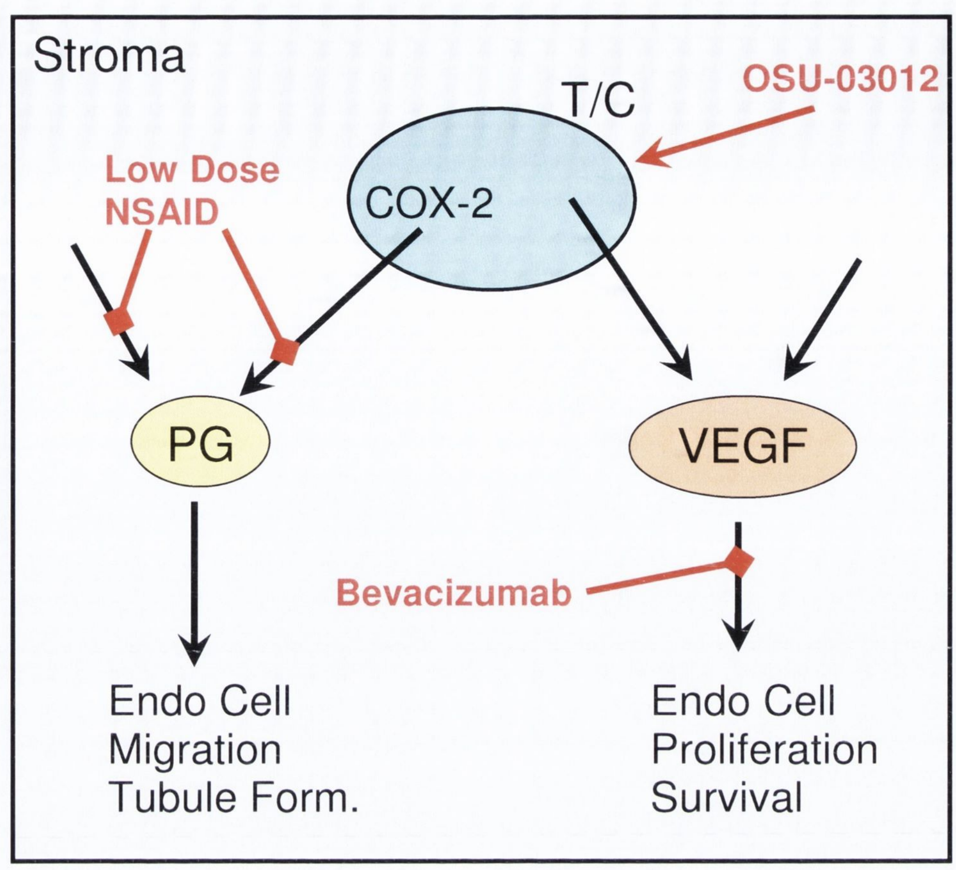


Figure 4.2 Potential Synergistic Therapies.

A combination of a low dose NSAID (to inhibit tumour cell and stromal COX-2 without detrimental effects), a Celecoxib derivative (to specifically target beneficial, COX-2 independent targets) and a VEGF inhibitor (as it is not regulated by COX-2) has potential in pancreatic cancer.

COX-2 – Cyclooxygenase-2; PG – Prostaglandins; VEGF – Vascular Endothelial Growth Factor; T/C – Tumour Cell; Endo Cell – Endothelial Cell; NSAID – Non Steroidal Anti-Inflammatory Drug; Black Arrow – Stimulates; Red Arrow – Stimulates Apoptosis; Red Diamond – Inhibits.

<u>Appendix A.</u>

Telephone: 8730700 Fax: 8730932

The Rotunda Hospital, Dublin

Founded 1745 Incorporated by Royal Charter 1756

Not for Prescription Purposes

19th October, 2006.

Dr. Joseph Murphy,
Trinity Centre for Health Sciences,
The Adelaide & Meath Hospital
incorporating the National Children's Hospital,
Tallaght,
Dublin 24.

Re: Collection of Portion of Umbilical Cord for Research Purposes

Dear Dr. Murphy,

Thank you very much for attending the Research Ethics Committee Meeting this morning. Whilst the Committee has no ethical difficulty with the study progressing there are a number of points that should be finalised before the study commences.

We need to have some indication as to the numbers you intend to study and the exclusions and inclusions of patients whose cords you will take.

It also should be borne in mind that even though these will be low risk cases occasionally the Pathology Department may need to retrieve the cord for further analysis. Under these circumstances the hospital should control the data linkage. Possibly you might link with both Dr. Gillan and Dr. Geary in order to ensure that this meets with Dr. Gillan's requirements and practicalities.

Finally you might also mention in the patient information leaflet that the cell lines will only have a life span of approximately one month and will have no commercial value. When these matters have been addressed and the Committee has been informed by you showing them the new protocol the study may proceed.

Yours sincerely,

Dr. Peter McKenna.
Chairman.
Research Ethics Committee.

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