INHIBITORS OF CYCLOOXYGENASE-2 (COX-2) AND PROSTATE CANCER: EFFECTS ON APOPTOSIS AND ROLE IN TUMOUR INHIBITION

By

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Summary

In comparison to other cancers, advanced prostate cancer is resistant to chemotherapy. There is a need to understand the mechanisms which are responsible for this resistance and find better treatments for this disease or methods to increase the efficacy of current treatments.

Cancer cells often evade apoptosis. Cyclooxygenase-2 (COX-2) is an enzyme reported to be elevated in prostate cancer, and has oncogenic properties, including apoptosis attenuation. Because of this, COX-2 inhibition could be beneficial for both prevention and treatment of cancer.

This study showed that COX-2 protein was not detected in LNCaP, PC-3 or DU145 cells. To assess the effect COX-2 has on the apoptotic sensitivity of prostate cancer cells, we transfected two cell lines (stable transfection in LNCaP, transient in PC-3) with the human COX-2 gene or empty control vector. We measured the effect on cell viability of COX-2 after treatment with a diverse set of agents e.g. etoposide, carboplatin, Fas, TRAIL, celecoxib and sulindac, using the MTT assay. We observed COX-2 dependent resistance to carboplatin, etoposide and celecoxib in LNCaP but not PC-3.

Carboplatin mediated reduction in cell viability was due to an S phase block and induction of apoptosis. COX-2 transfection in LNCaP cells attenuated both the cell cycle block and apoptosis. There was reduced p53 and p27KIP1 induction following carboplatin treatment in LNCaP-COX-2, compared to LNCaP-Neo. COX-2 transfection caused elevated cellular levels of anti-apoptotic proteins Bcl-2, Bcl-xL and survivin.
Celecoxib could not reverse the resistance seen in LNCaP-COX-2 to carboplatin, and PGE\textsubscript{2} could not increase the resistance in LNCaP-Neo cells, suggesting that COX-2 mediates an apoptotic resistance which is COX-2 enzymatic activity independent.

Other mechanisms were sought to reverse the carboplatin resistance. PI3K inhibitors wortmannin and LY294002 partially reversed the LNCaP-COX-2 resistance. These data suggests that COX-2 acts on the PI3K signalling pathway to mediate resistance in LNCaP. This was confirmed by Western blot findings of elevated levels of P-Akt\textsuperscript{ser473} in LNCaP-COX-2 compared with LNCaP-Neo. Celecoxib decreased levels of P-Akt\textsuperscript{ser473} in a manner similar to PI3K inhibitors, indicating that the PI3K inhibitors and celecoxib act in different ways on PI3K signalling. Because complete reversal of resistance does not occur with PI3K inhibition, COX-2 must be acting on other pathways also. However, unlike the PI3K inhibitors, celecoxib could not reverse resistance to carboplatin.

Wortmannin and LY294002 decreased levels of Bcl-2, Bcl-x\textsubscript{L}, survivin and, surprisingly, COX-2 protein. In contrast, celecoxib had little effect on Bcl-2 and increased COX-2 levels. Cyclin B1 levels were higher in LNCaP-COX-2 than LNCaP-Neo.

Gene microarray analysis confirmed the up-regulation of survivin in LNCaP-COX-2 and also showed elevation of cyclin I and fatty acid synthase and down-regulation of glutathione-S-transferase in LNCaP-COX-2.

In conclusion, stable COX-2 transfection causes a selective resistance to cytotoxic agents which is mediated via activation of the PI3K pathway and attenuation of p53 induction in LNCaP. COX-2 also causes the up-regulation of a number of anti-apoptotic factors and PI3K inhibition results in down-regulation of these proteins.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AIPC</td>
<td>Androgen independent prostate cancer</td>
</tr>
<tr>
<td>Akt/ PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptotic protease activating factor</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>AV</td>
<td>Annexin V</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B cell lymphoma-2</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign prostatic hyperplasia</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>COX-1/-2</td>
<td>Cyclooxygenase-1/-2</td>
</tr>
<tr>
<td>CRE</td>
<td>Cyclic AMP response element</td>
</tr>
<tr>
<td>Cyt c</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>DIABLO</td>
<td>Direct IAP binding protein with low pI</td>
</tr>
<tr>
<td>DISC</td>
<td>Death inducing signalling complex</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>DN</td>
<td>Dominant negative</td>
</tr>
<tr>
<td>(c) DNA</td>
<td>(complimentary) Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyl transferase</td>
</tr>
<tr>
<td>DR4/5</td>
<td>Death receptor 4/5</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECACC</td>
<td>European Collection of Cell Cultures</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EGF(R)</td>
<td>Epidermal growth factor (receptor)</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extra-cellular signal regulated kinase</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas associated death domain</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial adenomatous polyposis</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FLICE</td>
<td>FADD like interleukin 1β converting enzyme</td>
</tr>
<tr>
<td>FLIP</td>
<td>FLICE inhibitory protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GST (π)</td>
<td>Glutathione-S-transferase (Pi)</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vascular endothelial cells</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>50% inhibitory concentration</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin growth factor</td>
</tr>
<tr>
<td>IKB</td>
<td>Inhibitor of KB</td>
</tr>
<tr>
<td>IKK</td>
<td>IKB kinase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILK</td>
<td>Integrin linked kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KGF</td>
<td>Keratinocyte growth factor</td>
</tr>
<tr>
<td>LHRH</td>
<td>Luteinizing hormone release hormone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>LOX</td>
<td>Lipooxygenase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>Myeloid cell leukaemia-1</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>$M_w$</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NE</td>
<td>Neuroendocrine</td>
</tr>
<tr>
<td>NF-$\kappa$B</td>
<td>Nuclear factor-$\kappa$B</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDT</td>
<td>Population doubling time</td>
</tr>
<tr>
<td>PG($E_2$)</td>
<td>Prostaglandin ($E_2$)</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIA</td>
<td>Proliferative inflammatory atrophy</td>
</tr>
<tr>
<td>PIN</td>
<td>Prostatic intraepithelial neoplasia</td>
</tr>
<tr>
<td>PKA/C</td>
<td>Protein kinase A/C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidyl serine</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate specific antigen</td>
</tr>
</tbody>
</table>
Abbreviations

PTEN  Phosphatase and tensin homologue
Rb    Retinoblastoma
RIE   Rat intestinal epithelial
RNA   Ribonucleic acid
ROS   Reactive oxygen species
RT    Reverse transcription/ transcriptase
SAPK  Stress activated protein kinase
SD    Standard deviation
SDS-PAGE  Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SMAC  Second mitochondria derived activator of caspase
TBS   Tris buffered saline
TGF   Transforming growth factor
TNFα  Tumour necrosis factor α
TRAIL Tumour necrosis factor related apoptosis inducing ligand
VEGF  Vascular endothelial growth factor
VP-16 Etoposide
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CHAPTER 1

INTRODUCTION
Chapter 1 Introduction

1.1 Prostate Cancer

1.1.1 Incidence and Mortality

Prostate cancer is the sixth most common cancer in the world and the third most common in men. However, it is the most common non-dermatological cancer in men in Europe and North America (reviewed by Gronberg 2003), surpassing lung and colon carcinomas, comprising 32% of all cancers (Yoshimura et al. 2000). In the UK there are 30000 new cases annually, which constitutes 20% of all male cancers. 10000 men die from the disease annually in the UK, a figure which represents 12% of cancer related deaths in males (figures taken from Cancer Research UK website, April 2005). 40000 Americans die annually from prostate cancer, a figure which represents 15% of all cancer related deaths, making it the second leading cause of cancer deaths in males (reviewed by Denmeade et al. 1996). Annual mortality is declining however, and this could be due to better detection, more effective treatment and increased public awareness.

Prostate cancer poses one of the greatest health risks to men over 50, and in the US approximately 230,000 men will be diagnosed with the disease, a number which is growing annually. 60% of these diagnoses will be pathologically advanced, with no hope of cure (Steiner et al. 2001). Only palliative care can be given for advanced disease. Once a prostate cancer patient progresses to an androgen independent state (Chapter 1.1.4), the disease is usually fatal. Men diagnosed with cancer confined to the prostate can benefit from surgery or radiation therapy, but there is no curative treatment for metastatic prostate cancer (reviewed by Nelson et al. 2001).
Three quarters of diagnosed prostate cancers may not result in fatality, largely due to old age and the indolent nature of the cancer (reviewed by Horwich et al. 2002). Less than 0.1% of all diagnosed patients are less than 50 years of age, whereas 85% are diagnosed after the age of 65. Worldwide, the mean age of diagnosis is 72-74. Most men older than 85 have histological prostate cancer at autopsy. As the population ages, the impact of prostate cancer will increase, especially as the incidence of people dying from illnesses such as cardiovascular disease has decreased (reviewed by Tindall & Scardino 2001).

1.1.2 Prostate Physiology

The function of the prostate gland is to produce seminal fluid. The gland is made up of epithelial glands in a fibromuscular stroma. The epithelia has three cell types: basal, luminal secretory and neuroendocrine cells. A subset of basal cells may be stem cells for luminal cells. It is the epithelial cells which eventually give rise to cancer. Luminal cells secrete components of prostatic fluid, prostate specific antigen (PSA) and prostatic acid phosphatase (PAP), in an androgen dependent manner and express the androgen receptor. The fibromuscular stroma contains fibroblasts, smooth muscle cells, endothelial cells, dendritic cells, nerves and infiltrating cells such as masts and lymphocytes (reviewed by Feldman & Feldman 2001).

All cells are thought to originate from a putative stem cell population in the basal layer (reviewed by De Mulder et al. 2002). Tissue stem cells are thought to reside in the basal layer, where most cell division (renewal) occurs. Luminal secretory cells derive from these basal cells and perform androgen regulated differentiated functions such as PSA production and secretion (reviewed by De Marzo et al. 2001). Neuroendocrine cells and chromogranin A are often found in prostatic
adenocarcinoma and are implicated in tumour growth and invasiveness (reviewed by Hoosein 1998).

Growth and development of the prostate gland requires presence of sex hormones and it is the androgens which are the most important in this regard. Androgens stimulate proliferation and inhibit apoptosis (reviewed by Denmeade et al. 1996). The androgens are synthesised primarily in the Leydig cells of the testes under control of the hypothalamus-pituitary axis but also by conversion of adrenal steroids. An important step in androgen mediated action is testosterone conversion to dihydrotestosterone (DHT) by 5α-reductase within target cells (Castagnetta et al. 1994). 90% of testosterone is converted to DHT by 5α-reductase.

The action of androgens is effected by binding to its receptor, the androgen receptor, which has DNA binding and transcriptional activity upon ligand binding. The androgen receptor gene is located on the X chromosome. DHT has higher affinity for the androgen receptor than testosterone, it is a 100-fold more potent androgen. The androgen receptor in the basal state is bound to heat shock protein which prevents DNA binding. Testosterone is bound to sex hormone binding globulin (SHBG) in blood with only a small fraction free in blood (reviewed by Feldman & Feldman 2001). Upon ligand binding the receptor dimerises and binds to androgen response elements (ARE) in promoter regions of target genes. Testosterone stimulates growth of prostate cancer, partly by modulating activity of local growth factors. Cancer cells are known to produce epidermal growth factor (EGF), transforming growth factor (TGF-α, cachetin), basic fibroblast growth factor (bFGF) and nerve growth factor (NGF) (reviewed by Limonta et al. 1994).

As well as controlling growth rate, androgens induce enzymes involved in lipogenesis such as fatty acid synthase and cholesterol synthesising enzymes. Fatty acid synthase
Chapter 1 Introduction

is over-expressed in a number of cancers including prostate cancer and is associated with a poor prognosis (Swinnen & Verhoeven 1998).

There is continuous epithelial prostate cell turnover in the normal adult prostate. In androgen maintained rat prostate, a low cell proliferation rate of approximately 1-2% per day is balanced by an equally low apoptotic rate. In the normal prostate epithelium, cell proliferation balances with cell death, hence neither involution nor overgrowth occur (reviewed by Denmeade et al. 1996). Cancer growth is determined by the balance of this cell proliferation and cell death relationship. Tumour regression will only occur if the proliferation rate is less than the cell death rate. Any cancer can be seen as a disruption in homeostasis such that there is a net accumulation of atypical cells. Prostate cancer can be caused by uncontrolled cell proliferation, defective cell death (apoptosis), or a combination of both. Historically, cancer research has focused on cell proliferation, but recently much attention has been directed towards the role of apoptosis in cancer (reviewed by Riss 2001).

Upon castration or other androgen withdrawal methods e.g. anti-androgen treatment or luteinizing hormone release hormone (LHRH) analogues, serum testosterone levels drop and the prostate involutes due to a loss of prostate epithelial cells but not basal or stromal cells, as only the epithelial cells are androgen dependent and undergo apoptosis. Androgen withdrawal triggers apoptosis in normal prostate glandular epithelia and androgen dependent prostate cancer cells. However, androgen independent cells do not apoptose with androgen ablation, but the death cascade can be activated if sufficient cellular damage accrues (reviewed by Denmeade et al. 1996).

Following castration, within 24 hours there is a rapid drop in serum testosterone, cellular DHT, and changes in the androgen receptor, but apoptosis continues
approximately 2 weeks post castration, implying that reduction in androgen receptor occupancy by DHT is not sufficient alone to activate apoptosis. Thus, some other survival factors whose levels drop to a critical level, and are DHT-regulated, initiate apoptosis. Early events in androgen withdrawal are decreased glandular cell proliferation and cell atrophy. At this stage, events are reversible with exogenous androgen administration. Next there is change in nuclear chromatin structure after continued androgen withdrawal. There is an increase in intracellular calcium and it is thought that up-regulation of TGF-β1 mRNA may play a role in this. Endonucleases are enzymatically activated. Proteases degrade nuclear laminins after nuclear fragmentation, membrane blebbing and cellular fragmentation into apoptotic bodies occur. Apoptotic bodies are then phagocytosed by macrophages or neighbouring epithelial cells (reviewed by Denmeade et al. 1996).

Castration of rats and measurement on apoptotic rate show 1.2% glandular cells die pre-castration. 2-5 days after castration 17-21% die by apoptosis. These results show that glandular cell death is due to apoptosis and not necrosis. There is also a significant decrease in the percentage of cells entering cell cycle S phase following castration. There is thought to be no involvement of p53 in androgen ablation induced apoptosis (reviewed by Denmeade et al. 1996).

If androgen dependent prostate cancer cells are present in metastases, then the metastases will also be sensitive to androgenic stimulation. Most men with metastatic prostate cancer initially respond well with androgen ablation therapy. However due to the presence of androgen independent cells, which are unaffected by androgen ablation, prostate cancer cells will continue to proliferate (see Chapter 1.1.5). Daily rates of cell proliferation and cell death have been determined for normal, premalignant and cancerous prostate cells, and metastases in lymph node, soft tissue and
bone. Data shows that normal prostate glandular epithelial cells have extremely low proliferation and death rates in the rat. Localised prostate cancer cells arise from no further increase in proliferation but a decrease in rates of cell death (reviewed by Denmeade et al. 1996).

In the elderly male the prostate gland is often affected by benign prostatic hyperplasia (BPH), a non-malignant enlargement of the prostate, or by malignant disease (Chaudry et al. 1991). Before the onset of malignant disease, pre-malignant precursors are thought to develop first. Proliferation of epithelial cells involved in prostate cancer predominantly occurs in the peripheral zone, but can also rarely occur in the transition zone (reviewed by Agarwal 2000).

Low and high grade prostatic intraepithelial neoplasia (LGPIN and HGPIN) are prostatic lesions. HGPIN is thought to be a precursor lesion for prostate cancer. It is not certain yet if intermediate lesions exist. In HGPIN and prostate cancer, stem cell features appear in the secretory compartment also. Thus cells have morphological features of both stem and secretory cells (reviewed by De Marzo et al. 2001).

Prostatic epithelium could be a target for carcinogenesis because of chronic inflammation, with reactive oxygen species (ROS) released by inflammatory cells causing genomic damage. Inflammation may also be influenced by diet. Indeed, the activated macrophage is the hallmark of chronic inflammation and can be associated with carcinogenesis. Proliferative inflammatory atrophy (PIA) is another lesion thought to be a precursor for prostate cancer. It is also known as post atrophic hyperplasia, and is often associated with inflammation (Zha et al. 2001). Early inactivation of glutathione S-transferase (GST) is thought to play a role in PIA development as it is usually accompanied by chronic inflammation (reviewed by De
PIA is different from diffuse hormonal atrophy because it is not related to androgen withdrawal and is not associated with elevated levels of apoptosis. Inactivation of GST may serve as an initiating lesion for prostatic carcinogenesis and normal prostate cells may be susceptible to neoplastic progression mediated by dietary components as a result of GST deficiency. GST can inactivate carcinogens by conjugating them to reduced glutathione. GST can be inactivated by hypermethylation of residues near its regulatory sequences and such inactivation has also been reported in tumour suppressor genes such as Rb and p16 in other cancers. This increased methylation could occur as a result of increased DNA methyl transferase (DNMT) expression, which is reported in a number of cancer tissues. The model proposes that chronic inflammation (perhaps from infection) triggers the appearance of PIA lesions, which express high levels of GST and other protective enzymes, and the subsequent loss of GST function results in increased genome damage by the diet over decades. A possible therapeutic intervention is inhibition of DNMT (reviewed by Nelson et al. 2001). The putative role of GST in prostate cancer development is shown in Figure 1.1.
Figure 1.1 Proposed model for prostate carcinogenesis. A potential mechanism for the development of advanced prostatic disease, emphasising the importance of GST inactivation (Taken from Nelson et al. 2001).

This section described the non-pathological function and physiology of the prostate gland and the role that androgens and the androgen receptor play in growth and development of the gland. It also described the advent of pre-malignant lesions. Some of the molecular mechanisms which are involved in prostate carcinogenesis will be covered more fully in later sections.

1.1.3 Risk Factors and Chemoprevention

1.1.3.1 Risk Factors for prostate cancer

Prostate cancer is a multi-factorial disease involving both genetic and environmental risk factors such as age, race, family history, geography, androgenic status and diet (reviewed by Fleshner & Kucuk 2001). Risk factors or markers for the disease that could be used to identify suitable populations for chemoprevention are age greater
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than 50 years, familial history of prostate cancer, high serum testosterone, a high fat
diet, prostatitis and pre-malignant lesions (reviewed by Kelloff et al. 2001).
Age is the greatest risk factor for prostate cancer. By age of 80, more than 70% of all
men have prostate cancer. In the US this equates to more than 20 million men with the
disease. Age dependent appearance of prostate cancer appears not to be influenced by
race or geography.
There is a 70-fold difference in incidence between ethnic populations; it is lowest in
China and Japan, and highest in African-Americans (Figure 1.2). The incidence is
rising in both high and low risk populations. In the US this could be due to better
screening. However, death rates are significantly lower in Japan than the US,
suggesting that diet has a major impact not on the initial development of prostate
cancer but on its progression from a local neoplasia to metastatic cancer (Myers &
Ghosh 1999). Mortality rates of breast and prostate cancer are also much lower in
Asian countries compared to the US, and upon migration to US these rates increase.
As Asian countries adopt Western diets, the incidence of prostate and breast cancer
has started to rise. Further, early events in carcinogenesis and BPH appear to be the
same in US and Asia, suggesting that promotion and not initiation may be the
difference in the two geographical regions (reviewed by Coffey 2001).
Populations that consume large amounts of animal fat are considered to have an increased risk of prostate cancer. The disease is associated with a Western lifestyle, in particular a high fat and meat diet. Animal fats and meat are rich source of arachidonic acid, a desaturated product of dietary linoleic acid and a polyunsaturated fatty acid. Arachidonic acid is the most prevalent component of membrane phospholipids, and is converted to biologically active eicosanoids, which are known to be involved in carcinogenic processes. For example, PGE\textsubscript{2} promotes colon carcinoma cell growth and 12-HETE (a 12-lipoxygenase [LOX] product) promotes
cancer cell motility and invasion (Myers & Ghosh 1999). The cooking of red meat at high temperatures causes heterocyclic formation and these are known carcinogens. Low disease incidence in Asia is thought to be due to consumption of phyto-oestrogens such as soybeans and flavanoids. These could have several effects: an anti-oestrogenic effect via the estrogen receptor and reducing circulating androgens by increasing sex hormone binding globulin (reviewed by Gronberg 2003). For example, the phytoestrogens genistein, daidzein and coumestrol have been shown to have growth inhibitory properties in androgen-dependent (LNCaP) and -independent prostate cancer cell lines (PC-3) (Mitchell et al. 2000).

Chaudry et al. (1991) were the first to show differences in fatty acid consumption in patients with benign and malignant prostatic disease. Lower levels of arachidonic acid were found in areas of malignancy and these tissues had a greater capacity for eicosanoid synthesis. Animal but not plant products contain arachidonic acid and an initial epidemiological link between high animal fat intake and prostate cancer postulated the importance of the eicosanoids. However, it has been difficult to make the link between dietary fat and prostate cancer, as the specific fatty acids people consume over the many years the disease develops are difficult to ascertain (reviewed by Myers et al. 2001).

As explained above, growth and development of the prostate gland requires the presence of sex hormones, the androgens. Studies have shown that circulating testosterone in youth, which is higher in blacks than whites, could account for observed differences in incidence rates. Also important is 5α-reductase activity and Japanese men have the lowest worldwide (Steiner et al. 2001). Thus, both testosterone biosynthesis and metabolism can contribute to prostate cancer.
Ross et al. (1998) propose a genetic model for prostate cancer involving testosterone metabolism involving polymorphism in four groups of genes showing inter-racial allelic variation. The groups are those involved in testosterone biosynthesis, conversion of testosterone into DHT, the androgen receptor gene and those genes involved in testosterone metabolism. High fat diets also thought to be associated with high levels of androgens further implicate dietary fat in prostate cancer (reviewed by Fleshner & Kucuk 2001).

Mortality rates for prostate cancer are high in North America and North Western Europe but appear to be lower in Africa and central and South America, it is postulated that UV radiation could have a protective effect. It has also been hypothesised that vitamin D deficiency increases prostate cancer risk (Walczak et al. 2001). Vitamin D is produced by UV irradiation in the skin or can be obtained from the diet. Increased age leads to decreased serum vitamin D levels as the photoproduction efficiency decreases with age. Melanin content, and therefore skin pigmentation, also effects vitamin D production as melanin absorbs UV radiation. Japanese men also consume fish oil in their diet, which contains large amounts of vitamin D and this may contribute to the lower mortality observed for prostate cancer in Japanese men.

1.1.3.2 Chemoprevention of Prostate Cancer

It is important to prevent prostate cancer in the first place as no effective curative treatment is currently available for advanced disease. Many classes of cancer chemopreventive agents are being evaluated. Chemoprevention of malignant disease involves inhibiting the conversion of precancerous lesions to malignant carcinomas in the hope that it will lead to a reduction in cancer incidence.
Because of the contribution of environmental factors to prostate cancer progression, there is an opportunity to modulate or manipulate them to reduce mortality from the disease. The major environmental risk factor is diet and this could be easily modified to prevent carcinogenesis (reviewed by Nelson et al. 2001). Prostate cancer is also a realistic target for chemoprevention because of its long latency, as it is thought to take 20-40 years to develop. Current promising chemopreventive agents include anti-androgens and anti-oestrogens, steroid aromatase inhibitors, retinoids, vitamins D, E and analogues, lycopene, differentiation agents, apoptosis inducers and anti-inflammatories such as NSAIDs (the non-steroidal anti-inflammatory drugs) (reviewed by Kelloff et al. 2001).

1.1.3.2.1 Flavanoids

Progression of prostate cancer initiation to clinical cancer could be controlled as mortality is low in Japan and other Asian countries. This could be explained on the basis of dietary habits, as food which is rich in flavanoids, isoflavones (genistein) and flavanones (silymarin), present in yellow-green vegetables, fruits, soybeans and green tea, are shown to inhibit disease progression. These compounds have anticancer activity such as inhibiting TGFα mediated EGFR activation in prostate cancer cell lines (reviewed by Agarwal 2000).

1.1.3.2.2 Anti-oxidants

Oxidative damage induced by ROS could play a part in prostate carcinogenesis, by damaging biomolecules such as DNA, proteins and lipids. The diet is important in this respect because fats are a substrate for oxidative stress. Thus, it is not surprising that
antioxidants such as vitamin E and selenium are associated with reduced prostate
cancer incidence. Also, as inactivation of GST may be an important step in prostate
carcinogenesis (Fleshner & Kucuk 2001), supplemental vitamin E can have protective
effect on oxidative damage and can inhibit tumour progression in nude mice.
Glutathione peroxidase, an enzyme which protects cells against oxidative damage, is
selenium dependent, but the mechanism of action of selenium is unknown. Selenium
and vitamin E cancer prevention trial (SELECT) is a double blind placebo controlled,
multinational study being conducted from 2001 for men with normal digital rectal
examination and normal PSA. The trial is due to end in 2013 and the primary
endpoint is prostate cancer incidence (reviewed by Gronberg 2003).
Antioxidants such as β-carotene, α-tocopherol and ascorbic acid have also been shown
to have chemopreventive activity in experimental animal models. α-tocopherol may
reduce incidence of the disease but an increase in cerebral haemorrhage is also
observed (reviewed by Vainio 1999). Lycopene is a carotenoid present in tomatoes
and other fruits, and is a potent antioxidant also associated with a decreased risk of
prostate cancer (Fleshner & Kucuk 2001).

\section{1.1.3.2.3 Anti-inflammatories}

Inflammation is often linked to carcinogenesis and in this respect COX-2 is a key
inflammatory enzyme (see Chapter 1.2). Prostaglandins, the products of COX-2
enzymatic activity, have multiple carcinogenic effects. Thus, down-regulation or
inhibition of COX-2 could play an important role in cancer prevention. Because
COX-2 is over-expressed at an early stage in some cancers, it is a potential target for
chemopreventive agents (reviewed by Vainio 1999). Inhibition of prostaglandin
synthesis can inhibit tumourigenesis, as can use of anti-inflammatory steroids such as
glucocorticoids. Vitamin E and glucocorticoids inhibit PLA₂ activity, resulting in decreased amount of arachidonic acid available for metabolism to inflammatory prostaglandins (reviewed by Vainio 1999). The mechanisms by which COX-2 inhibitors may provide chemopreventive and anti-tumour activity will be discussed in greater detail in Chapter 1.2.7.1.

1.1.3.2.4 Anti-estrogens

Oestrogens can also cause inflammation in the prostate, as can a soy-free diet; chemicals present in soya include genistein and isoflavonoids, which are anti-inflammatory, although their mechanism of action is unknown. They could act as anti-oestrogens, anti-oxidants or tyrosine kinase inhibitors (reviewed by Coffey 2001).

Oestrogens have been implicated in the initiation of prostate cancer. The prostate contains oestrogen receptors in both the stroma and epithelium. They have DNA and ligand binding domains and bind oestradiol with equal affinity. Oestrogens can induce BPH only in the presence of androgens; they appear to increase the sensitivity of the prostate to androgens by up-regulating the androgen receptor. They stimulate cellular proliferation upon ER binding by inducing production of local stimulatory peptide growth factors such as TGFα, insulin growth factor (IGF) and EGF and inhibiting expression of local growth inhibitory factors such as TGFβ. Thus, selective estrogen receptor modulators (SERMs) would be expected to decrease stimulatory and increase inhibitory growth factors, inactivating local oestrogen regulated genes. Because androgen levels are unaffected, SERMs do not affect libido and sexual function. Thus, they are attractive as potential chemopreventive agents. Japanese men have the lowest serum oestrone and oestradiol levels and the lowest risk for developing the disease (Chapter 1.1.3.1) (Steiner et al. 2001).
To summarise, Chapter 1.1.3 described the various risk factors for prostate cancer and how the major environmental risk, diet, can be decreased by intervention measures. Chemoprevention is quite different in principle to disease treatment (Chapter 1.1.5) although both strategies may seek to disrupt similar signalling pathways. The potential role of NSAIDs and COX-2 inhibitors was briefly discussed, and as COX-2 is central to this research project these agents will be discussed in greater detail throughout this work. Further, inflammatory events are linked to prostate cancer development and COX-2 is a key mediator of inflammation, implicating COX-2 in the carcinogenic process.

1.1.4 Androgen Independent Prostate Cancer (AIPC)

Prostate tumour growth is initially androgen dependent. Thus, the therapeutic option of slowing tumour growth by stopping androgen production exists – this is the principle behind androgen deprivation therapy (Chapter 1.1.5). However, most advanced tumours eventually become resistant to the effects of androgen ablation and may grow even in the absence of androgens – they become androgen independent. After androgen ablation therapy, tumour cells adapt by using growth factors other than androgens. During the development of this process prostate cancer cells acquire the ability to survive and then proliferate in an androgen depleted environment. Several possible pathways for the development of androgen independent prostate cancer (AIPC) exist and genetic modification underlies its development. The mechanism by which prostate cancer cells survive androgen ablation and become androgen independent is not clear, but several possible routes exist (reviewed by Denmeade et al. 1996).
GST is expressed in the normal prostate but is lost in 90% of cancers via methylation of its promoter. As this protein is involved in detoxification of potential carcinogens it renders prostate cells susceptible to mutations. There will then be increased chance of 'multiple hits' of mutations which lead to androgen independence and malignancy. Some studies show that mutations exist even prior to androgen blockade, in untreated metastatic tumours. More mutations are seen in the androgen receptor in metastatic disease compared to primary prostate cancer. It is possible that androgen blockade may select for cells in which anti-androgen acts as an agonist rather than antagonist.

In a review by Feldman & Feldman (2001) five separate mechanisms of AIPC development are described. In the first mechanism, the hypersensitive pathway, prostate cancer cells need much lower levels of androgen for survival i.e. have increased sensitivity to androgens, but are still dependent on androgen. In this scenario, androgen receptor amplification will allow enhanced ligand occupancy even with reduced ligand. Indeed, 30% of tumours have an amplified androgen receptor gene. Furthermore, 5α-reductase activity or levels could be enhanced. To support this, it is known that after ablation testosterone levels fall by 95% but DHT levels fall by only 60%.

The second mechanism, termed the 'promiscuous pathway', concerns androgen receptor mutations. These mutations result in gain of receptor function. Non-classical ligands can then bind and activate the receptor e.g. other steroid hormones. LNCaP, a commonly used prostate cancer cell line, has a mutation in the ligand binding domain which allows it to be stimulated by other steroid hormones and flutamide, the androgen antagonist. It has also been reported that in LNCaP, estrogen mediated cell growth is via specific estrogen receptors (Castegnetta et al. 1995). Co-activators and co-repressors also act on the receptor. Over-expression of co-activators or loss of co-
repressors is a possible mechanism of enhancing receptor activity. For example, overexpression and amplification of the p160 co-regulator has been reported in human prostate cancers (reviewed by Scher et al. 2004).

A third mechanism, the outlaw pathway refers to steroid receptors activated by ligand independent means. IGF, keratinocyte growth factor (KGF) and EGF can also activate the androgen receptor in absence of androgen. Over-expression of HER2/ neu can activate androgen receptor dependent genes in the absence of androgen receptor ligand but not in absence of the receptor itself. Tyrosine kinase activation of these growth factor receptors activates mitogen activated protein kinase (MAPK) and phosphatidyl 3-kinase (PI3K) signalling, which phosphorylate and activate the androgen receptor.

In the fourth pathway there is no involvement of androgens or its receptor, but the principle is the same, namely cells can provide a substitute survival signal and could be selected by therapy. In the normal prostate, Bcl-2 (B cell lymphoma) is expressed by basal epithelial cells, neuroendocrine cells but not glandular epithelial cells and these Bcl-2 negative cells are the major androgen dependent cell type within the prostate gland and they are also the cells of origin of most human prostate adenocarcinoma (reviewed by Kyprianou et al. 2000).

There is increased Bcl-2 expression in prostate cancer cells and hormone refractory prostate cancer and an association between Bcl-2 expression and progression to androgen independence exists. However, Bcl-2 is not expressed in all androgen independent metastatic tumours, suggesting that there could be multiple pathways for progression to an androgen independent phenotype in prostate cancer. Mutations in p53, which triggers apoptosis in response to injury or DNA damage, may also be a
factor, as p53 is associated with progression of prostate cancer from localised to metastatic androgen independent phenotype (reviewed by Denmeade et al. 1996).

The fifth pathway postulates that a population of androgen independent cells exists even before therapy is given, so called ‘lurker’ cells, i.e. putative stem cells, which therefore have the properties of cancerous cells. There could also be increased bioavailability of androgens within the tumour itself (despite castration levels in the blood) via sequestration with steroid hormone binding globulin and increased expression of enzymes involved in steroid synthesis e.g. squalene epoxidase (reviewed by Scher et al. 2004).

Androgen independent cells in the tumour population could suppress the proliferation of androgen sensitive cells and gain growth advantage as a result, this could be due to androgen independent cells secreting interleukin-1 (IL-1), inhibiting growth of androgen sensitive cells. Exogenous IL-1 has no effect on androgen independent PC-3 or DU145 cells, suggesting that they have lost sensitivity to IL-1 growth inhibitory effects (reviewed by Hoosein 1998).

We have seen in this section that prostate cancer cells may acquire an androgen independent phenotype, which mediates their resistance to apoptosis not only following androgen deprivation but also to chemotherapy and radiotherapy (which is discussed in the next section). Changes in expression of apoptotic and cell cycle regulatory proteins underlie this phenotype. A central theme in this thesis is the potential role of COX-2 in modulating apoptotic proteins and mediating resistance to chemotherapy, although COX-2 is yet to be implicated in the development of androgen independence.
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1.1.5 Treatment Options

In prostate cancer, cell proliferation exceeds cell death, producing continuous tumour growth. One goal of prostate cancer therapy is to correct this imbalance. Androgen independent cells can still undergo apoptosis by means other than androgen ablation; this is a target for therapeutic intervention. As discussed in Chapter 1.1.4, androgen withdrawal therapy may select for androgen independent, Bcl-2 expressing cells in the tumour population (reviewed by Kyprianou et al. 2000). Advanced prostate cancer is incurable and only palliative treatment is given.

1.1.5.1 Early Localised Disease

There are broadly three management options for patients with early, localised disease. Firstly, active surveillance involves not administering a curative treatment. Many patients with either localised or locally advanced disease will not die from it, especially patients with well differentiated tumours. Conservative patient management is reasonable in individuals over 70 years, a Gleason score less than or equal to 6, PSA less than 10 ng/mL and PSA doubling time greater than 10 years (reviewed by Horwich et al. 2002). Secondly, there is the option of radical prostatectomy, the surgical removal of the gland. But there is a high risk of impotence with this procedure although a low risk of incontinence. Thirdly, the patient can undergo radiotherapy, either with either external beam radiotherapy (EBRT) or the implantation of radioactive seeds (brachytherapy). It is thought that seeds offer lower toxicity, but can be used only for small tumours with good prognosis. For EBRT and brachytherapy possible side-effects are cystitis and dysuria. Hormonal ablation therapy can improve radiotherapy results (reviewed by Horwich et al. 2002).
1.1.5.2 Androgen Deprivation Therapy

Inhibiting testosterone function is an important aspect of prostate cancer therapy due to its effect on cell proliferation and division. There are a number of methods available to either reduce circulating androgens or inhibiting their function. These include orchidectomy, androgen receptor antagonists, LHRH analogues and 5α-reductase inhibitors. Androgen ablation therapy is not always successful as metastatic prostate cancer in patients could have both androgen dependent and independent cells and this therapeutic strategy will be ineffective for pre-existing androgen independent cells (reviewed by Tapia-Vieyra & Mas-Olivia 2001).

Orchidectomy, the surgical removal of testes, is now largely replaced by pharmacological means of depleting androgens. Also, there are side effects with this therapy which make them unattractive for prostate cancer prevention; loss of libido, muscle mass, bone mass and impotence. Thus other ways have been developed to perturb androgen metabolism with fewer side effects.

Anti-androgens have been approved to treat prostate cancer. 5α-reductase is the enzyme which mediates intracellular metabolism of testosterone to DHT. Inhibition of this enzyme is a good target for prostate cancer prevention as men with genetic deficiency for type II 5α-reductase have never been reported to develop clinical prostate cancer. Inhibitors of 5α-reductase, are being tested to treat benign and malignant prostate disease, breast cancer, osteoporosis and alopecia. Agents such as finasteride have been shown to cause regression of the hyperplastic prostate and inhibit prostate growth, with a decrease in serum PSA levels (Trump et al. 2001).

Two sources of androgens are the testes which produce 95% of all androgens and adrenal glands, which produce dehydroandrostosterone, dehydroandrosterone sulphate
and androstenedione. Both sources are under control of the anterior lobe of the pituitary, which releases leutinizing hormone (LH). LH release from the pituitary is under control of LHRH (the releasing hormone), released by the hypothalamus. Hypothalamic LHRH is degraded in the pituitary; therefore circulatory levels are low. Serum testosterone acts as a negative feedback for LHRH release (reviewed by Droz et al. 2002).

LHRH analogues or agonists suppress LH release. Maximal androgen blockade combines an LHRH agonist with androgen receptor antagonist. Oestrogen treatment for prostate cancer was terminated with the advent of LHRH agonists (Corey et al. 2002). LHRH agonists are useful therapeutic agents in treating advanced prostate cancer, as they suppress androgen production. They also exert an anti-proliferative effect on the tumour directly by decreasing EGFR levels (reviewed by Motta et al. 1996). Thus, LHRH agonists inhibit tumour growth by suppressing pituitary-testicular axis activity. LHRH agonists produce an initial LH flare which can be suppressed with a short course of anti-androgens. Intermittent androgen blockade may delay the development of hormone refractory disease and reduce side-effects. Control and regulation of LHRH action is illustrated in Figure 1.3. This figure also illustrates the mechanism by which LHRH agonists mediate their therapeutic activity along the hypothalamic-pituitary-prostatic axis.
Figure 1.3 Control of androgen production by the hypothalamus-pituitary axis. LHRH and LH release is under control of the hypothalamus-pituitary axis (Taken from Denmeade & Isaacs 2002).

1.1.5.3 Chemotherapy for Advanced Disease

One problem of early prostate cancer treatment is the frequency of indolent tumours, which renders chemotherapeutic treatments less effective than in other cancers. Agents that are cytostatic or cytotoxic only affect cells that are proliferating. More
than 90% of prostatic cancer cells in a patient may not be actively proliferating and therefore will be resistant to cytotoxic chemotherapy (reviewed by Tapia-Vieyra & Mas-Olivia 2001). No chemotherapy has been demonstrated to improve survival in relapsed disease patients. Evidence is also lacking that chemotherapy prolongs survival in hormone refractory patients. No curative combination treatment for metastatic prostate cancer exists (reviewed by Droz et al. 2002). Thus, there is a need for cytotoxic therapy to kill androgen independent cells that does not require cell proliferation to be a mechanism of action.

Categories of agents tried as first line therapy include alkylating (cyclophosphamide), platinum-containing (cisplatin, carboplatin), pyrimidines (5-fluoro-uracil), anti-folates (methotrexate), intercalating (doxorubicin), anti-mitotic (etoposide, vinblastine), polyamine inhibitors and immunologic agents. Cyclophosphamide has surpassed doxorubicin as the standard cytotoxic agent for advanced prostate cancer (reviewed by Yagoda & Petrylak 1993). Estramustine, a conjugate of oestradiol and nitrogen mustard, is frequently used alone or in combination with docetaxol, vinblastine or etoposide (reviewed by Lara & Myers 1999).

Mitoxantrone has palliative effects but little effect on survival, but it is the recognised standard chemotherapy for advanced prostate cancer in the US. Docetaxel is attractive as it has wide-spectrum activity and synergism is shown with other agents. However, combinations may not be synergistic or even additive for prostate cancer cells; they could be antagonistic (Budman et al. 2002).

Bone is the most frequent site of metastasis and has major consequences in terms of pain and nerve compression, which affects treatment options. Hormone deprivation is the preferred treatment at this stage. Symptoms are either local or diffuse. For pain, radiotherapy or radiopharmaceuticals are used. Patients with hormone suppression can
develop osteoporosis, with combination of both osteoblastic and osteolytic metastases occurring (reviewed by Droz et al. 2002).

Trial regimens experimented for prostate cancer include prednisone, hydrocortisone, taxanes such as paclitaxel and vinblastine. Endpoints in such trials are typically survival, progression free survival, PSA response and quality of life. Due to absence of reliable biochemical and biological tumour markers for the disease, subjective analysis such as quality of life, weight changes and analgesic use for response criteria has had to be included (reviewed by De Mulder et al. 2002).

There are no consensus guidelines for second line therapies. Options include endocrine manipulation, chemotherapy alone or combined, or radiotherapy. The purpose of second line therapy is not to prolong life but to improve quality of life. For these patients quality of life is of greater importance than actual length of survival (reviewed by Mahler & Dennis 1992).

We have seen in this section the need for better treatment options for prostate cancer, particularly advanced, metastatic, androgen independent disease, which has no cure. COX-2 inhibitors, agents which form a central part in this current study, have not been discussed here because they do not form part of therapy for prostate cancer yet although their utility is currently being assessed. The anti-cancerous effects will be discussed in greater detail regularly throughout this thesis.

1.2 Cyclooxygenase-2 (COX-2)

1.2.1 Biochemistry

Cyclooxygenase (COX) is the rate limiting enzyme in the conversion of arachidonic acid to the eicosanoids. COX is bound to the endoplasmic reticulum (ER), but is also
found associated with the nuclear membrane, but not plasma or mitochondrial membranes of fibroblasts, as determined by immunocytochemical studies (reviewed by Needleman et al. 1986). The enzyme is found in virtually every cell; only subsequent arachidonic acid metabolism to biologically active prostanoids varies in different cells. The molecular weight ($M_w$) of the purified enzyme is approximately 72 kDa and it requires a haem molecule for maximal catalytic activity. COX is associated with lipid membranes; hence lipids derived from the membrane gain access to the COX active site without leaving their site of origin. A variety of polyunsaturated fatty acids (PUFAs) are substrates for COX. The rate of oxygenation depends upon the number of cis-double bonds and number of carbons in the FA. The most common COX substrate *in vivo* is arachidonic acid (reviewed by Smith & Marnett 1991).

There are three phases of prostanoid formation: (i) hormone mediated arachidonic acid release from cellular phosphoglycerides by lipases (ii) sequential conversion of arachidonic acid to prostaglandin endoperoxides $\text{PGG}_2$ then $\text{PGH}_2$ by COX (iii) either isomerisation or reduction of $\text{PGH}_2$ to biologically active derivatives $\text{PGD}_2$, $\text{PGE}_2$, prostacyclin ($\text{PGI}_2$), $\text{PGF}_{2\alpha}$ and $\text{TxA}_2$. After their synthesis, they exit the cell via facilitated diffusion (reviewed by Smith *et al.* 1996).

Lipase systems which are activated when cell surface receptors interact with appropriate stimuli are $\text{PLA}_2$ (which acts on phosphatidyl ethanolamine/phosphatidylcholine) and PLC and DAG lipase (which act on phosphatidyl inositol derivatives). Arachidonic acid is often mobilised by both pathways in stimulated cells. $\text{PLD}$ is a third form of phospholipase. Arachidonic acid used for prostanoid formation is derived primarily from membrane phospholipids, but may also come from cholesterol esters, phospholipids of LDL or the triglyceride pool. A number of $\text{PLA}_2$
isoforms exist: type I is secretory and pancreatic. Type II is secretory and from platelets and the liver. Three types of cytosolic PLA2 have also been characterised (reviewed by Smith 1992).

The COX enzyme contains 2 moieties: a COX moiety which converts arachidonic acid to PGG2 (the oxygenation reaction) and an endoperoxidase moiety which converts PGG2 to PGH2 (the cyclisation reaction). PGG2 and PGH2 are cyclic endoperoxides. Because PGH2 is the end product of COX, COX is also known as prostaglandin endoperoxide synthase (PES) or PGH synthase.

The first reaction is the synthesis of PGH2 by COX, after membrane phospholipids are released by phospholipases. COX exhibits both a bis-oxygenase or cyclooxygenase activity, catalysing PGG2 formation and a peroxidase activity, catalysing a 2 electron reduction of PGG2 to PGH2. COX and peroxidase activities of the enzyme are on spatially distinct sites of the enzyme and both activities require haem. Treatment with aspirin or indomethacin competitively inhibit arachidonic acid binding, blocking COX activity but not the peroxidase activity. Similarly, mutants of COX exist that have COX activity but not peroxidase activity. For example, Tyr-385 is essential for COX activity; replacement with Phe-385 by site-directed mutagenesis produces an enzyme with no detectable COX activity (Smith et al. 1996). There are two major differences between COX-catalysed and spontaneous oxidation of arachidonic acid; firstly, the increased rate and secondly the stereochemical control, as only 1 of 64 isomers predominates, i.e. PGH2. A mechanism must exist whereby COX produces a single stereoisomer of PGG2 from arachidonic acid. Due to their unstable nature and short half-life, continued synthesis of prostanoids is needed to facilitate their diverse biological effects (Ristimaki et al. 1994).
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PGH₂ is then converted by cell specific synthases to give products such as prostaglandins and thromboxanes. Most of the synthases that further metabolise PGH₂ are also present on the endoplasmic reticulum – these are PGD, PGE, PGF, PGI and TxA synthases. Most prostaglandin forming cells produce only a single prostaglandin product because of the predominance of a single PGH₂ metabolising enzyme e.g. platelets contain TxA synthase and normally form TxA₂ but not other prostanoids, whereas endothelial cells produce mainly PGI₂. Thus, the particular prostaglandins produced depend upon the enzymatic machinery present on the ER in a particular cell type (reviewed by Dubois et al. 1998). Formation of PGF₂α from PGH₂ involves a 2 electron reduction, whilst all other products are formed from non-redox isomerisation reactions. Figure 1.4 illustrates the enzymatic reactions involved in the conversion of arachidonic acid to two biologically active prostanoids, TxA₂ and PGI₂.
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Phospholipids

\[ \text{Arachidonic Acid} \]

\[ \text{Cyclooxygenase} \quad \text{COX-Reaction} \]

\[ \text{PGG}_2 \]

\[ \text{Cyclooxygenase} \quad \text{Peroxidase-Reaction} \]

\[ \text{Leukotrienes} \]

\[ \text{Thromboxane synthase} \]

\[ \text{Thromboxane A}_2 (\text{TXA}_2) \]

\[ \text{PGH}_2 \]

\[ \text{Prostacyclin synthease} \]

\[ \text{Prostacycline (PGI}_2 \]

**Figure 1.4 The COX pathway schematic.** The enzymes involved in the conversion of arachidonic acid to two biologically active prostanoids (\(\text{TXA}_2\) and \(\text{PGI}_2\)). Arachidonic acid, upon release from phospholipids via phospholipases is converted first to \(\text{PGG}_2\) (the COX reaction) then to \(\text{PGH}_2\) (the peroxidase reaction). \(\text{PGH}_2\) is the substrate for cell specific synthases, which catalyse the formation of the biologically active prostanoids.

The COX enzyme is inactivated by an autocatalytic mechanism; the product \(\text{PGG}_2\) activates it (positive feedback) i.e. little \(\text{PGG}_2\) results in little COX activity. However, since \(\text{PGG}_2\) is converted to \(\text{PGH}_2\) continually in normal tissue, there is little COX activity normally. Only at very high \(\text{PGG}_2\) concentrations is there activation of COX; thus there is a \(\text{PGG}_2\) window for normal COX function. It is thought that \(\text{PGE}_2\) also activates COX2 expression i.e. a positive feedback loop exists where the product further downstream activates the initial rate limiting enzyme responsible for its
synthesis. Oxygen levels are not limiting, except in anoxic tissue. Arachidonic acid concentration in the tissue is well above the $K_m$ level of COX for arachidonic acid. COX has a requirement for a hydroperoxide activator. If these levels are reduced COX activity can be inhibited. It is not certain what the hydroperoxide activator is. Many cells also contain reduced glutathione (GSH) and GSH peroxidase that also inhibit COX activity (reviewed by Smith & Mamett 1991).

During the two reactions of the COX enzymes, free radicals are generated which can oxidise xenobiotics to form mutagens. PGH$_2$ can also break down to malondialdehyde, a mutagen (Kirschenbaum et al. 2000). The peroxidase activity of COX, in particular can generate radicals in the cell, which could be mutagenic or carcinogenic. The peroxidase component of COX can oxidise a wide range of chemical carcinogens. The peroxy radicals formed during arachidonic acid metabolism can cause epoxidation of carcinogens to more reactive forms.

### 1.2.2 COX isoforms

Prior to 1991, only one COX isoform was identified, but two COX isoforms are now known to exist and they differ in terms of their expression, prostaglandin synthesis site, C-terminus 18 amino acids, aspirin acetylation site, chromosome location and protein, gene and mRNA sizes. COX-1 and -2 are structurally similar but are encoded for by two different genes; conserved regions between them include the active site (Tyr-371), glycosylation sites and transmembrane regions (reviewed by Badawi 2000).

Human COX-2 is very similar in structure to sheep COX-1, with enzymatic residues being conserved in the two enzymes. COX-1 and COX-2 genes have 61% sequence homology; COX-1 is located on chromosome 9, COX-2 on chromosome 1 (reviewed
by Fosslien 2000). The murine COX-1 gene is 22.5 kb in length and the COX-2 gene is 8 kb. The locations of the intron/ exon boundaries are the same for both but the non-coding introns in the genes are much smaller for murine COX-2 than COX-1 (reviewed by Smith 1992).

Residue 523 (isoleucine in COX-1; valine in COX-2) is the only difference in the active site lining between the two isoforms, which means that the COX-2 binding site is larger and therefore results in biochemical differences between the two enzymes: COX-2 oxygenates a wider array of fatty acid substrates than COX-1 (reviewed by Taketo, 1998a).

COX-1 has 599 amino acids, COX-2 has 604. Although the M_w of both COX isoforms is approximately 72 kDa, as determined by SDS-PAGE, the M_w calculated from the primary sequence is approximately 65.5 kDa. The difference is probably due to the attachment of a number of oligosaccharides per enzyme. The enzyme exists as a homodimer in detergent solution, but it is not known if the enzyme can function as a monomer. It is not certain how COX is associated with cellular membranes because it does not have extensive hydrophobic domains – one possibility is that the enzyme is membrane associated through a specific anchor protein (reviewed by Smith et al. 1991).

In cultured cells, COX-1 is typically expressed at constant levels throughout the cell cycle – hence it is known as the constitutive isozyme. COX-1 forms prostanoids that act locally through G protein coupled receptors (GPCRs) outside the cell to mediate ‘housekeeping’ functions in response to circulating hormones relating to renal, gastric and platelet functions.

The rapid induction of COX-2 mRNA parallels the expression of c-fos and it is thus classified as an early immediate or early response gene. That is, it is a gene which is
activated in cellular stimulation by mitogens, hormones, growth factors or phorbol esters. COX-2 was originally identified as a gene product inducible by v-src in chicken fibroblasts and a phorbol ester early gene product in murine 3T3 cells (reviewed by Smith 1992). COX-2 is likely to have 2 roles: COX-2 co-localised with COX-1 in the ER could augment the actions of COX-1. COX-2 in the nuclear membrane forms prostanoids that act through nuclear targets associated with cell differentiation and replication (reviewed by Smith et al. 1996).

1.2.3 Functions of the Cyclooxygenases

Prostaglandins act via prostanoid receptors to modulate second messenger levels. They are impermeable to the cell membrane. Prostanoids have both autocrine and paracrine functions and when they exit the cell, interact with receptors on either the parent cell or neighbouring cells (reviewed by Smith & Marnett 1991). Prostanoids can be thought of as local hormones formed in response to circulating hormones.

Two classes of prostaglandin receptor transduce signal upon ligand binding; GPCR and nuclear peroxisome proliferator activator receptors (PPAR) which act directly as a transcription factor upon ligand binding.

The GPCRs are termed TP, IP, EP1/2/3/4, FP and DP for TxA2, PGI, PGB, PGF, and PGD respectively. Functionally, these receptors can be put into 3 categories: relaxant (increase in cAMP), contractile (increase in Ca^{2+}) and inhibitory (decrease in cAMP). Table 1.1 summarises the functions and organ expression of the GPCR prostaglandin receptors.
<table>
<thead>
<tr>
<th>PG and receptor</th>
<th>GPCR</th>
<th>Effect</th>
<th>Organs</th>
<th>Physiological Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGE₂</td>
<td>EP₁</td>
<td>Unknown</td>
<td>Kidney, lung, stomach</td>
<td>Various, organ dependent</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EP₂</td>
<td>Gₛ</td>
<td>Abundant</td>
<td>Various, organ dependent</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EP₃</td>
<td>Gᵢ</td>
<td>Kidney, brain, GI tract</td>
<td>Various, organ dependent</td>
</tr>
<tr>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td></td>
<td>EP₄</td>
<td>Gₛ</td>
<td>Abundant</td>
<td>Various, organ dependent</td>
</tr>
<tr>
<td>PGI₂</td>
<td>IP</td>
<td>↑ cAMP and PI metabolism</td>
<td>Vascular endothelium</td>
<td>Vasodilation, inhibit platelets</td>
</tr>
<tr>
<td>PGF₂</td>
<td>FP</td>
<td></td>
<td>Corpus luteum</td>
<td>Uterine contraction and ovulation</td>
</tr>
<tr>
<td>PGD₂</td>
<td>DP</td>
<td></td>
<td>Small intestine</td>
<td>Allergic reactions</td>
</tr>
<tr>
<td>TxA₂</td>
<td>TP</td>
<td>Gᵢₛ</td>
<td>Platelets</td>
<td>Blood clotting</td>
</tr>
</tbody>
</table>

Table 1.1 Functions and expression of the GPCR family of prostaglandin receptors. The table summarises 5 major prostaglandins, the receptors that mediate their biological function, the G protein associated with them and the major organs in the body where these receptors are located.

Prostaglandins not only play a central role in inflammation but are also induced in physiological functions such as blood clotting, ovulation, bone metabolism, nerve growth and development, wound healing, kidney function, blood vessel tone and immune responses (reviewed by DuBois et al. 1998 and summarised in Table 1.1).

COX-2 is normally undetectable in most tissues. It is inducible by a number of inflammatory agents and is concerned with prostaglandin production in inflammatory sites, and in cell proliferation and differentiation (reviewed below). COX-2 is dramatically up-regulated when there is increased cell replication or differentiation (see Chapter 1.2.4).
Initially, COX-2 was thought of as an inflammatory, inducible enzyme, however it appears to have significance in other disease states. COX-2 as well as COX-1 is found in the kidney, however interaction between the two are not clear. COX-2 null mice show severe disruption of kidney development. COX-2 appears to be involved in regulating brain function and neural development. Prostaglandins cause rhythmic contraction of the uterus and thus help induce labour (reviewed by Narumiya et al. 1999). The LH surge also produces prostaglandins and this is accompanied with COX-2 mediated ovulation. COX-2 is also involved in embryo implantation in the uterine endometrium. COX-2 null mice show multiple failures in reproductive function, including ovulation, fertilization and implantation, confirming the importance of prostaglandins in these processes (reviewed by Fosslien 2000). Prostaglandins stimulate both bone resorbing cells (osteoclasts) and bone forming cells (osteoblasts) and thus, in the context of bone metabolism, its effects appear contradictory (reviewed by DuBois et al. 1998).

Animal models of inflammatory arthritis suggest that increased expression of COX-2 is responsible for the increased prostaglandin production seen in inflamed tissues. Synovial fluid taken from patients with rheumatoid arthritis has COX-2 overexpression (reviewed by DuBois et al. 1998). COX-2 is central to the inflammatory process and COX-2 inhibition can arrive at the same therapeutic endpoints as less specific inhibitors that also target COX-1. Prostaglandins sensitise pain receptors to lower levels of stimulus. Thus, COX action causes hyperalgesia – an effect countered by pain relieving NSAIDs and COX-2 inhibitors. COX-2 inhibitors effectively suppress pain without gastrointestinal lesions. Inflammatory mediators and microglia result in chronic inflammation that causes neural damage. COX-2 expression in neurones also leads to apoptosis (reviewed by DuBois et al. 1998). The roles that
COX-2 plays in carcinogenesis are described in Chapter 1.2.5. Figure 1.5 shows the differential expression, function and regulation of COX-1 and COX-2.

Figure 1.5 Differential expression, induction and functions of COX-1 and COX-2. The 2 isoforms of COX are expressed in different tissues, are regulated differently and are involved in different functions (Taken from Hussain et al. 2003).

1.2.4 Induction and Regulation of COX-2

There is little similarity between regions regulating gene expression of COX-1 and COX-2. COX-2 is a highly inducible gene product expressed in response to processes such as growth (Tjandrinawata et al. 1997), differentiation and inflammation. It can be induced by hormones (Shimada et al. 2000), growth factors, cytokines (Xu et al. 1999), inflammatory factors (Bradbury et al. 2004) and stresses such as UV light.
(Tang et al. 2001), radiation, oxidative stress (Adderley et al. 1999), heat and hyperosmolarity. The promoter and enhancer regions regulating COX-2 reflect this. The 5' flanking region of the COX-2 gene has a TATA box; the COX-1 gene does not, and this enables p53 regulation of COX-2. COX-2 does not seem to be expressed in most unstimulated tissues, but is rapidly induced in response to these stimuli. Thus, although COX-1 and COX-2 catalyse the same reaction, they are regulated independently (reviewed by DuBois et al. 1998).

COX-2 expression is regulated primarily at the transcriptional level, with activation of transcription factors acting on sites such as SP-1, AP-1, AP-2, CRE, NF-IL6 (CCAAT enhancer binding protein or C/EBP), or NFKB within the COX-2 promoter but also at the post-transcriptional level by mRNA stability. This induction occurs in a cell type and stimulus dependent fashion. Compounds which inhibit AP-1 mediated COX-2 transcription include: retinoic acid, PPAR agonists and agents that target HER-2/neu, EGFR, protein kinase C (PKC), PI3K, MAPK (reviewed by Subbaramaiah & Dannenberg 2003). If COX-2 promoter activity is elevated it is either due to an over-expressed or overactive transcription factor, or a negative regulator of COX-2 transcription is lost or mutated (McGinty et al. 2000).

COX-2 expression also correlates with activity of signalling pathways such as the MAPK family (the extracellular regulated kinases [ERK], p38 and stress activated protein kinases/ c-Jun N terminal kinases [SAPK/ JNK]) and PI3K. COX-2 transcription can also occur via PKC and ras/ MAPK mediated signalling. PKC can activate Raf-1, which regulates MAPK activity. The ERK pathway is activated primarily by mitogens of the RTK family and utilised by a number of different extracellular stimuli, such as cytokines and growth factors that lead to cell growth and differentiation. SAPK/JNK and p38 MAPK are preferentially activated by
inflammatory cytokines (IL-1β and tumour necrosis factor α (TNFα)) and stress processes, and these two pathways are implicated in the inflammatory process. Their activation leads to growth inhibition and apoptosis. JNK phosphorylates c-Jun on its N-terminal activating domain, leading to c-Jun and c-fos heterodimers and transcriptional activity of genes via AP-1 and CRE sites. ERK is thought to activate NF-IL6 by phosphorylation NF-IL6. Activation of ERK1/2 not only stimulates COX-2 transcription but also stabilises COX-2 mRNA. COX-2 mRNA is unstable with a half-life of only minutes (Jang et al. 2004). COX-2 mRNA at the 3' untranslated region contains AU enriched elements, which confer instability. But binding of HuR, an mRNA binding protein increases mRNA stability (Subbaramaiah & Dannenberg 2003). Some of the signalling pathways, transcription factors and gene regulatory elements are shown in Figure 1.6.
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Figure 1.6 Regulation of the COX-2 gene in cancer. Some important ligands, receptors, signalling pathways and transcription factors involved in COX-2 gene induction and regulation (Taken from Subbaramaiah & Dannenberg 2003).

COX activity is also increased in some transformed cells. Subbaramaiah et al. (1996) investigated the effects of transformation (both viral and Ras oncogene) on COX-2 expression and prostaglandin biosynthesis in mammary epithelial cells. They showed
that cellular transformation was associated with increased COX-2 expression and increased PGE\(_2\) production.

Because hypoxia induces human umbilical vascular endothelial cells (HUVEC) to increase prostacyclin production, Schmedtje et al. (1997) investigated if COX-2 was induced by hypoxia and demonstrated that it was induced by NF-\(\kappa\)B p65, one of the NF-\(\kappa\)B transcription factor family. NF-\(\kappa\)B typically dimerises with p50 and p65 subunits, enters the nucleus and binds to DNA consensus sites in COX-2 promoter region and initiates transcription. Hypoxia increased the levels of COX-2 mRNA and protein in HUVEC cells.

Microtubule interfering agents (MIAs) are widely used to treat cancer. These agents work by interfering with microtubule assembly, impairing mitosis and disrupting the cytoskeleton. Subbaramaiah et al. (2000) showed that paclitaxel, vinblastine and colchicine also stimulate expression of the COX-2 gene and increase PGE\(_2\) production in human mammary epithelial cells, mediated by ERKs 1 and 2 and p38 MAPKs and AP-1. Inhibitors of MAPK and p38 blocked MIA induced COX-2 induction. It is possible that MIA mediated COX-2 induction can decrease efficacy of these compounds. Because of COX-2 inducing activities of MIAs, COX-2 inhibitors may need to be given to enhance anti-tumour activities.

Wadleigh & Herschman (1999) showed that there is a requirement for CRE (cis-acting or cAMP response element) and NFIL6 sites and the c-Jun transcription factor in the COX-2 promoter for COX-2 induction by serum, bFGF, PDGF, PGE\(_2\) or TNF\(\alpha\) plus IL-1\(\beta\) in a murine osteoblast line. Dominant negative forms of JN and MEKK blocked COX-2 induction but c-Jun over-expression enhanced COX-2 expression by the above stimuli. CRE transcription is usually mediated by CREB or members of the
ATF family but c-Jun can also activate COX-2 expression via the CRE site. Mutation of both NFIL6 reduced COX-2 promoter activity by all inducers.

Subbaramaiah et al. (1999) investigated the effects of p53 on COX-2 expression. Mouse embryo fibroblasts which expressed p53 showed 10-fold decrease in PGE2 compared to those cells expressing mutant p53, and COX-2 protein and mRNA levels were suppressed and this effect was due to decreased COX-2 transcription and activity of COX-2 promoter was significantly reduced. The region of the COX-2 promoter that responds to p53, found using murine COX-2 promoter deletion constructs, was the first 40 base pairs of the 5' transcription start site. These results could be important in understanding why COX-2 levels are undetectable in normal cells but expressed in many tumours, which are frequently p53 defective. Thus, p53 represses COX-2 transcription, and mutant p53 may contribute to increased COX-2 expression seen in malignant tissue. p53 suppresses cellular growth and transformation and can induce apoptosis (Chapter 5). Inactivation of p53 can lead to deregulation of the cell cycle, DNA replication, selective growth advantage and tumour formation. p53 acts on a number of target genes via particular consensus sequences (Chapter 5).

Xu et al. (1999) investigated the effects of aspirin and sodium salicylate on COX-2 expression in human umbilical vein endothelial cells and foreskin fibroblasts. Both compounds inhibited COX-2 mRNA and protein induced by IL-1β. Salicylic acid is formed from aspirin in the blood, but does not have inhibitory activity against purified COX-1 or COX-2, and fails to inhibit prostaglandin synthesis in intact cells. Thus, one possible mechanism is that it inhibits COX-2 gene transcription. Sodium salicylate had no effect on COX-2 mRNA degradation. The mechanism could involve inhibition of NF-κB or AP-1 mediated gene transcription.
In this section, experimental evidence for the role of various stimuli, signalling pathways, transcription factors and DNA promoter regions involved in induction and regulation of COX-2 transcription in a number of non-cancerous cell types has been discussed. The same mechanisms may or may not be responsible for regulating COX-2 transcription in cancer cells. Interactions between COX-2, p53 and PI3K will be explored in Chapters 4 and 5, respectively.

1.2.5 Effects of COX-2 in Cancer

Cancers are known to form more prostaglandins than the normal tissues from which they arise (Chapter 1.2.6). As COX-2 and not COX-1 is reported to be elevated in many cancers (Chapter 1.2.6), COX-2 is likely to be responsible for the overproduction of prostaglandins in malignant tissue (Subbaramaiah et al. 1996). Experimental and epidemiological evidence provide strong clues for the involvement of COX-2 and prostaglandins in the multistage carcinogenic process, in both early (initiation) and later (promotion) stages of tumour progression (discussed here).

The role of COX-2 in cancer was suggested by the observation that patients taking NSAIDs had a reduced risk of colon cancer. Epidemiological studies show that there is a lower rate of colorectal adenomas and carcinomas in subjects who have taken NSAIDs over a period of time, suggestive of a pathogenic role for COX in colonic tumourigenesis (Sheehan et al. 1999). As NSAIDs have inhibitory effect on carcinogenesis, this further implicates prostaglandin synthesis in cancer development (Badawi 2000).

It is known that when oncogenes are introduced into the cell, COX2 protein and mRNA is expressed (Subbaramaiah et al. 1996), suggesting that COX2 may be an important factor for carcinogenesis. Studies reviewed here show that COX-2
transfected or induced in a variety of cell types causes pro-carcinogenic phenotypes. These include increased cell proliferation, resistance to apoptosis, pro-angiogenic effects, inflammatory effects, enhanced metastatic capability and changes in the cell cycle.

1.2.5.1 Increased Cell Proliferation

Tjandrinawata et al. (1997) showed that exogenous dmPGE$_2$ caused increased cell growth and PGE$_2$ production, COX-2 mRNA and DNA content in PC-3 and LNCaP cells. The NSAID fluribprofen reduced COX-2 mRNA levels. Munkarah et al. (2002) showed that ovarian epithelial cancer cells treated with dmPGE$_2$ resulted in increases in COX-2, Bcl-2 and Bax mRNA. This was associated with increased proliferation flow cytometry and reduced apoptosis, as assessed by TUNEL.

Kawamori et al. (2003) used the AOM colon carcinogenesis model to assess the effects of exogenous PGE$_2$ in rats over 25 weeks on proliferation and apoptosis. PGE$_2$ was associated with increased multiplicity and tumour volume. Kinoshita et al. (1999) showed that COX-1 and COX-2 transfection into a colon carcinoma line doubled the growth rate and elevated EGFR levels. Indomethacin inhibited this increased growth, EGFR induction and DNA synthesis. Also, PGE$_2$ has been shown to induce COX-2 activity by activating tyrosine kinase activity of EGFR. Using knock out mice for prostanoid receptors or EP antagonists in the diet in the AOM mouse colon carcinogen model. Mutoh et al. (2002) assessed aberrant crypt foci formation. EP4 knock out mice developed 50% fewer tumours and there were fewer tumours in mice fed the EP4 antagonist.
Chen & Hughes-Fulford (2000) showed that administration of arachidonic acid stimulated c-fos mRNA within minutes and \( \text{PGE}_2 \) production remained elevated for hours in PC-3, although increased growth was not demonstrated. Flurbiprofen lowered both c-fos and \( \text{PGE}_2 \) levels. Use of a PKA inhibitor confirmed its role in this induction.

COX-2 has also been showed to be an anti-proliferative. Zahner et al. (2002) showed that COX-2 transfection into rat mesangial cells caused reduced cell proliferation and this was associated with elevated p53, \( p21^{\text{WAF1}} \) and \( p27^{\text{KIP1}} \). NS398 could not prevent the COX-2 induced anti-proliferative effect or change in cell cycle check proteins. Thus, COX-2 was shown to have the opposite effects to other studies reviewed here, namely an anti-proliferative and therefore anti-cancerous effect.

### 1.2.5.2 Resistance To Apoptosis

Subbarayan et al. (2001) showed that TNF-\( \alpha \) caused an increase in COX-2 mRNA over 4 hours in LNCaP, DU145 and PC-3 cells, although no increase in LNCaP \( \text{PGE}_2 \) was detected, and this was associated with reduced apoptosis as assessed by PI staining.

Tsujii & Dubois (1995) transfected COX-2 into rat epithelial cells and observed altered ECM adhesion and resistance to butyrate induced apoptosis, increased Bcl-2 levels, decreased expression of E-cadherin and the TGF\( \beta \)2 receptor, which is important in transducing signals modulating apoptosis. Sulindac sulphide, an NSAID, was able to reverse the inhibition of apoptosis and the increased ECM adhesion.

Lin et al. (2001) transfected COX-2 into lung adenocarcinoma cells and demonstrated resistance to vinblastine induced apoptosis. Myeloid cell leukaemia-1 (Mcl-1) protein levels but not Bcl-2, Bcl-\( x_L \) or Bax were elevated due to transfection. Use of anti-
sense Mcl-1 vector restored normal apoptotic sensitivity, confirming the role of Mcl-1 in this resistance.

Nzeako et al. (2002) showed that in cholangiocarcinoma cells either transfecting or inducing COX-2 with a cytokine cocktail (TNFa, IL-1β, IFN-γ) inhibited Fas (but not TNF-α or tumour necrosis factor related apoptosis inducing ligand [TRAIL]) mediated apoptosis. NS398 inhibited COX-2 mediated Fas resistance to apoptosis and PGE$_2$ restored the resistance. Mcl-1 but not other anti-apoptotic markers was upregulated by COX-2 expression.

Tang et al. (2002) transfected COX-2 into colorectal HCT-115 cells and assessed the effect on TRAIL induced apoptosis. COX-2 cells had lower DR5 mRNA and elevated Bcl-2 mRNA and less caspase-3, -8 and -9 activation. Sulindac sulphide pre-treatment restored DR5 levels and also reduced cell viability when combined with TRAIL synergistically.

Sun et al. (2002) used colon carcinoma HCT-115 cells, which do not express COX-2 or PGE$_2$. After COX-2 transfection they observed a reduction in apoptotic response to NS398, sulindac sulphide and 5-FU. Bcl-2 mRNA levels were increased and there was reduced caspase-3 and -9 activation. Lower doses of NS398 reduced PGE$_2$ levels but did not reverse COX-2 mediated apoptotic resistance.

Jabour et al. (2002) induced COX-2 in bladder epithelial cells with IL-1α to assess its effect on camptothecin induced apoptosis. NS398 abolished PGE$_2$ production caused by increased COX-2. PGE$_2$ abolished camptothecin induced apoptosis and this was likely to have been mediated through cAMP since forskolin (a potent AC activator) had the same effect.
Shimada et al. (2000) showed that COX-2 induction by hepatocyte growth factor (HGF) caused a resistance to ceramide induced apoptosis in gastric epithelial cells, and this resistance was inhibited by 20 μM NS398.

Han et al. (2002) used various cancer cells to demonstrate that p53 can induce COX-2 via the Ras/ Raf/ MAPK pathway and this reduced apoptosis in COX-2 expressing cells. This effect appears contrary to the role of p53, which is to induce and not inhibit apoptosis. The authors propose that this is to counter the pro-apoptotic effects of p53.

Ishaque et al. (2003) performed adenoviral transfer of the COX-2 gene into rat mesangial cells to assess the effects on TNF-α and VP-16 mediated apoptosis. They also induced COX-2 with IL-1β. This conferred protection against apoptosis to TNF-α only, but not VP-16. NS398 restored TNF-α mediated apoptosis and PGE₂ and PGI₂ protected against TNF-α mediated apoptosis.

McGinty et al. (2000) showed that COX-2 induced by IPTG in pheochromocytoma cells resisted apoptosis caused by NGF withdrawal. Pre-administration with NS398 reversed this resistance. Caspase-3 activity was reduced due to COX-2 and PGE₂ treatment in control cells also decreased caspase-3 activity.

Krysan et al. (2004) transfected COX-2 into non-small cell lung cancer cells (NSCLC) cells in both sense and antisense directions. There was enhanced survivin protein levels in the COX-2 over-expressing cells and PGE₂ had the same effect. The COX-2 inhibitor SC-58236 administered prior to staurosporine increased apoptosis. Survivin levels (but not other inhibitor of apoptosis [IAPs]) proteins were reduced with antisense COX-2 transfection. They found that survivin ubiquitination was reduced in COX-2 sense levels, stabilising survivin levels.
1.2.5.3 Angiogenesis

COX-2 is thought to be involved in hypoxia induced tumour angiogenesis. PGE₂ is reported to stimulate angiogenesis. Hypoxia is one of the most potent stimulators of vascular endothelial growth factor (VEGF). Concomitant up-regulation of COX-2 is seen with VEGF in the PC-3 ML subline. Use of NS398 suppressed cobalt chloride induced VEGF expression, but administration of PGE₂ restored ability of cobalt chloride to induce VEGF (Kirschenbaum et al. 2001).

Masferrer et al. (2000) showed in an in vivo study using the Lewis lung carcinoma model, dose dependent dietary celecoxib reduced primary tumour growth and decreased number and size of lung metastases. COX-2 was detected in the neovasculature. In another corneal angiogenesis model, the same group demonstrated that celecoxib suppressed corneal blood formation, implicating COX-2 in the angiogenic process.

Fujita et al. (2002) transfected COX-2 into LNCaP cells and observed greater growth rates and larger tumour volumes in these cells versus mock transfectant when introduced into SCID mice. Also, the COX-2 transfectant produced higher levels of the angiogenic factor VEGF.

1.2.5.4 Inflammation and oxidative damage

COX-2 activity can generate free radicals (Chapter 1.2.1), and if detoxifying enzymes such as GST are down-regulated or deactivated, then damage to biomolecules and DNA in particular can occur. During the two reactions of the COX enzymes, free radicals are generated which can oxidise xenobiotics to form mutagens (Chapter 1.2.1). PGH₂ can also break down to the mutagen malondialdehyde.
Inflammatory cytokines such as TNF-α, IL-1α and IL-1β, induce COX-2 enzyme expression, which increase synthesis of pro-inflammatory prostaglandins (reviewed by Fosslien 2000).

Cellular proliferation in the prostate could also be associated with chronic inflammation (Chapter 1.1.2). Development of some cancers have inflammatory involvement. In prostate, these lesions are highly proliferative and exhibit reduced apoptosis. The anti-death gene Bcl-2 is over-expressed in these lesions and there is decreased expression of the cell cycle regulatory gene p27KIP1. Luminal cells induced to express COX-2 by inflammation have an increased expression of Bcl-2 and a decreased expression of p27KIP1 (Chapter 5). There is increased COX-2 expression in most PIN cells (Chapter 1.2.6). TNF-α and IL-6 induce COX-2 expression and PGE₂ secretion in these cells.

1.2.5.5 Invasion and Metastasis

Attiga et al. (2000) assessed effects of PLA₂, COX and LOX inhibitors on the ability of PC-3 and DU145 cells to invade through a reconstituted basement membrane and on cell survival, motility and secretion of matrix metalloproteinases (MMPs). Ibuprofen and NS398 inhibited cell invasiveness and this was reversed with PGE₂, although PGE₂ alone did not induce invasiveness itself. NS398 treatment resulted in reduction in MMP2 and MMP9 levels.

Tsuji et al. (1997) transfected COX-2 into Caco-2 colorectal cells to assess the effect on invasive potential. MMP2 levels were increased after transfection and sulindac sulphide inhibited PGE₂ production and reversed the ability of the COX-2 transfectant to invade a membrane.
Nithipatikom et al. (2002) showed that COX-2 levels were 3 times higher in PC-3 high invasive cells compared to PC-3 low invasive cells and PGE$_2$ levels were 2.5 times higher. Indomethacin and NS398 inhibited PGE$_2$ production and the ability to invade through a basement membrane. However, PGE$_2$ alone did not induce invasion, it only enhanced it, and PGE$_2$ reversed the ability of NS398 to inhibit invasion. Vadlamudi et al. (1999) showed that neu differentiation factor binding to HER2/3 led to an increase in COX-2 protein and mRNA levels and c-myc levels. NDF promoted ECM survival and cell invasion and this was inhibited with NS398, which also inhibited PGE$_2$ production.

1.2.5.6 Cell Cycle

DuBois et al. (1996) showed that transfection of COX-2 into rat epithelial cells delayed G$_1$ progression and this was associated with a reduction in cyclin D and CDK4 expression. These cells grow slower because G$_1$ duration increased from 8 to 24 hours. These findings oppose the work reviewed in section 1.2.5.1, which describes an enhanced growth effect of COX-2 in other cell types. One consequence of this G$_1$ delay is to increase the time abnormal cells with mutations to accrue i.e. for enhanced tumourigenesis to occur.

Trifan et al. (1999) showed that COX-2 transfection into various cells produced a G$_0$/G$_1$ block and reduction in cells in the S phase. NS398 could not reverse this effect and COX-2 mutants which are catalytically defective could also induce this cell cycle arrest, suggesting a prostaglandin independent mechanism. Why COX-2 should produce anti-proliferative effects in these cells is not certain, but it could be a cell-type or context specific effect.
In this section we have described the reported oncogenic effects of COX-2 in various cell types after experimental approaches such as transfection, enzyme induction and administration of the main prostaglandin normally expressed in the experimental cell system.

### 1.2.6 COX-2 Levels in Prostate Cancer

COX-2 has been reported to be elevated in many cancers, including prostate, compared to the non-cancerous tissues from which they arise. A consensus has not been reached on which cell types over-express COX-2, what the magnitude of overexpression is and at which stage of the carcinogenic process the enzyme becomes elevated. Contradictory data exists in the literature concerning the relevance of COX-2 protein and mRNA levels in non-cancerous and cancerous regions of the prostate. Studies show that levels of prostaglandins in many tumours are higher than surrounding normal tissue, and the major prostaglandin is PGE₂ (reviewed below). This will be discussed below.

Kirschenbaum et al. (2000) showed that COX-2 was expressed in the smooth muscle cells of both normal and cancerous prostates. In non-cancerous epithelia, COX-2 was expressed only in the basal cell layer and smooth muscle. In prostatic inflammation, COX-2 is induced in luminal epithelial cells by lymphocytes (reviewed by Kirschenbaum et al. 2001).

Kirschenbaum et al. (2000) also investigated the differential expression of COX-1 and COX-2 in prostate cancer, BPH and precancerous lesions, using immunohistochemistry. They found highest COX-2 expression in the smooth muscle of the prostate. COX-2 was also expressed in most basal epithelial cells and in luminal
cells of higher grade PIN. COX-1 was also up-regulated in prostate cancer, but not to the same extent as COX-2.

Gupta et al. (2000) used pair matched benign and prostate cancerous tissue from the same patients to minimise inter-individual variation and showed there was significantly greater COX-2 mRNA and protein in cancerous tissue compared to benign tissue. Immunohistochemical analysis showed that there was mild staining in benign tissue and strong staining in tumour and stromal cells, with staining in stroma greater than that in tumour cells in 2 of 12 tissues.

Lee et al. (2001) performed a study to assess COX-2 expression in 22 BPH and 18 prostate cancer samples using immunohistochemistry. The intensity of staining for COX-2 correlated with tumour grading as assessed by Gleason score. In most prostate cancer tissues, strong COX-2 expression was detected in glandular epithelial cells and weaker staining was found in the stroma. There was weak COX-2 staining in adjacent normal tissue. A much smaller percentage of positivity was observed for COX-2 in BPH tissue.

Madaan et al. (2000) assessed COX-1 and COX-2 distribution and expression in 82 prostate cancer and 30 BPH samples using immunohistochemistry and immunoblotting. COX-1 was constitutively expressed in the fibromuscular stroma of all samples, with only a weak luminal expression. There was no COX-2 expression in the stroma of BPH specimens. They detected slightly greater COX-1 expression in tumour cells compared to BPH. Immunoblotting showed four times more COX-2 in prostate cancer than BPH but no difference for COX-1. There was also elevated COX-2 expression with increasing Gleason score. COX-2 expression in BPH was predominantly in luminal glandular epithelial cells, and in neoplastic epithelial cells for prostate cancer samples.
Edwards et al. (2004) used 89 prostate cancer (all staged T1-T4 at diagnosis) and 28 BPH samples. HER2 is over-amplified in ovarian and breast tumours and is associated with poor prognosis, but it is uncertain if HER2 is amplified in prostate cancer or if it is correlated with high Gleason score or poor prognosis. They performed IHC for COX-2 and HER2 and fluorescent in situ hybridisation (FISH) to assess HER2 over-amplification. COX-2 levels in prostate cancer were significantly higher than in BPH, but they were not related to Gleason score, PSA or overall survival in this cohort. But COX-2 levels were greater in T3 and T4 versus organ confined T1 and T2 tumours. There were similar COX-2 levels between T1 and T2 and BPH samples. No significant over-expression of HER2 was observed in either cancerous or BPH samples.

Uotila et al. (2001) used prostate cancer specimens from 12 patients and compared them to control prostates from 13 patients with bladder cancer. COX-2 staining and NOS-2 was stronger in cancer cells compared to the epithelia of control prostates. No difference for COX-1 was detected. This suggests that inducible genes COX-2 and NOS-2 are stimulated in human prostate adenocarcinoma and may also be expressed in PIN, considered a precursor for malignant disease.

Yoshimura et al. (2000) performed a study to investigate expression of COX-1 and COX-2 in prostate cancer, BPH, PIN and normal prostate specimens. Via immunohistochemistry and RT-PCR, they detected COX-1 mRNA in all samples, but COX-2 was highly expressed in cancer samples only. COX-2 was also detected in blood vessels and stromal tissue of cancer samples, but not in BPH, PIN and normal prostate samples. No significant difference in COX-2 staining between prostate cancer grading groups was found. Figure 1.7 illustrates the potential involvement of COX-2 in the various stages of carcinogenesis.
Chapter 1 Introduction

Prostate Epithelial Cells

\[ \text{COX-2} \rightarrow \text{Inflammation} \]

High Grade PIN

\[ \text{COX-2} \rightarrow \]

Clinically Significant Prostate Cancer

\[ \text{COX-2} \rightarrow \]

Metastatic Prostate Cancer

Figure 1.7 Possible involvement of COX-2 in multistage prostate carcinogenesis. COX-2 has been implicated in various stages of prostate carcinogenesis (Taken from Kirschenbaum et al. 2001).

COX2 is also reported to be over-expressed in several other tumour tissues such as colon (Sheehan et al. 1999), cervical (Kulkami et al. 2001), gastric, breast (Hwang et al. 1998), gallbladder (Asano et al. 2002), hepatocellular, lung, oesophageal and pancreatic carcinomas (Yoshimura et al. 2000).

Some studies do not find an elevation of COX-2 in prostate cancer compared to normal tissue. For example, Zha et al. (2001) showed that by immunohistochemistry normal prostate tissue was negative for COX-2 staining, prostate cancer and HGPIN
stained only weakly but there was strong COX-2 staining in PIA, particularly in mononuclear macrophages. There was no correlation between COX-2 staining and Gleason score, which opposes the findings of Lee et al. (2001). Even aggressive tumours which metastasised to lymph nodes were COX-2 negative. RT-PCR analysis suggested COX-2 mRNA was down-regulated in prostate cancer compared to normal tissue. Thus, they hypothesised that PIA may be a precursor for prostate cancer and is associated with acute and sometimes chronic inflammation.

Tanji et al. (2000) investigated the localization of COXs and Bcl-2 expression in prostatic adenocarcinoma after androgen blockade, using 12 samples from patients with cancer and 3 from prostatic hyperplasia by immunohistochemistry. They showed that COX-1 expression occurred mainly in the stromal cells of all samples. Many infiltrating cells in prostate cancer also expressed COX-1, suggesting it may also play a role in tumour development. The study did not find significant correlation between COX-2 and Bcl-2 expression. However, there was no COX-2 protein detected in normal human prostate and prostate adenocarcinoma, in contradiction to the findings of many publications reviewed here.

We have seen in this section that COX-2 mRNA and protein is reported to be elevated in prostate cancer, although some studies do not show elevation over non-cancerous tissue. In Chapter 1.2.5 the carcinogenic effects of COX-2 were presented. Because of this, COX-2 inhibitors are thought to be potentially useful agents in the management of prostate cancer. This is briefly discussed below.
1.2.7 COX inhibition

NSAIDs are among the most widely used therapeutic agents, taken for a range of pathophysiological conditions, including cardiovascular prophylaxis, analgesia for headaches and minor injuries and severe pain alleviation caused by inflammatory diseases such as rheumatoid arthritis. However, NSAID use can lead to ischemia, mucosal damage and ulceration as prostaglandins alter blood flow in the gastric mucosal microcirculation (reviewed by DuBois et al. 1998). Due to the discovery of the second COX isoform, COX-2, and the belief that it was responsible for exacerbation of inflammatory disease symptoms but not responsible for gastric protection, COX-2 inhibitors were developed to be as clinically useful as non-COX specific NSAIDs but to spare the gastric toxicity. Focus was put on COX-2 inhibitors, as COX-2 was observed to be present in increased amounts in tumour tissue compared to normal tissue, whereas COX-1 expression is low in both normal and cancer specimens (Chapter 1.2.6).

As will be discussed elsewhere in this thesis in greater detail, some COX-2 inhibitors at concentrations greater than those needed to inhibit prostaglandin production have anti-cancer activity in cell lines, in animal models of cancer and in human disease. Some studies suggest that COX-2 inhibition is an important factor in the ability of COX-2 inhibitors to induce growth arrest and apoptosis. Evidence given for this is the inability of COX-2 inhibitors to affect the viability of COX-2 non-expressing cells and the ability of prostaglandins to modulate the effect of the COX-2 inhibitor (Hsu et al. 2000, Kamijo et al. 2001).

The evidence that COX-2 inhibitors affect cell viability independent of COX-2 levels or expression is more convincing. The concentration of COX-2 inhibitor needed to induce growth arrest or apoptosis is typically 1000 times higher than that needed to
inhibit the enzyme (Liu et al. 1998, Johnson et al. 2001, Arico et al. 2002). Also, COX-2 inhibitors with similar IC$_{50}$ values in COX-2 inhibition have varying efficacies in apoptosis induction (Zhu et al. 2002), implying involvement of different molecules. Further, these agents can affect cell viability in cells which do not express COX-2. This observation does not necessarily minimise the potential role of COX-2 in promoting carcinogenesis. Firstly, non-catalytic effects i.e. protein binding, of COX-2 may be partly responsible for mediating apoptotic resistance. Furthermore, COX-2 can cause numerous other carcinogenic effects (Chapter 1.2.5) in addition to its effects on apoptosis, so although concentrations of COX-2 inhibitors in excess of those needed for enzyme inhibition, these agents may have anti-angiogenic or anti-invasive effects at enzyme inhibitory levels.

This complicates the question as to whether COX-2 expression is useful as a predictive factor for either therapeutic or chemopreventive measures. With regards to apoptosis, COX-2 mediates a RESISTANCE, not complete INHIBITION. If COX-2 is over-expressed in a particular malignancy, inhibiting COX-2 therefore may not necessarily cause apoptosis of COX-2 expressing cancer cells. As the relative importance of prostaglandin-dependent and -independent effects of COX-2 in carcinogenesis is fully understood, the potential usefulness of assessing COX-2 expression as an indicator for response prediction is still not confirmed.

Non-COX-2 targets of COX-2 inhibitors have been recently identified (Figure 1.8). These include proteins involved in PI3K and ERK signalling and the NF-$\kappa$B pathway. The result of COX-2 inhibitor mediated anti-proliferative activity is the up-regulation or activation of growth arrest or pro-apoptotic proteins or the down-regulation or inactivation of anti-apoptotic proteins, or a combination of both. These mechanisms will be discussed in greater detail in Chapters 5 and 6.
Figure 1.8 The vast array of identified cellular targets for NSAI\(\text{D}\)s and COX-2 inhibitors. The diagram summarises some of the known targets of NSAI\(\text{D}\)s and COX-2 inhibitors, which results in growth inhibition or apoptosis. Signalling pathways which are implicated include PI3K/ Akt, MAPK, NF-\(\kappa\)B and cyclin/ CDK complexes (Taken from Tegeder et al. 2001).
There have been several attempts to classify COX inhibitors, using different criteria. These include kinetic behaviour, such as competitive or covalent binding (reviewed by Fosslien 2000), selectivity of the COX isoforms (reviewed by Flower 2003), or chemically, referring to the individual groups as belonging to the salicylates, pyrazolones, fenamates, propionates, oxicams and indomethacin.

Below, we briefly describe some characteristics of NSAIDs and then COX-2 inhibitors that possess anti-proliferative or apoptotic potential and, therefore, anti-cancer activity.

### 1.2.7.1 Non-selective COX inhibitors

Before the introduction of COX-2 inhibitors in 1999, all NSAIDs inhibited both COX-1 and COX-2. Aspirin was the first member of the NSAID family. Whereas aspirin inactivates COX activity of COX-1 by covalent binding, it modifies COX activity of COX-2 without covalent inhibition. Aspirin has non-COX inhibition related activities, which could be useful in anti-tumourigenesis; it can inhibit the NF-κB transcription factor (reviewed by Taketo 1998a).

Sulindac is an analogue of indomethacin and is a pro-drug that needs to be metabolised into sulindac sulphide to become biologically active (reviewed by Chau & Cunningham 2002). This can occur either hepatically or by colonic bacteria. Sulindac can also be converted to the sulphone metabolite, but this does not exhibit COX inhibitory activity. Sulindac compounds inhibit growth and kill prostate cells in vitro, even in cells which over-express Bcl-2, a known mediator of cellular resistance to chemotherapy and radiotherapy (Lim et al. 1999)
1.2.7.2 COX-2 selective inhibitors

DuP-697 was the first COX-2 inhibitor described in the literature (reviewed by Flower 2003). DuP-697 irreversibly and competitively inactivates COX-2 (Copeland et al. 1994). In a study in rats, Gans et al. (1990) showed that DuP-697 had comparable anti-inflammatory, anti-pyretic and analgesic activity in inflammation induced pain to piroxicam, indomethacin and sulindac, but did not produce gastrointestinal toxicity or altered renal blood flow.

Celecoxib (Celebrex®, Searle-Monsanto and Pfizer) was the first COX-2 inhibitor to be approved by the FDA for the treatment and management of inflammation in rheumatoid arthritis and osteoarthritis in 1998. A year later, its use was expanded as an adjunct to usual care in familial adenomatous polyposis (FAP). Celecoxib is a benzene sulphonamide. Reported IC$_{50}$ values for the COX isoforms are: COX-1 15 µg/mL, COX-2 0.04 µg/mL (Kulp et al. 2004). In cells expressing either COX-1 or COX-2 IC$_{50}$ values are: 13-15 and 0.05 µg/mL respectively i.e. celecoxib has approximately 375 selectively for COX-2 over COX-1 (reviewed by Davies et al. 2000). Commercially, oral doses of 100 mg and 200 mg are available. Celecoxib has known in vitro and in vivo anti-tumour activity. This will be explored further in Chapters 4, 5 and 6.
Figure 1.9 The celecoxib molecule. The chemical structure of the COX-2 inhibitor celecoxib.

NS398 was the second COX-2 inhibitor described in the literature. NS398 was reported as a compound that had similar anti-inflammatory and analgesic properties as indomethacin but minimal stomach lesions (Reviewed by Taketo 1998a). Copeland et al. (1994) provided evidence that NS398 irreversibly and competitively inactivates COX-2, in a manner similar to Du-P697. This agent has been shown to cause apoptosis in a variety of cancer cell lines and to decrease in vivo xenograft tumour growth (Liu et al. 1998, 2000).

Rofecoxib, like celecoxib has pro-apoptotic activity, but it less potent and is slower acting. Whereas celecoxib can induce cell kill within a few hours in prostate cancer and colon cancer cells, higher doses of rofecoxib take days to cause the same effect (Johnson et al. 2001, Yamazaki et al. 2002). Rofecoxib has 10 times more selectivity for COX-2 than celecoxib (reviewed by Gasparini et al. 2003).
1.3 Aims of the Project

We have seen that COX-2 protein is reported to be elevated in several cancers, although not unequivocally so in the literature for prostate cancer (Chapter 1.2.6). However, COX-2 has tumourigenic properties (Chapter 1.2.5). One of these properties is to attenuate the apoptotic response to a variety of cytotoxic stimuli (Chapter 1.2.5.2).

We, therefore, wished to assess the effect, if any, of COX-2 on the apoptotic or growth inhibitory response of prostate cancer cells following cytotoxic insult. In order to answer this question we used two approaches to elevate COX-2 protein in our cells, both stable and transient transfection of human COX-2 cDNA and pharmacological induction of COX-2. If COX-2 expressing cells were found to be resistant to apoptosis or growth inhibition then the nature of the resistance would be investigated.

Further, we performed gene microarray experiments in LNCaP COX-2 null and overexpressing cells to identify genes which were differentially expressed due to COX-2. It should also be noted that COX-2 is not always reported to be expressed in prostate cancer cells (discussed in Chapter 3) and it could be argued that the rationale for this study is weakened. We have used primarily late-stage disease, metastatic cell lines and (as discussed in Chapter 3) COX-2 expression may be temporal during the inflammatory phase of the carcinogenic process and reduced in end-stage disease.
CHAPTER 2

MATERIALS AND METHODS
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2.1 Reagents

Agilent Technologies, West Lothian, UK
RNA NanoLabchip®

Alexis Biochemicals, Notts, UK
SuperKiller™ human recombinant TRAIL

Ambion (Europe Ltd.), The RNA Company
RNase-free microfuge tubes, RNase Zap

Amersham Biosciences
Cy®3 and Cy®5 dCTP fluorescent cDNA labels

Bio-Rad Laboratories Ltd, Herts, UK
Bio-Rad DC protein assay kit

BioSource International Inc., Nivelles, Belgium
Polyclonal antibodies to total Akt and phosphor-Akt ser473

CalbioChem (Merck Biosciences), Beeston, Nottingham, UK
Wortmannin, goat anti-mouse IgM-HRP conjugate
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*Cayman Chemical, (IDS Ltd)*, obtained from Alexis Corp.
16,16-dimethyl Prostaglandin E₂; LY294002, antibody to COX-2, COX-2 protein standard

*Corning Incorporated, Schiphol, Netherlands*
UltraGAPSTM slides for gene microarray

*DakoCytomation, Glostrup, Denmark*
Anti-mouse and anti-rabbit HRP-conjugated immunoglobulins

*Eastman Kodak Company, (distributed by Sigma Aldrich, Poole, UK)*
Kodak BioMax Light Film

*ECACC, UK, Salisbury, UK*
PC-3 and DU145 cell lines

*Genosys Inc., Haverhill, UK*
Sense and anti-sense primers for COX-2 and β₂-microglobulin

*Immunotech Coulter, Bucks, UK*
CD95 Fas IgM (CH-11 clone)

*Invitrogen-Gibco Life Technologies, Paisley, UK*
NuPAGE® Novex Bis-Tris gels, MOPS electrophoresis running buffer, Western blotting transfer buffer, foetal bovine serum (FBS), neomycin antibiotic
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Oncogene Research Products, (distributed by CN Biosciences, Beeston, UK)
Antibody to β-actin, Annexin V-FITC (Fluorescein isothiocyanate) apoptosis detection kit

Pharmacia, NY, USA
Celecoxib

Pierce, IL, USA
SuperSignal® West Pico Chemiluminescent Substrate

Promega Corporation, Southampton, UK
ImProm-II™ Reverse Transcriptase, Recombinant RNasin® Ribonuclease Inhibitor oligo(dT)₁₅ primer, dNTPs, reverse transcription buffer, Taq DNA Polymerase, Mg²⁺-PCR buffer, diethylpyrocarbonate (DEPC) H₂O

Qiagen Inc, West Sussex, UK
HiSpeed Plasmid Midi Kit, Cancer Oligos for gene microarray

Roche Diagnostics Corporation, East Sussex, UK
FuGene 6 Transfection reagent

Santa Cruz Biotechnology Inc (Autogen Bioclear), Wiltshire, UK
Antibodies to Bcl-2, Bcl-xL, cyclin B1, p27kip1, p53, survivin
Schleider and Schuell BioScience, (distributed by VWR International, Lutterworth, UK)

HybriSlip™ plastic cover slips

**Sigma, Poole, Dorset, UK**

Tri reagent™, 0.4% Trypan blue, L-glutamine, Porcine Trypsin-EDTA, RPMI-1640 and DMEM cell culture media, Ribonuclease A, propidium iodide (PI), Etoposide (VP-16), Sulindac, Carboplatin, Tween®20 detergent (polyoxyethylene sorbitan monolaureate), Nonidet® P-40 detergent, sodium dodecyl sulphate (SDS), phenylmethyl sulphonyl fluoride (PMSF), aprotinin, leupeptin, sodium orthovanadate (NaVO₄), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ethidium bromide, Lipopolysaccharide (LPS) from Salmonella abortus equi

**Stratagene, Cedar Creek, TX, USA**

Universal human reference for gene microarray

### 2.2 Solutions and buffers

**Phosphate buffered saline (PBS)**

For x1 strength: 80.0g NaCl, 11.5g Na₂HPO₄, 2.0g KCl, 2.0g KH₂PO₄ in 10L deionised water.

**Tris buffered saline – tween (TBS-T)**

For x5 strength: 12.1g Tris base (C₄H₁₁NO₃), 40.0g NaCl, in 1L de-ionised water, adjusted to pH7.6 with concentrated HCl, 0.05% Tween®20 detergent.
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Cell lysis buffer

50mM Tris-HCl (C₄H₁₀NO₃.HCl), 150mM NaCl, adjusted to pH7.0 with concentrated
HCl, in de-ionised water. This was supplemented with 1% Nonidet® P-40 detergent,
0.2% SDS, 1 mM phenylmethyl sulphonyl fluoride (PMSF), 10 μg/mL aprotinin, 10
μg/mL leupeptin and 1 mM sodium orthovanadate (NaVO₄), made fresh on ice, prior
to cell lysis.

Electrophoresis running buffer

Purchased as x20 strength solution (Invitrogen Corp), diluted with de-ionised water:
0.05M 3-(N-morpholino) propane sulphonic acid (MOPS), 0.05M Tris base
(C₄H₁₀NO₃), 3.45mM SDS, 1.0mM EDTA, pH7.7.

Western blotting transfer buffer

Purchased as x20 strength solution (Invitrogen Corp), diluted with de-ionised water:
pH 8.3, supplemented with 10% methanol for 1 gel transfer or 20% methanol for 2
gels.

Nitrocellulose membrane stripping buffer

2% w/v SDS, 62.5mM Tris-HCl adjusted to pH6.8 with NaOH, 100mM β-
mercaptoethanol in 200 mL de-ionised water.

2.3 Cell culture

Prostate cancer cell lines PC-3 and DU145 were obtained from the European
Collection of Cell Cultures (ECACC). LNCaP stable transfectants (Neo and COX-2)
were a kind gift from Dr Atsushi Mizokami (Kanazawa University, Japan). Stock cells of LNCaP transfectants were treated every third passage with 0.35 mg/mL neomycin to select for cells which maintained the plasmid containing either the Neo or COX-2 cDNA. All prostate cancer cells were grown in RPMI-1640 medium, supplemented with 10% FBS and 2 mM L-glutamine. HeLa cells, a kind gift from Dr George Kass, University of Surrey, were grown in DMEM medium, also supplemented with 10% FBS and 2 mM L-glutamine. FBS was heat inactivated at 55°C for 2 hours prior to use.

Cells which were not used for experiment were treated at each passage with 20 μg/mL ciprofloxacin for prophylaxis against mycoplasma infection.

Cells were grown in a humidified incubator with 5% CO₂ at 37°C. At each passage, cells were detached from the culture flask with porcine trypsin-EDTA solution. Cells were counted using a haemocytometer prior to plating for experiments.

### 2.4 Cell cytotoxicity assays

Cell cytotoxicity assays were performed via MTT assay, using the method described by Mosmann (1983). Drugs were all dissolved in dimethyl sulphoxide (DMSO) as stock solutions and stored at -20°C. All cells were plated at 3x10⁴/mL in 96 well plates in a volume of 200 μL. Cells were allowed to grow and attach in the humidified incubator for 24 hours at 37°C and 5% CO₂, unless stated otherwise. Typically, eight different concentrations of drugs were used, with a 2-3 log fold range (0.5-100 μM for celecoxib, 0.2-50 μM for etoposide, 5-1000 μM for sulindac). Drug sensitivity data was obtained in both 10% FBS and serum-free conditions. For assessment of drug sensitivity in 10% serum, 50 μL of drug was added to 200 μL of medium already present in the wells. For drug sensitivity in serum-free conditions, drugs were
dissolved in serum-free RPMI medium and 250 µL of it replaced the 10% medium. Cells were incubated with drug for 48-72 hours (equivalent to 3 or 4 cell doublings). The assay was terminated when the cells were incubated with 0.05 mg/mL MTT for approximately 4 hours at 37°C. The medium was removed and the formazan crystal products were dissolved in 200 µL DMSO. The absorbance of each well was read at 540 nm using an automated plate reader (Labsystems Multiskan RC plate reader) with Genesis 3.05 software. A graph of cell viability against drug concentration was produced from the mean absorbance values, by calculating the percentage growth of drug treated cells against control cells. For each drug concentration treatment was set up in triplicate or quadruplicate, which were averaged and the percentage of control growth calculated. The IC₅₀ for each experiment was interpolated from this dose-response curve. The IC₅₀ values from a minimum of three independent experiments were averaged and reported here, along with the standard deviation.

For experiments requiring Trypan blue exclusion cell counts, cell pellets were resuspended in serum free RPMI-1640 medium and equal volumes of cell suspension and Trypan blue solution were mixed and applied to the haemocytometer and counted. This was repeated in triplicate for control and all drug treated cells. Percentage of viable cells at each drug concentration was calculated by dividing the percentage of viable cells at a particular drug concentration by the percentage of viable control cells.

2.5 Western blot analysis

Cells were grown in either T25 or T75 flasks, washed in PBS, trypsinised as per normal, centrifuged at 1200 rpm for 5 minutes, resuspended in ice cold PBS and centrifuged again. The supernatant was removed and the cell pellet was incubated for
10 minutes on ice with freshly prepared cell lysis buffer. Cells were lysed using ultrasonication with a MSE Soniprep 150 sonicator, on ice for 20 seconds.

Cell protein content was estimated using a modified Lowry method, the Bio-Rad DC protein assay, according to manufacturer's instructions. SDS was added to 1-5% NaOH solution in ratio 1:50 and 25 µL was added to cell lysate samples or BSA protein standard in the range 0.2–1.5 mg/mL in a 96-well plate. 200 µL Folin reagent was added, mixed, allowed to stand at room temperature for 15 minutes. The absorbance was taken at 690 nm in a Labsystems Multiskan RC plate reader. Protein concentration of cell lysates was estimated by interpolation of the BSA protein standard curve.

Cell lysates were prepared in 0.5 M dithiothreitol (DTT) reducing buffer (protein load ranged from 25 to 100 µg), heated for 5 minutes at 85°C then subject to electrophoresis. The protein content was separated on an SDS-PAGE NuPAGE® Novex Bis-Tris gels (either 10%, 12% or 4-12% gradient gel and transferred to a nitrocellulose membrane in an assembly provided by the manufacturer. Non-specific binding sites on the membrane were blocked with a protein solution ranging from 2 – 5 % BSA or milk in TBS-Tween®20.

Primary antibodies (either mono- or polyclonal), dissolved in a protein solution at the concentration recommended by the manufacturer, were used to probe the nitrocellulose membrane for 1 hour at room temperature or at 4°C overnight by gently shaking. Non-specific Ig antibodies in place of primary antibodies were not used as negative controls. Appropriate secondary horse radish peroxidase (HRP) conjugated IgG or IgM antibodies (anti-mouse, goat or rabbit) were used against the primary antibody, in a protein solution recommended by the manufacturer for 1 hour at room temperature. The membrane was washed after exposure to both the primary and
secondary antibody with TBS-T 3 times for 5 minutes. The signal was detected using X-ray film via enhanced chemiluminescence (ECL) reagents. To demonstrate equivalent protein loading, the same membrane was stripped by incubating the membrane at 60°C for 30 minutes in 2% w/v SDS, 62.5 mM Tris-HCl pH 6.8, 100 mM β-mercaptoethanol, and gentle shaking. Then the membrane was exposed to a murine anti-β-actin IgM antibody at a 1:10000 dilution. A goat anti-mouse IgM conjugated to HRP was used at a 1:2000 dilution.

### 2.6 RT-PCR mRNA analysis

Total RNA was extracted using a guanidine thiocyanate/phenol method. TRI reagent™ was added to adherent cells to dissociate nucleoprotein complexes. Chloroform was added to separate RNA, DNA and protein phases. After 15 minutes centrifugation at 13000 rpm and 4°C, RNA was decanted with isopropanol addition. After a brief ambient temperature incubation, samples were centrifuged at 13000 rpm and 4°C, the RNA pellet washed with 75% ethanol and dissolved in RNase free water. The PCR machine used for both reverse transcription (RT) and polymerase chain reaction (PCR) was the Techne Touchgene (supplied by Jencons-PLS). Total RNA was converted to cDNA using ImProm-II™ reverse transcriptase kit from Promega Corp (250 mM Tris-HCl pH 8.3, 375 mM KCl, 50 mM DTT, dNTPs, 5 mM MgCl₂ and a ribonuclease inhibitor RNasin. Priming for the RT reaction was done with oligo(dT)₁₅ primers. As a negative control no RNA was added to the oligo(dT) for priming. RNA was primed with 50 ng/mL random hexamers, 10 mM dNTPs and DEPC water for 5 minutes at 65°C, followed by at least one minute on ice. RT buffer, 0.1 M DTT and RNase inhibitor were then added and incubated at 25°C for 2 minutes. 200 units of Superscript II reverse transcriptase was added, incubated at
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25°C for 10 minutes, then 42°C for 50 minutes and the reaction terminated at 70°C for 15 minutes. RNaseH was added and the reaction mix incubated at 37°C for 20 minutes. Samples were then subject to the PCR reaction.

cDNA was amplified using Taq Polymerase (5 units/µL), buffer containing 500 mM KCl, 100 mM Tris-HCl (pH 9.0), 1% Triton® X-100 and 15 mM MgCl₂. 10 mM dNTPs. 10 µM sense and anti-sense primers were obtained from Genosys Inc.

To ensure equivalent loading of cDNA for PCR, total RNA was approximated by performing RT-PCR of the β₂-microglobulin housekeeping gene. The sense strand was 5’-ACCCCACTGAAAAAGATGA-3’; anti-sense sense strand was 5’-ATCTTCAACCTCCATGATG-3’. After an initial denaturation step of 94.0°C for 3 minutes, 35 cycles of PCR were performed: denature at 94.0°C for 45 seconds, anneal at 54.0°C for 45 seconds and chain extension at 72.0°C for 90 seconds. This gave rise to a 120 bp product which was separated on a 1.2% agarose gel and visualised with ethidium bromide. The strength of the bands were used to approximate levels of total RNA and cDNA loading for COX-2 PCR was adjusted accordingly.

2.7 Plasmid amplification and purification

Before PC-3 cells could be transfected with either pBOSNeo (mock transfectant) or pBOSNeoCOX-2 (COX-2 transfectant), the plasmids had to be amplified. The construct of the plasmid is shown in Figure 2.1. These plasmids were a kind gift from Dr Atsushi Mizokami (Kanazawa University, Japan).
Figure 2.1 pBOSNeo Plasmid Gene Construct. Map of the plasmid constructs used for both transient (PC-3) and stable (LNCaP) transfection. For our studies either no cDNA or COX-2 cDNA had been inserted in the Xbal restriction sites. Taken from Fujita et al. (2002).

Amplification was performed in the competent E. Coli strain DH5α. 0.5 µg pBOSNeo and pBOSNeoCOX-2 plasmid were added to 50 µL E. Coli and kept on ice for 30 minutes. The cells were heat shocked at 42°C for 45 seconds then left on ice for 5 minutes. 200 µL Luria broth was added and cells were incubated at 37°C for 60
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minutes. A further 50 mL Luria broth was added which contained 0.125 μg/mL ampicillin, and left to agitate overnight at 37°C. The cells were then centrifuged at 3000 rcf for 12 minutes at room temperature. The supernatant was removed and the cells frozen at -20°C prior to plasmid purification.

Plasmid was isolated using a plasmid DNA isolation kit from Qiagen Ltd, a modified alkaline lysis procedure, according to the manufacturer’s instructions. Briefly, transfected cells were reconstituted in 50 mL Luria broth and harvested by centrifugation at 6000g for 15 minutes at 4°C. After removing the supernatant, cell lysis was achieved using 6 mL of a resuspension buffer containing RNase A. Precipitation of genomic and plasmid DNA was achieved with 6 mL of lysis buffer. The precipitate was filtered through the manufacturer’s cartridge. After neutralisation and wash, DNA was precipitated with isopropanol. This eluate was forced through the manufacturer’s precipitator and washed with 70% ethanol. The membrane was dried, and DNA eluted with 1 mL DEPC water.

Plasmid yields were quantified by agarose gel analysis. 1 μL of the purified plasmids were subject to electrophoresis in a 1.2% agarose gel (0.2 μg/mL ethidium bromide) at 90V. Lambda (λ) standards in the range 200 – 1000 ng were used along with a 10 – 0.25 kbp DNA ladder.

2.8 Transient transfection

PC-3 cells were plated at 1x10^5/mL in 96 well plates in 200 μL of 10% serum supplemented RPMI-1640 medium. The cells were allowed to attach and grow overnight. After 24 hours the medium was replaced with serum-free medium. Concentrations of plasmid DNA and transfection medium and duration of transfection were determined by optimisation studies to determine the conditions which produced
the greatest level of COX-2 transfection. FuGene 6 transfection reagent was mixed with serum-free media and either Neo or COX-2 plasmid in sterile eppendorf tubes and allowed to mix at room temperature for 15 minutes. The DNA/ transfection media complex was added drop wise to the cells whilst shaking gently. The cells were incubated with plasmid DNA and transfection medium for 4 hours at 37°C. This created either PC-3-Neo or PC-3-COX-2 transient transfectants. The medium was then changed back to 10% serum containing cytotoxic drugs at the specified concentrations. To ensure transfection was successful, the procedure was scaled up in 6 well plates. Cells were recovered by trypsinisation, and COX-2 protein levels assessed by Western blot (see Chapter 2.4 for Western blot protocol).

2.9 Flow cytometric cell cycle analysis

LNCaP-Neo and LNCaP-COX-2 cells were plated at 3x10⁴/mL in T25 flasks and allowed to adhere for 24 hours. The cells were treated for either 24 or 48 hours with either 50 μM or 100 μM carboplatin. Both floating and attached cells were harvested, centrifuged at 1300rpm for 3 minutes and then washed in ice cold PBS. The samples were vortexed and ice cold 75% ethanol in PBS was added drop-wise. The samples were then stored at 4°C for a minimum of 24 hours. The cells were centrifuged at 1300rpm for 3 minutes and washed in ice cold PBS and centrifuged again. The cells were resuspended in 500 μL PBS which contained 0.8mg/mL ribonuclease A and 10μg/mL PI. The samples were incubated at 37°C for 30 minutes in the dark, and analysed using a Beckman Coulter Epics XL flow cytometer (argon laser, excitation wavelength 488 nm). A minimum of 20000 events were acquired and analysed in FL-3 (PI detector, 620 nm).
2.10 Annexin V/PI apoptosis assay

Apoptosis was detected by flow cytometric analysis of AV/PI binding using a kit from Oncogene, following the manufacturer's instructions. Briefly, LNCaP-Neo and LNCaP-COX-2 cells were plated at 3x10^4/mL in T25 flasks and allowed to adhere for 24 hours. The cells were treated for either 24 or 48 hours with either 50 μM or 100 μM carboplatin. Both floating and attached cells were harvested and resuspended in a media binding reagent, which enhanced AV binding to PS in tissue culture media. AV-FITC was added to a concentration of 0.002% and incubated at room temperature for 15 minutes in the dark then centrifuged for 5 minutes at 1000 rpm. The media was removed and the cells resuspended in ice cold binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 2.5 mM CaCl\(_2\), 1 mM MgCl\(_2\), 4% BSA). PI was added to a concentration of 0.02%, on ice and away from light and analysed using a Beckman Coulter Epics XL flow cytometer (argon laser, excitation wavelength 488 nm). A minimum of 20000 events were acquired analysed in FL-3 (PI detector, 620 nm).

2.11 Gene Microarray Analysis

RNA was extracted from LNCaP-Neo and LNCaP-COX-2 cells as described in section 2.6. The quality of the RNA was checked with an Agilent 2100 Bioanalyser using an RNA NanoLabchip® according to the manufacturer’s instructions. The RNA was also diluted 1 in 10 with RNase free water and quantified using a nanodrop spectrophotometer (Agilent).

5 μg total RNA, 3 μg/μL random primers, 2 μg/μL oligo(dT) primers, 2 μL spike mix (universal ScoreCard, as analytical control) were prepared to a final volume of 20 μL.
with nuclease free water, then incubated at 70°C for 10 minutes and placed on ice. The reaction was also prepared with 5 μg total RNA from a universal human reference (Stratagene), and used as an internal control for the normalization of the microarray data.

Cy®3 (for the test sample) and Cy®5 (for the universal human reference) labelling mixes were prepared, protected from light, both at 0.02 mM, using ChipShot™ RT buffer and RT, 5 mM MgCl₂, dNTP mix and nuclease free water to a final volume of 20 μL. This labelling mix was added to each primed RNA solution respectively, spun briefly, incubated at 25°C for 10 minutes, then at 42°C for 2 hours. RNase treatment was performed after labelling total RNA, by adding 1 μL RNase H (1.5 U/μL) and 0.35 μL RNase solution and incubating at 37°C for 15 minutes, away from light.

cDNA was purified using a Spin-X® column following the manufacturer's instructions. Briefly, binding particles, 3 M sodium acetate and binding solution were added to 40 μL of labelled cDNA, vortexed gently for 10 seconds and incubated at 22-25°C for 1 minute. The entire contents were applied to the column and centrifuged at 10000g for 1 minute. The flow through was discarded and the column washed with 500 μL of 80% ethanol and centrifuged at 10000g for 1 minute. This wash step was repeated 3 times, with the flow through being discarded. cDNA was eluted with 60 μL of elution buffer and stored away from light at -20°C. The amount of Cy®3 and Cy®5 incorporation (FOI) in the cDNA was quantified using the nanodrop.

Printing of 3000 cancer oligos (from Qiagen Inc.) onto microarray slides was performed by the microarray group facility, led by Professor Colin Smith.

Prior to hybridization, the arrays were prepared according to manufacturer's instructions. They were immersed in sodium borohydride solution at 42°C for 20 minutes and then transferred to a wash solution at ambient temperature for 30
Chapter 2 Materials and Methods

seconds. This last step was repeated. The arrays were then incubated in a pre-
hybridization solution at 42°C for 15 minutes and washed at ambient temperature
twice, then transferred to nuclease free water and incubated at ambient temperature
for 30 seconds and dried by centrifugation at 2500 rpm for 2 minutes.

30 pmoles of sample cDNA and human reference were mixed in a sterile 1.5 mL
Eppendorf and samples were dried using a speed-vacuum centrifuge (UniScience) at
1200 rpm for 45 minutes. Then the cDNA was dissolved in 32 µL of the
manufacturer’s hybridization solution and incubated at 95°C for 5 minutes. The array
was placed in a hybridisation chamber and the 32 µL labelled cDNA was pipetted
onto the array and a plastic cover slip (HybriSlip™, Schleider and Schuell
BioScience) was carefully placed onto the array. The chamber was sealed and
incubated at 42°C for 16 hours overnight.

Following hybridization, the arrays were washed in manufacturer’s solutions at 42°C
to remove the plastic cover slip from the array, followed by several washes at ambient
temperature, according to the manufacturer’s instructions. The arrays were dried by
centrifugation at 2500 rpm for 2 minutes and stored away from light in preparation for
scanning. The arrays were scanned on an Affymetrix 428 array scanner and the data
were then imported into analysis software: BlueFuse (BlueNome, Oxford, UK). The
16bit TIFF images were processed with normalisation per cDNA spot relative to the
internal reference control (Cy5 labelled). This was carried out by dividing control
channel per chip and data were normalised to the 50th percentile. Data were omitted
with p values >0.05. BlueFuse data were then imported into GeneSpring (version 4.2,
Silicon Genetics Inc/Agilent Technologies UK Ltd, Stockport, UK) for hierarchical
clustering to produce a ‘gene tree’ using Spearman’s correlation (unsupervised). The
arrays were analysed using Blue Fuse software and the data was exported into
GeneSpring for the statistical analysis. GeneSpring uses the ‘centroid’ clustering method. This is based on the distance between 2 clusters being regarded as the distance between the averages of the data points under one branch and the averages of the data points under another.

### 2.12 COX-2 Induction with PMA

PC-3 cells were plated at $3 \times 10^4$/mL in 10% serum in 96 well plates and allowed to grow/attach for 24 hours. The media was then changed to serum free and drug was added at the indicated concentration either alone (this constituted the control) or concomitantly with 100 nM PMA. Cells and drugs with or without PMA were incubated for 72 hours at 37°C in a humidified incubator. Cell viability was determined with the MTT assay, as described in section 2.4.

### 2.13 Statistical analysis

Data was expressed as mean ± standard deviation. Significance was determined by the 2-tailed, paired, Student’s t-Test using Microsoft Excel. P-value < 0.05 indicated a significant difference.
CHAPTER 3

DEVELOPMENT OF CELL LINE MODELS OVER-EXPRESSING COX-2
Chapter 3 Development of Cell Line Models Over-expressing COX-2

3.1 Aims

To address the main hypothesis of this study, that COX-2 expression effects apoptotic sensitivity to cytotoxic agents and to other apoptotic inducing agents, the levels of COX-2 enzyme in the cell lines had to be determined. We assessed both the constitutive (basal) protein and mRNA levels of COX-2 using Western blotting and RT-PCR respectively in LNCaP, PC-3 and DU145 cells. As COX-2 protein could not be detected in any of our prostate cancer cell lines, mechanisms to elevate cellular COX-2 levels were sought. These included both stable and transient transfection and pharmacological induction. This chapter describes the rationale and development of these cell line models over-expressing COX-2.

3.2 Introduction

Cancer cell lines can either be derived from primary cancer or metastatic sites. Cell selection problems are minimised if tissue from metastatic sites selected for in vitro study. Therefore, metastatic lines have proved to be more reliable models for study. In our studies the three most commonly used prostate cancer lines (PC-3, DU145 and LNCaP, which are all metastatic) were used.

The PC-3 cell line was established by Kaighn et al. (1979) from tumour tissue obtained from lumbar vertebra removed from a 62 year old Caucasian who presented with urinary retention, weight loss and anaemia. The PDT was reported to be
approximately 33 hours. A biopsy revealed poorly differentiated prostatic adenocarcinoma. Hence, PC-3 is a prostate cancer cell line derived from bone metastases of human prostatic cancer.

Stone et al. (1978) established the DU145 cell line from a 69 year old Caucasian who presented with lymphocytic leukaemia and had been given diethylstilbestrol hormone treatment. An autopsy revealed metastasis to the femoral neck, vertebral column, liver, lungs and brain. The histological appearance of the prostate and brain tumours was similar, both poorly differentiated. Tissue was taken from a metastatic CNS lesion and taken for in vitro culture. PDT is reported to be 34 hours. Hence, DU145 is a metastatic human prostate cancer line, as for PC-3, but it is derived from a CNS lesion.

The LNCaP cell line was obtained from a 50 year old Caucasian with moderately differentiated adenocarcinoma by Horoszewicz et al. (1980). The patient had rapidly advancing disease which was resistant to hormonal therapy and subsequently to chemotherapy. Cell culture was started from a lymph node needle aspiration. The cells were initially reported to have a PDT of approximately 72 hours. Thus, LNCaP is cell line also of human origin from lymph node metastases of prostatic adenocarcinoma.

The levels of COX-2 in human prostatic cancer samples was discussed in Chapter 1.2.6, where it was concluded that a consensus on the levels, temporal nature and mechanisms of COX-2 elevation in prostate carcinogenesis has not been reached. We assessed the levels of COX-2 mRNA and protein in our cells. The results are reported in this chapter.

Transfection of the COX-2 gene into cell lines is the preferred method to elevate COX-2 protein above basal levels in order to assess the biological effects of this
protein. Although transfection is a 'clean' method for elevating a cellular protein, it has the disadvantage that the expression of the transfected protein is not under control of normal cellular control mechanisms, as it is constitutively expressed via a viral promoter. We used LNCaP cells stably transfected with a plasmid mammalian expression vector called pBOS containing human COX-2 cDNA (pBOS-Neo-COX-2) or empty plasmid (pBOS-Neo). The resultant cell lines were designated LNCaP-COX-2 and LNCaP-Neo respectively. The plasmid contains a gene which confers resistance to neomycin, thus allowing transfected cells to be selected. A genetic map of the plasmid construct is shown in Chapter 2.7. These LNCaP cells and the plasmids alone were a kind gift from Dr Atsushi Mizokami (Kanazawa University, Japan).

We also performed a transient transfection of PC-3 using these plasmids, to provide us with an additional cell line over-expressing COX-2 via transfection. It should be noted that this method (i.e. without long term selection of transfected cells with neomycin, as performed when establishing a stably transfected line) produces an increase in transfected gene product over a short period of time and often the increase in protein elevation is not as great as that observed following stable transfection.

Conversely, another cellular approach to elucidate COX-2 function is to deplete cellular COX-2 levels in a controlled fashion. Song et al. (2002) cloned COX-2 anti-sense cDNA into a tetracycline response element and performed a stable transfection in PC-3, DU145 and LNCaP. In this model, the addition of tetracycline mediates expression of anti-sense COX-2 mRNA which interferes with and reduces the levels of endogenous mRNA. Thus, cellular COX-2 protein levels can be effectively switched on or off in the presence or absence of tetracycline, the agent which controls the expression of the gene cloned to the promoter.
In addition to transfection, COX-2 can be induced pharmacologically i.e. use of endogenous or chemical agents to induce COX-2 gene expression. This method is considered less precise than transfection because in addition to COX-2 induction, a variety of other genes may also be expressed concomitantly, making the true biological response of COX-2 more difficult to interpret. A number of endogenous and pharmacological inducers of the COX-2 gene are known (Chapter 1.2.4). We attempted to induce COX-2 in PC-3, LNCaP and DU145 using phorbol 12-myristate 13-acetate (PMA) and LPS.

PMA is a diacylglycerol analogue and therefore an activator of PKC and it is a known tumour promoter. PMA has been used to induce COX-2 in a variety of different cell types, for example, prostate cancer cells (Zha et al. 2001), oesophageal cancer cells (Souza et al. 2000), human umbilical cells (Ristimaki et al. 1994) and human foreskin fibroblasts (Gilroy et al. 2001).

LPS is a component of gram negative bacteria cell walls and a known pyrogen (Inoue et al. 1995). It performs a protective function for bacteria such as Salmonella abortus equi but it also stimulates human mononuclear cells to produce TNF and activates macrophages. As described in Chapter 1.2.4, TNF can induce COX-2, and in this context it is part of the host inflammatory response against the bacteria in immunological cells. Inoue et al. (1995) in vascular endothelial cells and Monick et al. (2002) in human alveolar macrophages have used LPS to induce COX-2 protein.

Thus, we have attempted to establish a minimum of two models of COX-2 overexpression using widely used prostate cancer cell lines and the techniques of transfection and gene induction, with the understanding that each technique has limitations and disadvantages not only when compared with each other but also with normal physiological induction and regulation of COX-2.
3.3 Results – Cellular COX-2 mRNA and protein levels

3.3.1 LNCaP stable transfectant

In order to assess the effect COX-2 may have on the cellular response to cytotoxic treatment, the amount of COX-2 mRNA and protein expressed by three prostate cancer cells PC-3, DU145 and LNCaP had to be assessed. RT-PCR was used to detect cellular COX-2 mRNA levels and Western blotting was used to detect cellular COX-2 protein levels. Materials and Methods are described in Chapter 2. DU145 and parental LNCaP (LNCaP-Neo) do not express COX-2 mRNA, whereas a little was detected in PC-3 (Figure 3.1). There was strong COX-2 mRNA expression in LNCaP-COX-2.
Figure 3.1 COX-2 mRNA levels in prostate cancer cells. Levels of COX-2 mRNA in prostate cancer cells PC-3, DU145 and stable LNCaP transfectants -Neo and -COX-2. Bands show 300 base pair COX-2 PCR and 120 base pair β2-microglobulin products. Isolated RNA was reverse transcribed, amplified by PCR using specific primers as described in Materials and Methods, run on 1.2% agarose gel and visualised with ethidium bromide.

As can be seen in Figure 3.2 COX-2 protein could not be detected in any parental prostate cancer cell line, even when 100 μg of protein was used (data not shown). Thus, these cell lines in their parental forms do not express COX-2 protein. However, stable transfection of LNCaP with COX-2 plasmid produces a cell line with a significant increase in COX-2 protein compared to LNCaP-Neo (Figure 3.2).
3.3.2 PC-3 transient transfectant

Having established that PC-3, LNCaP or DU145 do not express COX-2 protein in their basal states, but stable transfection of LNCaP can produce a cell line which expresses high levels of COX-2 mRNA and protein, we transiently transfected the PC-3 line with either the empty plasmid or COX-2 plasmid to create PC-3-Neo or PC-
3-COX-2 transient transfectants. LNCaP and PC-3 are cell lines with different characteristics. They differ in their dependence of androgen; PC-3 is androgen independent whilst LNCaP is androgen dependent. Another important difference is their p53 status; PC-3 does not express p53 whilst LNCaP has wild type p53.

Figure 3.3 shows the increase in COX-2 protein after transfection with the COX-2 plasmid. Optimisation experiments showed that maximal induction occurred between 36 and 48 hours post transfection and by 72 hours COX-2 protein could not be detected. In contrast, transfection with the empty Neo plasmid did not result in an increase in COX-2 protein over the same time period (data not shown). It should be stressed that immunoblots with 100 μg of protein had to be run to detect COX-2 protein in this PC-3 model, whereas protein loads of 50 μg and less, and up to 15 passages, consistently demonstrated strong COX-2 expression in LNCaP-COX-2 (Figure 3.2).
Chapter 3 Development of Cell Line Models Over-expressing COX-2

3.3.3 COX-2 induction in PC-3

As well as elevating cellular COX-2 levels by both transient (PC-3) and stable (LNCaP) transfection, we attempted to induce COX-2 protein by inducing the gene pharmacologically or by using agents which stabilise COX-2 mRNA or protein. LPS has been shown to induce COX-2 in a variety of cell types (see section 3.2), but we were unable to demonstrate COX-2 induction in any of the prostate cancer cell lines with this agent. PMA is also a well-documented inducer of COX-2 via its ability to

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Figure 3.3 Levels of COX-2 protein in PC-3 cells following transient transfection. PC-3 cells were lysed following transient transfection as described in Materials and Methods. 100 μg protein was electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose membranes and exposed to murine IgG monoclonal COX-2 antibody (Cayman Chemical) at a 1:1000 dilution and a murine IgM β-actin antibody (Oncogene) at a 1:10000 dilution. HRP conjugated antibodies to detect murine IgG (Sigma) and IgM (Calbiochem) were used at a 1:2000 dilution. Bands were detected using ECL (Pierce). 50 ng of purified COX-2 (Cayman Chemical) served as a positive control.
activate PKC. Figure 3.4 shows the effect of 100 nM PMA (a dose determined by optimisation experiments) on the PC-3 line. PMA produced an increase in COX-2 with maximal induction observed at 72 hours. Thus, there was a narrow time window in which COX-2 was over-expressed using this method. This was a similar time window as that observed for transient transfection. It should be noted that 100 µg was needed for COX-2 detection. This effect was observed in serum free media only and not if cells were treated in serum supplemented media. The mechanisms as to why serum should influence the COX-2 gene induction process were not investigated. We were unable to induce COX-2 with PMA in DU145 and LNCaP (data not shown).

Figure 3.4 COX-2 protein levels in PMA treated PC-3 cells. Cells were lysed as described in Materials and Methods, at 4, 24 and 72 hours after 100 nM PMA treatment in serum free medium. 100 µg protein was electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose membranes and exposed to murine IgG monoclonal COX-2 antibody (Cayman Chemical) at a 1:1000 dilution and a murine IgM β-actin antibody (Oncogene) at a 1:10000 dilution. HRP conjugated antibodies to detect murine IgG (Sigma) and IgM (Calbiochem) were used at a 1:2000 dilution. Bands were detected using ECL (Pierce).
The results of transfected COX-2 both stably and transiently and gene induction are summarised in Table 3.1.

<table>
<thead>
<tr>
<th>Cell line model</th>
<th>mRNA</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>DU145</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LNCaP-Neo</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LNCaP-COX-2</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>PC-3</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PC-3-Neo</td>
<td>N.D.</td>
<td>-</td>
</tr>
<tr>
<td>PC-3-COX-2</td>
<td>N.D.</td>
<td>+</td>
</tr>
<tr>
<td>PC-3 plus PMA</td>
<td>N.D.</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 3.1 Summary of COX-2 mRNA and protein levels. The levels of COX-2 detected in our DU145, LNCaP and PC-3 cells. - indicates none detected, + and +++ indicate weak and strong expression respectively, N.D. is not done.

3.4 Discussion

The data presented in this Chapter shows that our prostate cancer lines have low levels of COX-2 mRNA and protein. We were unable to detect COX-2 protein in PC-3, DU145 and LNCaP prostate cancer cells. Small amounts of COX-2 mRNA were present in PC-3 but not in DU145 or LNCaP. We did not investigate why COX-2 mRNA in PC-3 did not translate into protein. Possible explanations include an mRNA
transcript with reduced stability or rapid degradation of COX-2 protein in PC-3. This may also be the case for LNCaP-Neo and DU145.

Different research groups report a variation in the amounts of COX-2 mRNA and protein that these prostate cancer cells express. Zha et al. (2001) showed that both COX-2 mRNA and protein could not be detected at all in PC-3, LNCaP and DU145. Patel et al. (2005) and Lim et al. (1999) also report undetectable levels of COX-2 protein in PC-3 and LNCaP. Subbarayan et al. (2001) report all three lines to be negative for both COX-2 mRNA (measured by both Northern blotting and RT-PCR) and protein. However, Nithipatikom et al. (2002) found moderate levels of COX-2 protein in PC-3 and Fujita et al. (2002) demonstrated some COX-2 protein and mRNA in LNCaP.

In contrast to our findings, Tjandrinawata et al. (1997) showed that PC-3 cells were negative for COX-2 mRNA but mRNA was detected in LNCaP. Hsu et al. (2000) found strong COX-2 protein in LNCaP and PC-3 and Song et al. (2002) also detected COX-2 protein in PC-3. The findings in the current study, namely that COX-2 protein could not be detected in any of our three parental cell lines, agree with some but not all the published research.

There are several possible reasons for these differences. The actual reported cell lines themselves may not be the same. Slight differences in cell culture conditions, such as the type of serum, antibiotic or culture media used may effect the expression of COX-2 or the stability of the mRNA or protein. Also, there are differences in detection methods used in these published studies, for example, immunoblotting,
immunohistochemistry or mRNA analysis. Even if the same method is employed, use of different antibodies with varying performance could also be a factor.

Because COX-2 protein could not be detected in any of these three cell lines, methods were needed to elevate COX-2 protein in one or more of these cells. Transfection is a method of introducing sustained increases in cellular levels of a protein of choice. The gene of the desired protein is inserted into a suitable vector and the synthesis of protein (COX-2 in this instance) is under the control of a viral promoter.

Another method to elevate protein levels is to induce protein expression pharmacologically, using agents which are known to either enhance transcription of the gene or to stabilise the mRNA transcript or protein. We attempted to induce COX-2 in these cells using PMA and LPS. We achieved weak COX-2 induction with PMA but were unable to demonstrate any induction with LPS.

Zha et al. (2001) showed that 0-80 nM PMA for 6 hours induced COX-2 protein and mRNA in a dose-dependent manner in PC-3 and TSU cells, but not in DU145 or LNCaP. These findings are similar to our own in that PMA could induce COX-2 in PC-3 cells but not LNCaP. However, the authors did not give data for a time dependent PMA response. Souza et al. (2000) used 50 ng/mL PMA (equivalent to approximately 80 nM) for 4.5 hours on oesophageal carcinoma lines to induce COX-2. However, increased COX-2 expression was only possible in cell lines which had basal COX-2 protein expression. Ristimaki et al. (1994) showed that 10 ng/mL PMA (equivalent to 16 nM) at 4 and 24 hours elevated COX-2 mRNA levels human umbilical cells. Also, PMA had a growth inhibitory effect in these cells. Gilroy et al. (2001) used 100 nM PMA over 1-24 hours in serum free conditions and found a peak COX-2 mRNA induction at 7 hours in human foreskin fibroblasts. De Ledinghen et al. (2002) used 50 ng/mL (80 nM) PMA for 6 hours to induce COX-2 protein and
mRNA in transformed rat hepatocytes. Adderley et al. (1999) demonstrated a rise in PGE$_2$ levels following PMA treatment of rat cardiac myocytes, an effect which was abolished by the COX-2 inhibitor NS398. Thus, PMA can induce COX-2 in a variety of cell types, including cancer cells.

An indirect method of assessing COX-2 mediated changes in cell phenotype is to introduce the major COX-2 catalytic product into the cell. In the case of cancerous epithelial cells, this is the prostaglandin PGE$_2$. This approach has been used by Chen & Hughes-Fulford (2000) and Nithipatikom et al. (2002) in prostate cancer lines, Jabbour et al. (2002) in bladder epithelial cells and Munkarah et al. (2002) in ovarian epithelial cancer cells. However, this technique would not take into account the effects that the presence of the enzyme would have, for example by binding to other proteins and modulating their activity.

In summary, this chapter described the development of a cell line models with elevated cellular COX-2 protein levels. Elevation was required because COX-2 protein could not be detected in either PC-3, LNCaP or DU145 cells. The methods employed to elevate cellular COX-2 were transfection and pharmacological induction. Having established these models, the next chapter is concerned with what effect this elevated COX-2 has on the response of these cells to cytotoxic agents.
CHAPTER 4

EFFECTS OF COX-2 OVEREXPRESSION ON SENSITIVITY TO CYTOTOXIC AGENTS
Chapter 4 Effects of COX-2 Over-expression on Sensitivity To Cytotoxic Agents

4.1 Aims

Having elevated COX-2 levels via either transfection or chemical induction in LNCaP and PC-3 cells, we wished to assess the sensitivity of these transfected prostate cancer cell lines to COX-2 non specific and specific inhibitors in addition to chemotherapeutic agents. The aim of this approach was to assess if cell viability correlated with cellular COX-2 expression levels. Cytotoxic agents can affect cell viability by mechanisms such as growth inhibition, apoptosis or a combination of both. Therefore, we have tried to use more than one technique in order to assess processes relating to growth inhibition or cell death.

4.2 Introduction

In Chapter 3 the development of suitable models of prostate cancer cell lines over-expressing COX-2 were described. Following on from this, we wished to assess the effect that COX-2 over-expression had on the sensitivity of those prostate cancer cells to cytotoxic agents. In this Chapter the effect of COX-2 on cellular response to two disparate groups of cytotoxic agents, chemotherapeutic drugs and COX inhibitors, is described.

The evidence that COX-2 mediates a pro-survival, anti-apoptotic phenotype was reviewed in Chapter 1.2.5.2. This effect has been reported in a variety of cell lines, both cancerous and non-cancerous and with a diverse group of cytotoxic agents. These include cell lines for prostate cancer (Subbarayan et al. 2001), lung cancer (Lin
et al. 2001 and Krysan et al. 2004), colon cancer (Sun et al. 2002 and Tang et al. 2002), cholangiocarcinoma (Nzeako et al. 2002), bladder cancer (Jabbour et al. 2002) and rat intestinal (Tsuji & DuBois 1995) and gastric epithelial cells (Shimada et al. 2000). Transfection was not the sole method used to elevate COX-2 levels in these studies. Some studies used an approach of enzyme induction via inflammatory cytokines and growth factors.

The fact that clinically advanced prostate cancers frequently develop resistance to conventional chemotherapy was discussed in Chapter 1.1.5. Etoposide and carboplatin are two chemotherapeutic agents, which have been used to effectively treat several different cancers, but have little efficacy in the treatment of prostate cancer. As will be discussed in Chapter 5, prostate cancer cell lines may also exhibit a drug resistant phenotype. Possible mechanisms for this resistance will be discussed in the Chapters following, but it can be hypothesised that COX-2 may exacerbate this inherent resistance further.

Etoposide (VP-16) is a topoisomerase II inhibitor, and an established anti-cancer agent. Topoisomerase II is an enzyme which plays an important role in the unwinding and integrity of DNA, by making transient breaks in double stranded DNA. These enzymes are essential for replication, transcription and recombination of DNA (reviewed by Hande 1998). It has been combined clinically with hormonal manipulations in the treatment of prostate cancer (Berchem et al. 1995). Interestingly, etoposide induced cell death may be proliferation independent (Salido et al. 1999).

Carboplatin is a second generation analogue of cisplatin. Both are platinum containing DNA interactive agents. Cisplatin forms DNA adducts which contain DNA-protein or DNA intrastrand and DNA-interstrand cross links. This leads to a distortion in the DNA helix, causing a cell cycle arrest in S phase, and this may manifest in a G2M
blockade and apoptosis (Di Felice et al. 1998). Repair of this damaged DNA can occur by nucleotide excision repair, which is p53 regulated. Cisplatin has been shown to induce apoptosis via caspase-3 activation (Nakamura et al. 2004). Carboplatin is better tolerated than cisplatin, with reduced nausea, vomiting and neuro- and nephrotoxicity side effects but resistance is frequently observed to this agent (Siemer et al. 1999). Thus, both etoposide and carboplatin are classical anti-cancer agents in the sense that they mediate their toxicity by targeting DNA and its replication.

Various studies have reported the sensitivity of prostate cancer cell lines to many anti-cancer agents. Throughout the literature the data sometimes appear contradictory. For example, McEleny et al. (2002) showed that PC-3, LNCaP and DU145 were all resistant to etoposide but previously Salido et al. (1999) reported that etoposide caused cell cycle block and apoptosis in these three prostate cancer cell lines. Lebedeva et al. (2000) showed that Bcl-x<sub>L</sub> down-regulation, an anti-apoptotic protein, sensitised LNCaP and PC-3 to etoposide, mitoxantrone and carboplatin, but Vilenchik et al. (2002) showed the exact opposite, namely Bcl-x<sub>L</sub> down-regulation mediated a resistance to similar cytotoxic agents.

Yee et al. (1998) showed that carboplatin did not cause apoptosis in LNCaP and PC-3 although DNA fragmentation was detected. In the same study these cells were shown to be relatively resistant to paclitaxel also, in comparison to HeLa cells, which were sensitive to the effects of both carboplatin and paclitaxel. Gibbons et al. (2000) demonstrated resistance to etoposide in LNCaP and PC-3 after brief temperature elevation and heat shock protein induction. Haldar et al. (1996) showed that DU145 were sensitive to paclitaxel, but PC-3 and LNCaP were resistant. Wang et al. (1999) reported that LNCaP were sensitive to camptothecin whilst PC-3 were resistant. The
role of PTEN and PI3K signalling will be explored further in Chapter 6, but we can mention here that Yuan & Whang (2002) showed that PTEN transfection into LNCaP gave rise to sensitisation to mitoxantrone, amongst other apoptotic stimuli. Grunwald et al. (2002) used the same principle to sensitise PC-3 to doxorubicin, whilst DU145 did not require this sensitisation as they were inherently sensitive.

This brief review above highlights the difficulty in determining an accurate assessment of the sensitivity of LNCaP, PC-3 or DU145 to chemotherapeutic agents. The reasons for these differences are likely to be similar to those given in Chapter 3, for differences in COX-2 levels in the same cell lines, namely variations in cell line strains, cell culture conditions, the use of different techniques and large differences in passage number, or indeed different clones of the same cell line used.

Sulindac is a non-selective COX inhibitor, an analogue of indomethacin which has been shown to cause apoptosis in prostate cancer cells (Lim et al. 1999). The most commonly used non-specific COX inhibitor, or NSAID is aspirin. As well as inhibiting both COX isoforms, other cellular targets for sulindac and aspirin have been reported. With regards to anti-cancer activity, these include inhibition of IKB kinase (IKK), the inhibitory protein of NF-κB, a transcription factor which can act to attenuate apoptosis (reviewed by Karin et al. 2002). Sulindac is a pro-drug and is metabolised hepatically in vivo to sulphide and sulphone derivatives, but interestingly, sulindac sulphone is devoid of either COX-1 or COX-2 inhibitory activity (Goluboff et al. 1999).

Epidemiological evidence has suggested long term aspirin and other NSAID use provides a protective effect against the development of colorectal cancer e.g. in those individuals with rheumatoid and osteoarthritis who take NSAIDs to relieve the pain.
Effects of COX-2 Over-expression on Sensitivity To Cytotoxic Agents

Chapter 4

and inflammation associated with these conditions (reviewed by Fosslien 2000). This effect appears to be dose and duration dependent. These anti-cancer properties of aspirin in colon cancer are also partly a result of suppression of COX-2 gene expression and not just inhibition of COX-2 activity (Xu et al. 1999).

The adenomatous polyposis coli (APC) gene is located on human chromosome 5q21 and was discovered in FAP patients. Mutations in this gene are responsible not only for FAP, but also other colorectal cancers, and in stomach and oesophagus. An inherited germ line mutation results in the formation of hundreds of polyps. The gene encodes a large protein of 2840 amino acids located on the plasma membrane and is involved in cell migration. Almost all patients with FAP develop colorectal cancer (Steinbach et al. 2000). Early studies demonstrated disappearance of polyps in FAP patients given sulindac resulted in their disappearance and drug discontinuation caused return of the polyps. Subsequently, randomised, placebo controlled studies have been conducted with similar results, however side effects common to other NSAIDs are observed, such as ulceration and bleeding (reviewed by Taketo 1998b).

COX-2 over-expression in prostate and other cancers was reviewed in Chapter 1.2.6. Celecoxib is a COX-2 selective inhibitor and was the first COX-2 inhibitor to be approved by Food and Drug Administration (FDA), in the USA, for the treatment of pain management of rheumatoid arthritis and osteoarthritis in 1998. It has not been licensed yet for analgesia (Davies et al. 2000). However, there are concerns over cardiovascular safety with COX-2 inhibitors as a whole (reviewed by Warner & Mitchell 2004).

As COX-2 is over-expressed in cancer and the enzyme has pro-carcinogenic properties (Chapter 1.2.5), it appears to be a valid target for therapy. The utility of COX-2 inhibitors in cancer therapy is currently being assessed, even though celecoxib
has been licensed for use as an adjunct to standard treatment and surgery in patients with FAP (reviewed by Chau & Cunningham 2002).

Because of the reported resistance observed in both clinical tumours and prostate cancer cell lines to chemotherapeutic agents and the fact that COX-2 is reported to be over-expressed in prostate cancer, the data in this Chapter describe the rationale for assessing the effect of COX-2 over-expression on prostate cancer cell viability. As COX inhibitors also have growth inhibitory and pro-apoptotic activity, and they inhibit the COX isoforms with differing specificities, we have assessed if COX-2 over-expression mediates a differential response to these agents.

**4.3 Results**

**4.3.1 Effect of COX-2 on chemotherapeutic drugs**

We assessed the effect that COX-2 over-expression had on the response of DU145, LNCaP and PC-3 lines to chemotherapeutic drugs. These included a number of drugs which have been tested as potential agents in the treatment of prostate cancer, although their clinical benefit in the treatment of this disease remains unclear.

Although PC-3 expresses some COX-2 mRNA whereas DU145 and LNCaP-Neo do not (Figure 3.1) we have not attempted to correlate the sensitivity of these three lines to their COX-2 mRNA levels. This is because they are genetically different and, therefore, their differential sensitivities to cytotoxic agents are likely to be mediated by a diverse range of other proteins and genes and not by COX-2 specifically. Therefore, we only attempted to correlate the effect of COX-2 on the stable LNCaP and transient PC-3 transfectants and COX-2 induction models. The initial screen for resistance was performed using the MTT cell viability assay.
Figure 4.1 An example of a dose-response curve plotted from a single cell cytotoxicity experiment. The table shows the absorbance values from an MTT assay of DU145 cells treated with VP-16, over the range 0.25-50 μM. The line drawn vertically down represents the IC\textsubscript{50} for this particular experiment.

Figure 4.1 shows an example of a cell cytotoxicity MTT assay. The tabulated data shows the individual absorbance values (in quadruplicate) over 0.25-50 μM VP-16 concentration range. This data was used to plot the graph depicted in Figure 4.1. from this graph, the IC\textsubscript{50} value is interpolated. As described in Chapter 2 Materials and Methods, IC\textsubscript{50} values obtained from a minimum of three experiments were averaged and reported here, along with the SD. This method was used to obtain all IC\textsubscript{50} data presented in this thesis.
Chapter 4 Effects of COX-2 Over-expression on Sensitivity To Cytotoxic Agents

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Etoposide</th>
<th>Carboplatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-3</td>
<td>18.9±6.7</td>
<td>–</td>
</tr>
<tr>
<td>DU145</td>
<td>1.8±0.2</td>
<td>–</td>
</tr>
<tr>
<td>LNCaP-Neo</td>
<td>2.7±1.4</td>
<td>39.0±10.1 **</td>
</tr>
<tr>
<td>LNCaP-COX-2</td>
<td>3.2±1.8</td>
<td>123.0±28.4 **</td>
</tr>
</tbody>
</table>

**p=0.006 Student's t-Test, 2-sided, paired

Table 4.1 Sensitivity of prostate cancer cell lines to chemotherapeutic drugs. Figures show IC$_{50}$ values ($\mu$M) ± S.D. for etoposide and carboplatin as determined by MTT assay. Cells incubated with drug for 72 hours in presence of 10% serum. All experiments were repeated a minimum of 4 times.

Table 4.1 shows the IC$_{50}$ values of parental PC-3 and DU145 cells and the stable LNCaP transfectants to etoposide and carboplatin. In LNCaP, COX-2 over-expression caused a slight but insignificant resistance to etoposide in the presence of 10% serum. However, COX-2 mediated a much larger and significant (p=0.006) resistance to carboplatin. LNCaP-COX-2 is found to be three-fold resistant to carboplatin than LNCaP-Neo, with IC$_{50}$ values of 123.0 $\mu$M ± 28.4 and 39.0 $\mu$M ± 10.1 respectively, as determined by the MTT assay.
Chapter 4 Effects of COX-2 Over-expression on Sensitivity To Cytotoxic Agents

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Etoposide</th>
<th>Carboplatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-3 parental</td>
<td>18.9±6.7</td>
<td>-</td>
</tr>
<tr>
<td>PC-3-Neo</td>
<td>22.3±5.9</td>
<td>85.3±18.1</td>
</tr>
<tr>
<td>PC-3-COX-2</td>
<td>24.6±6.2</td>
<td>84.0±6.0</td>
</tr>
</tbody>
</table>

Table 4.2 Sensitivity of transiently transfected PC-3 cells to chemotherapeutic drugs. Figures show IC\textsubscript{50} values (µM) ± S.D. for etoposide and carboplatin as determined by MTT assay. Parental cells indicate untransfected cells. Cells incubated with drug for 72 hours in presence of 10% serum. All experiments were repeated a minimum of 3 times.

Table 4.2 shows the IC\textsubscript{50} values of transiently transfected PC-3 cells to etoposide and carboplatin. No resistance was observed in PC-3-COX-2 in comparison to either PC-3-Neo or parental PC-3 cells. Thus, although resistance to 72 hour carboplatin treatment is observed in COX-2 over-expressing LNCaP cells, it is not evident in COX-2 over-expressing PC-3 cells. Therefore, sensitivity to carboplatin does not always correlate to levels of COX-2 expression.

Although resistance to etoposide was not observed in LNCaP-COX-2 compared to LNCaP-Neo with 72 hour exposure and in 10% serum, significant resistance to etoposide was observed under some (Figure 4.3) but not other conditions (Figure 4.2).
Figure 4.2 LNCaP-COX-2 cells are not resistant to etoposide over 3 days. Sensitivity of LNCaP-Neo and LNCaP-COX-2 to etoposide for 72 hours in serum free medium. Figures show IC\textsubscript{50} values (\textmu M) ± S.D. determined by MTT assay. Cells were plated in 96 well plates at 3x10\textsuperscript{5} mL, allowed to grow for 48 hours then incubated with etoposide for 72 hours in serum free medium. All experiments repeated a minimum of 4 times. p=0.1, 2-sided, paired Student’s t-Test.

Figure 4.2 shows the difference in IC\textsubscript{50} values for LNCaP-Neo and LNCaP-COX-2 cells when treated with etoposide in serum free medium for 72 hours. No difference in sensitivity to etoposide is detected under these conditions (p=0.1), a situation similar to that observed when cells are grown in 10% serum (Table 4.1). The IC\textsubscript{50} value for etoposide in LNCaP-COX-2 (50.0 \textmu M ± 12.5) is significantly higher than that for LNCaP-Neo (26.0 \textmu M ± 12.2) when cells are incubated in the absence of serum.
Chapter 4 Effects of COX-2 Over-expression on Sensitivity To Cytotoxic Agents

Figure 4.3 **LNCaP-COX-2 cells are resistant to etoposide over 6 days.** Sensitivity of LNCaP-Neo and LNCaP-COX-2 to etoposide for 6 days in serum free medium. Figures show IC$_{50}$ values (µM) ± S.D. determined by MTT assay. Cells were plated in 96 well plates at 3x10$^4$/mL, allowed to grow for 48 hours then incubated with etoposide for 6 days in serum free medium. All experiments repeated a minimum of 4 times. **p=0.01 2-sided, paired, Student’s t-Test.

Figure 4.3 shows the difference in IC$_{50}$ values for LNCaP-Neo and LNCaP-COX-2 cells when treated with etoposide in serum free medium for an extended period of time, 6 days. 6 day drug exposure results in a much greater difference in sensitivity to etoposide (p=0.01), with IC$_{50}$ values of 2.8 µM ± 0.7 and 13.0 µM ± 4.2 for LNCaP-Neo and LNCaP-COX-2 respectively.
4.3.2 Effect of COX-2 on response to COX inhibitors

The third type of pharmacological agent we exposed prostate cancer cells to were the COX inhibitors. Both celecoxib and sulindac have growth arrest and apoptotic inducing abilities (Zweifel et al. 2002, Wu et al. 2003, Lim et al. 1999, Goluboff et al. 1999). We wished to assess if celecoxib, a COX-2 inhibitor in particular, had a differential effect in COX-2 expressing or non-expressing cells.

The sensitivity of all prostate cancer cell lines to COX inhibitors is shown in Table 4.3.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Celecoxib</th>
<th>Sulindac</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-3</td>
<td>75.1±7.9</td>
<td>232.2±48.0</td>
</tr>
<tr>
<td>PC-3-Neo</td>
<td>72.6±9.9</td>
<td>–</td>
</tr>
<tr>
<td>PC-3-COX-2</td>
<td>67.2±9.1</td>
<td>–</td>
</tr>
<tr>
<td>DU145</td>
<td>74.9±7.7</td>
<td>410.0±81.9</td>
</tr>
<tr>
<td>LNCaP-Neo</td>
<td>58.3±16.1</td>
<td>235.0±76.0</td>
</tr>
<tr>
<td>LNCaP-COX-2</td>
<td>63.6±12.6</td>
<td>233.8±75.7</td>
</tr>
</tbody>
</table>

Table 4.3 Sensitivity of prostate cancer cells to COX inhibitors. Figures show IC_{50} values (μM) ± S.D. for celecoxib and sulindac as determined by MTT assay. Parental cells indicate untransfected cells. Cells incubated with drug for 72 hours in presence of 10% serum. All experiments were repeated a minimum of 3 times.
PC-3 expresses some COX-2 mRNA, whereas none is detected in DU145 or LNCaP-Neo (Figure 3.1). Thus, no correlation exists between the levels of COX-2 mRNA or protein and sensitivity to the COX-2 inhibitor celecoxib. It can also be seen from Table 4.3 that COX-2 transfection of LNCaP (which produces elevated COX-2 protein – Figure 3.2) does not mediate a difference in sensitivity to either the COX-2 inhibitor celecoxib or the non-selective COX inhibitor sulindac, as measured by the MTT assay.

As demonstrated in Table 4.3 transient transfection of COX-2 in PC-3 does not mediate a resistance to the effects of celecoxib, despite the fact that COX-2 protein is expressed up to 48 hours post transfection (Figure 3.3). It should be noted again that levels of COX-2 protein in this transient method of transfection in PC-3 cells were appreciably lower than the levels consistently measured in LNCaP. Unlike LNCaP-COX-2, where COX-2 mediates a marked resistance to the growth inhibitory effects of carboplatin (Table 4.1), COX-2 does not have the same effect in PC-3. A possible explanation for this is the much lower levels of COX-2 in the PC-3 model. We did not test if COX-2 expression gave rise to a change in celecoxib sensitivity in PC-3 as observed in LNCaP. However, we hypothesised that it would not for two reasons: COX-2 expression is not as strong and is transient and PC-3 is does not express p53. Possible involvement of p53 in COX-2 mediated apoptotic resistance is discussed further in Chapter 5.

As we had assessed the potential effect of COX-2 on sensitivity to COX inhibitors using only a single assay (MTT) and this assay failed to detect a difference in sensitivity to celecoxib in LNCaP- or PC-3 Neo or COX-2 cells, we performed another assay, Trypan blue exclusion cell counts, to confirm this. The results of these in the LNCaP stable transfectant are shown in Figure 4.4.
Figure 4.4 LNCaP-COX-2 cells are resistant to celecoxib – Trypan blue assay. Percentage of viable cells as determined by Trypan blue counts after 72 hours celecoxib treatment of LNCaP-Neo and LNCaP-COX-2. There are significantly more non-viable LNCaP-Neo cells than LNCaP-COX-2 cells after 100 μM celecoxib (**p=0.004) 2-sided, paired, Student’s t-Test.

This graph shows that at higher celecoxib concentrations (100 μM) a significantly higher percentage of viable cells existed after 72 hour celecoxib treatment in LNCaP-COX-2. With 80 μM celecoxib the viability of LNCaP-Neo and LNCaP-COX-2 are 66% and 93% respectively (p=0.06) and with 100 μM celecoxib the difference in cell viability increases with values of 18% and 40% (p=0.004). These results appear to contradict data from Table 4.3 which show little difference in IC₅₀ between LNCaP-Neo and LNCaP-COX-2 following 72 hour celecoxib treatment. Thus, COX-2 over-
expression causes a resistance to the growth inhibitory effects of celecoxib in LNCaP in a Trypan blue cell viability assay but not the MTT cell viability assay.

### 4.3.3 COX-2 induction in PC-3

It was established that PMA produced a time dependent increase in COX-2 protein in PC-3 cells (Figure 3.4) under serum free conditions. To test if this induction had an effect on the apoptotic sensitivity of PC-3 cells, we treated these cells with celecoxib, sulindac and etoposide, agents which had previously shown to have a growth inhibitory effect in those cells as measured by the MTT assay (Table 4.1 and Table 4.2).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>100 nM PMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Celecoxib</td>
<td>31.8±3.9</td>
<td>34.5±1.3</td>
</tr>
<tr>
<td>Sulindac</td>
<td>128.6±62.6</td>
<td>129.0±57.7</td>
</tr>
<tr>
<td>Etoposide</td>
<td>44.9±14.9**</td>
<td>76.8±19.0**</td>
</tr>
</tbody>
</table>

*^p=0.02 Student’s t-Test, 2-sided, paired

Table 4.4 PMA treatment of PC-3 cells mediates resistance to etoposide. Sensitivity of PC-3 cells to COX non-specific and COX-2 specific inhibitors and etoposide after induction of COX-2 with 100 nM PMA. Figures show IC\textsubscript{50} values (\mu M) ± S.D. as determined by MTT assay. Cells incubated with drug for 72 hours in serum free medium. All experiments were repeated a minimum of 3 times.
Following PMA induction of COX-2 in PC-3 cells in serum free media, no significant differences in IC$_{50}$ for celecoxib or sulindac (Table 4.5) were observed. However, PC-3 demonstrated a significant resistance to etoposide (p=0.02) after 100 nM PMA treatment, as the IC$_{50}$ increased from 44.9 µM in control cells to 76.8 µM in PMA treated cells.

### 4.3.4 Inability of COX-2 enzymatic activity to modulate carboplatin resistance

To investigate the modulation of the sensitivity to carboplatin we see in LNCaP-COX-2, the enzymatic activity of COX-2 was probed. We assessed if PGE$_2$, the major prostaglandin metabolite in these epithelial cells, could cause a resistance in LNCaP-Neo to the effects of carboplatin, in the same way that COX-2 transfection did.
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Figure 4.5 PGE₂ does not increase sensitivity of LNCaP-Neo to carboplatin. Modulation of LNCaP-Neo sensitivity to 10 µM carboplatin by PGE₂ for 72 hours. Figure shows the effect on cell viability of 0.1-20 µg/mL PGE₂ ± S.D. when administered concomitantly with 10 µM carboplatin, as determined by MTT assay. Cells were plated in 96 well plates at 3x10⁶/mL, allowed to grow for 24 hours then incubated with PGE₂ and carboplatin for 72 hours. All experiments were repeated a minimum of 3 times.

Figure 4.5 shows the effect of PGE₂ over a wide, biologically relevant concentration range, on the cell viability of LNCaP-Neo following 72 hour treatment with 10 µM carboplatin treatment. This dose of carboplatin has been previously shown to be cytotoxic to these cells (Table 4.1). However, PGE₂ was not able to significantly modulate the effects of carboplatin in LNCaP-Neo (Figure 4.5). These results shed doubt on the importance of COX-2 enzymatic activity in mediating the observed resistance to carboplatin.
Figure 4.6 Celecoxib does not sensitise LNCaP-COX-2 to carboplatin. Modulation of LNCaP-COX-2 sensitivity to 100 μM carboplatin by celecoxib for 72 hours. Figure shows the effect on cell viability of 1-20 μM celecoxib ± S.D. when administered concomitantly with 100 μM carboplatin, as determined by MTT assay. Cells were plated in 96 well plates at 3×10^3/mL, allowed to grow for 24 hours then incubated with carboplatin and celecoxib for 72 hours. All experiments were repeated a minimum of 3 times.

To confirm if COX-2 catalytic activity played an insignificant role in mediating resistance to carboplatin in LNCaP, the reverse experiment was also performed, namely concomitant COX-2 inhibition with 100 μM carboplatin treatment in LNCaP-COX-2. The results are summarised in Figure 4.6. Celecoxib, at doses at which it is COX-2 inhibitory, rather than cytotoxic, cannot significantly sensitise LNCaP-COX-2 to 72 hours exposure of 100 μM carboplatin treatment. Similar experiments with 24 hour pre-treatment of celecoxib yielded the same result (data not shown).
4.4 Discussion

In this Chapter we have shown that COX-2 mediates a selective resistance to the growth inhibitory or cytotoxic effects of some but not all chemotherapeutic agents and COX inhibitors. Changes in experimental conditions have indicated that loss of drug sensitivity is sometimes dependent on cell culture conditions. COX-2 over-expression in LNCaP, but not PC-3, caused resistance to the effects of carboplatin, etoposide and celecoxib. There may be three possible explanations for this. Firstly, COX-2 levels are lower and are transient only in the PC-3 transfectant in comparison to the stable LNCaP transfectant. Thus, if COX-2 levels in PC-3 could be elevated to the same extent as in LNCaP then it would be interesting to see if the same resistance to carboplatin occurs. Secondly, LNCaP is wild-type for p53, whereas PC-3 is mutant for p53. The role of p53 will be explored in Chapter 5, but it can be hypothesised that any effects COX-2 may have had on growth inhibition or apoptosis in PC-3 may not occur as p53 is not functional in PC-3. Thirdly, as COX-2 (via PGE2) activates aromatase activity (reviewed by Bundred & Barnes 2005), and that estrogens in turn induce COX-2, this positive feedback may occur in LNCaP, but is unlikely to occur in PC-3 (as we did not get strong COX-2 expression and aromatase has not been reported to be detected in PC-3 cells). A review of other cell types in which COX-2 has mediated resistance to specific apoptotic stimuli was reviewed in Chapter 1.2.5.2.

In a standard 72 hour MTT assay where control and drug treated cells are allowed to grow under normal growth conditions, i.e. in the presence of serum, resistance to etoposide in both COX-2 over-expressing LNCaP (Table 4.1) and PC-3 cells (Table 4.2), and also in the absence of serum (Figure 4.2) for LNCaP, is not observed. However, in the absence of serum, conditions in which cell proliferation is reduced in LNCaP, the effects of COX-2 on 6 day (Figure 4.3) etoposide treatment becomes
significant i.e. COX-2 mediates a resistance. A possible explanation for these findings will be elaborated further in Chapter 6, when the role of PI3K/ Akt signalling will be considered.

It is known that a particular cytotoxic drug may require specific culture conditions for cytotoxic effects to be manifest. For example, Chang & Weng (2001) showed that the COX-2 inhibitor NS398 induced apoptosis in a lung adenocarcinoma cell line in serum free conditions. However, in the presence of 10% serum NS398 caused growth arrest rather than apoptosis. It should be noted that NS398 and etoposide are two very different cytotoxic agents and serum may affect their activity in different ways. We do, however, know that agents such as etoposide and celecoxib are extensively protein bound (>90%) based on widely reported in vivo studies. We have not assessed if the effects of switching from 10% serum to a serum free environment causes a change in the mechanism of cell viability reduction in LNCaP cells, but COX-2 appears to change the sensitivity of the cells only in the absence of serum in our cells.

Resistance to the effects of carboplatin were seen in the LNCaP-COX-2 stable transfectant, but not the PC-3 transient transfectant. The MTT assay demonstrated a significant difference in IC$_{50}$ values between COX-2 non- and over-expressing LNCaP cells, with values of 39.0 $\mu$M and 123.0 $\mu$M respectively (Table 4.1). The mechanisms which may be responsible for the difference in sensitivity between these two transfectant lines will be elaborated in Chapters 5 and 6.

Yee et al. (1998) showed that 10 $\mu$M carboplatin, an IC$_{50}$ dose for both LNCaP and PC-3 cells, caused DNA fragmentation but not apoptosis. However, in the same experiments, HeLa cells readily apoptosis. HeLa cells were also considerably more sensitive to paclitaxel than the two prostate cancer cell lines, suggestive of an inherent
resistance mechanism. This dose of carboplatin was lower than the doses we needed to reduce cell viability by 50%.

Siemer et al. (1999) demonstrated apoptosis in testicular carcinoma cells with 800 µM carboplatin but as little as 33 µM cisplatin had the same effect. Itoh et al. (2000) demonstrated apoptosis with as little as 8 µM carboplatin in squamous cell carcinoma lines over 48 hours. Di Felice et al. (1998) also confirmed enhanced potency of cisplatin versus carboplatin, with IC_{50} values of 30 and 200 µM over 4 days, in retinoblastoma cells. Thus, in vitro cell culture experiments show that carboplatin has varying efficacy in different cancer cell lines. In Chapters 5 and 6, the mechanisms of carboplatin induced toxicity in LNCaP COX-2 non- and over-expressing cells will be investigated further.

Surprisingly, inhibition of COX-2 in LNCaP-COX-2 cells, with sub-cytotoxic doses of celecoxib, using drug combination studies with MTT assays and isobologram analysis, did not sensitise this cell line to the effects of carboplatin (Coley HM, personal communication). Similarly, addition of PGE\textsubscript{2} did not cause a carboplatin resistance in LNCaP-Neo cells. These data strongly support the notion that COX-2 is responsible for mediating a prostaglandin independent mechanism that gives rise to resistance to carboplatin. We can postulate that this effect is due to COX-2 binding to other proteins to either enhance or attenuate their activities. Candidate molecules for activation could be any of a number of pro-oncogenic, anti-apoptotic or anti-growth arrest proteins, or COX-2 may bind to and inactivate tumour suppressor gene products, for example p53. We will continue to develop this theme in Chapter 5. Both Trifan et al. (1999) and Zahner et al. (2002) also report COX-2 dependent, but prostaglandin independent effects of the COX-2 enzyme.
Chapter 4 Effects of COX-2 Over-expression on Sensitivity To Cytotoxic Agents

Other groups have demonstrated that NSAIDs and COX-2 inhibitors are able to sensitise cancer cells to chemotherapeutic agents. For example, Ricchi et al. (2002) showed that 24 hours pre-administration of NS398 enhanced apoptotic effects of irinotecan and etoposide in COX-2 expressing Caco-2 cells. 10 and 50 μM NS398 treatment was associated with lower Bcl-2 levels. Hida et al. (2000) used the COX-2 inhibitor nimuselide at sub IC_{50} doses to enhance the sensitivity of various cytotoxic drugs, including etoposide, in lung cancer lines. They also demonstrated that cells expressing COX-2 were more sensitive to nimuselide.

However, the effect of COX-2 over-expression on sensitivity to celecoxib yielded different findings when the Trypan blue exclusion assay was utilised (Figure 4.4). The MTT assay is a colorimetric, absorbance based measure of cell viability and is dependent on mitochondrial metabolism of a tetrazolium salt via dehydrogenase enzymes to generate a signal, i.e. the absorbance measured at 540 nm (Mosmann 1983). Actively proliferating cells with a higher energy requirement will have greater mitochondrial activity than quiescent, growth arrested cells, and will generate a larger signal too, although this assay cannot distinguish between these two processes. In addition, if cells are swollen due to drug treatment this can sometimes give an increased formazan signal. The Trypan blue assay is conceptually very different from the MTT assay. The dye is taken up by non-viable cells only, viable cells exclude the dye, and thus it is an indicator of membrane integrity. Viable and non-viable cells can be visualised under a hematocytometer and counted. However, unlike in the MTT assay in which quiescent and proliferating cells provide a differential signal, the Trypan blue assay discriminates only between viable and non-viable cells. A diagrammatic representation which offers a possible explanation for the different
celecoxib sensitivity data that the MTT and Trypan blue exclusion assays provides is shown in Figure 4.7.

Figure 4.7 The MTT assay versus Trypan blue exclusion. Celecoxib induces a different effect on COX-2 non- or over-expressing LNCaP cells. The pie charts show how the proportion of cells which are proliferating, quiescent or non-viable may be apportioned after 72 hour celecoxib treatment in 10% serum. The actual percentages have not been measured.

The signal generated by the MTT assay for any particular celecoxib concentration, is the same for LNCaP-Neo and -COX-2, and this is indicated by similar IC\textsubscript{50} values of around 60 µM. The total contribution of the two cells in various stages of growth arrest and/or cell death must, in theory, be identical. At 80 and 100 µM celecoxib in LNCaP-Neo there are populations of proliferating and non-viable cells, but for LNCaP-COX-2 there are likely to be fewer proliferating and non-viable cells. We can
postulate the presence of a third population comprising quiescent cells, in LNCaP-COX-2 which provides a smaller signal to compensate for the fewer proliferating cells. However, the Trypan blue assay suggests that there are differences in the cell populations between LNCaP-Neo and LNCaP-COX-2 but these give rise to an identical MTT signal. As summarised in Figure 4.7, celecoxib treatment results in a larger percentage of cell kill in LNCaP-Neo. There are fewer non-viable cells amongst LNCaP-COX-2 as some undergo growth arrest. It should be noted that the proportion of cells shown in Figure 4.7 have not actually been accurately measured, it serves only to illustrate the point that the data from the two assays combined indicates the existence of three cell populations following celecoxib treatment.

PMA was shown previously to induce COX-2 in PC-3 (Chapter 3.3.3). Concomitant treatment of PMA and cytotoxic agents in serum free media in PC-3 cells caused significant resistance to the effects of etoposide, but not celecoxib or sulindac (Table 4.4). This scenario is similar to that observed when using serum free media with the transfected LNCaP lines, as the COX-2 over-expressing cells appeared resistant to etoposide in the absence of serum. As mentioned in Chapter 3.2, PMA mimics the actions of DAG in vitro and therefore is a PKC activator. PMA is also a recognised, established tumour promoter. The significance and specificity of PC-3 resistance to etoposide after PMA induced COX-2 induction is not clear. PMA treatment in these cells may induce many other proteins in addition to COX-2, and these may play a more significant role in resistance to etoposide than COX-2 itself. Furthermore, COX-2 inhibitory doses of celecoxib were not able to reverse the resistance to etoposide caused by PMA induction of COX-2 (data not shown).

Our experiments showed that sulindac was the least potent cytotoxic agent in reducing the viability of prostate cancer cells. COX-2 over-expression in LNCaP cells did not
significantly change the sensitivity to sulindac (Table 4.3). Both PC-3 and LNCaP cells were more sensitive to this NSAID than DU145 cells which had an IC50 of 410 μM. Sulindac was typically 4-5 times less potent than celecoxib in reducing the viability of the prostate cancer cells used in this study (Table 4.3).

Sulindac compounds have previously been shown to induce apoptosis in human colon cancer and prostate cancer cell lines. For example, Lim et al. (1999) demonstrated an apoptotic effect of sulindac metabolites in LNCaP and PC-3 cells and these effects were independent of Bcl-2, Bcl-xL, and COX-2 as two metabolites are devoid of COX-2 inhibitory activity, suggesting that these compounds affect targets other than COX-2. It should be stressed that blood serum levels of 0.5 mM for the sulindac compounds represents values that are ten times higher than those obtained after administration of maximum recommended doses (Andrews et al. 2002). Hanif et al. (1996) used colorectal cell lines which were either COX negative or positive and found that 200 μM sulindac sulphide or 900 μM piroxicam for 72 hours induced apoptosis in both. Further, PGE2 did not rescue the cells from apoptosis, but did increase the proliferation rate. Huang et al. (2001) demonstrated apoptosis in DU145 cells with 200 μM sulindac sulphide and sulphone. Yamamoto et al. (1999) showed a growth inhibitory effect of 1000 μM sulindac and sulindac sulphone and 200 μM sulindac sulphide in COX deficient colorectal cells. It should be noted that they do not report an apoptotic effect of these compounds, even at these doses.

Other potential NSAIDs which have demonstrable apoptotic activity in cancer cells are ibuprofen and naproxen. Andrews et al. (2002) showed that these two agents induced apoptosis in LNCaP and DU145 cells at clinically achievable doses; 1 mM ibuprofen in vitro equates to 3200 mg/day, the maximum clinically acceptable dose.
Goluboff et al. (1999) inoculated LNCaP into athymic nude mice to assess the effects of exisulind, sulindac sulphone, the non-COX inhibitory metabolite of sulindac, on tumour volume and markers for apoptosis and proliferation. Exisulind-fed mice showed significantly smaller tumours with higher apoptotic bodies and lower proliferation.

We have not measured the conversion of sulindac to its biologically active metabolites in this study. Indeed, it is not certain if this occurs at all in our in vitro experiments and this may be an explanation for its lack of potency. Although we have found that sulindac is less potent in mediating a reduction in prostate cancer cell viability and the effects of this NSAID are independent of COX-2 levels, other in vitro cell culture, in vivo animal experiments and clinical data from FAP patients suggest a role for sulindac and its metabolites in the management of prostatic neoplasias.

Celecoxib was virtually equipotent in reducing cell viability of all parental and COX-2 or mock transfected cell lines, with an IC\textsubscript{50} ranging from 60-75 \textmu M. These MTT data suggest that the cytotoxic mechanism of celecoxib is independent of cellular COX-2 expression, p53 status or androgen sensitivity. However, as discussed below, Trypan blue experiments suggested celecoxib may mediate a different effect in COX-2 non- and over-expressing LNCaP cells.

Various in vitro cell culture studies have also demonstrated a beneficial anti-cancer effect of COX-2 inhibitors and a diverse set of molecular mechanisms have been reported. Because COX-2 inhibitors can induce apoptosis in both androgen-dependent (LNCaP) and -independent (PC-3 and DU145) prostate cancer cell lines, the possibility exists that these agents could be efficacious in biologically diverse prostate
cancers. Celecoxib is not the only agent with these properties: NS398 and SC58125 are also two widely used experimental COX-2 inhibitors.

Several studies suggest that COX-2 is a key target for COX-2 inhibitor induced cell kill in prostate cancer cells. Liu et al. (1998) showed that NS398 caused apoptosis in LNCaP cells which expressed COX-2 mRNA, but not in human foetal prostate fibroblasts, which were COX-2 negative. Hsu et al. (2000) demonstrated that 50 μM celecoxib caused apoptosis in COX-2 expressing PC-3 and LNCaP within 3-4 hours, but the COX-1 selective inhibitor piroxicam had no effect. Furthermore, normal prostate cancer cells which did not express COX-2 were insensitive to celecoxib. Although 10 μM celecoxib in this Hsu study reduced the viability of both PC-3 and LNCaP cells, 800 mg/day celecoxib (levels greater than therapeutic levels) equates to less than 10 μM in the blood (Andrews et al. 2002). Kamijo et al. (2001) also demonstrated apoptosis in PC-3 and LNCaP cells with the COX-2 inhibitors etodolac and NS398 and non-COX-2 expressing normal prostate cells were insensitive to these agents. Minter et al. (2003) showed that 75 μM NS398 over 3 days was needed for consistent growth inhibition of oral carcinoma cells, whereas 20 μM only was needed for PGE₂ production inhibition. However, in a 6-12 day assay 5 μM NS398 exhibited growth inhibition, which could be modulated by PGE₂, suggesting COX-2 dependent effects of NS398. Sumitani et al. (2001) showed that 25 μg/mL NS398 over 4 days inhibited the viability of oral cancer cell lines which expressed COX-2 only. 100 pg/mL PGE₂ suppressed the anti-proliferation effect of NS398. COX-2 anti-sense oligonucleotides also inhibited cell proliferation. Hashitani et al. (2003) showed that 10 μM celecoxib caused growth inhibition only in COX-2 expressing head and neck carcinoma cells and not in COX-2 negative cells. 100 pg/mL PGE₂ pre-treatment prevented celecoxib mediated reduction in cell proliferation. Sulindac was
significantly less potent. Souza et al. (2000) showed that 0.1-10 μM NS398, a dose range which was selective for COX-2 inhibition, induced apoptosis in oesophageal adenocarcinoma cells which expressed COX-2 but not in a cell line which did not, whereas a COX-1 inhibitor, flurbiprofen, had no effect in either line. Wu et al. (2003) used both COX-2 expressing and negative cholangiocarcinoma cell lines to assess the apoptotic properties of 40 μM celecoxib. It caused apoptosis only in COX-2 expressing cells and 200 pg/mL PGE$_2$ rescued these cells only from the effects of celecoxib.

It is also accepted that COX-2 inhibitors cause apoptosis in cells in which COX-2 protein or mRNA cannot be detected. Williams et al. (2000) showed that 40 μM celecoxib reduced cell viability of various colorectal cancer lines over 12 hours and induced apoptosis. These effects were COX-2 independent as similar effects observed in COX-2 positive or negative fibroblasts. Grosch et al. (2001) used colon cancer cells which were either COX-2 negative or positive but celecoxib induced apoptosis in both. They also showed that a COX-1 selective inhibitor, SC-560 was less potent than celecoxib. Arico et al. (2002) demonstrated time and dose dependent celecoxib mediated apoptosis in a COX-2 negative colorectal cell line. Yamazaki et al. (2002) assessed the effect of six different COX-2 inhibitors on COX-2 positive and negative colon adenocarcinoma lines. Only celecoxib induced apoptosis, within 6 hours, although all six agents inhibited PGE$_2$ production.

Zhu et al. (2002) provided evidence for COX-2 inhibitory independent apoptotic activity of celecoxib, by synthesising alternative forms of celecoxib and rofecoxib with modified chemical structures. By creating a library of related compounds, they were able to identify chemical groups within the molecules which were responsible for COX-2 inhibition and those that induced apoptosis in PC-3 and LNCaP cells.
There was no correlation between a compound’s ability to inhibit COX-2 or induce apoptosis. Song et al. (2002) also found no correlation between the COX-2 inhibitory and apoptotic activity of celecoxib derivatives in PC-3, LNCaP and DU145 cells. Furthermore, COX-2 ablation did not cause apoptosis, whereas 50 µM celecoxib did. Johnson et al. (2001) showed that 100 µM celecoxib caused rapid apoptosis in PC-3 cells within 2 hours, whereas other COX-2 inhibitors rofecoxib, NS398 and DuP697 were less potent, taking 48 hours. Non selective and COX-1 specific inhibitors did not have this effect. Although COX-2 levels were not assessed, non-COX-2 targets of celecoxib were identified. These targets will be explored further in Chapters 5 and 6.

Data from animal model also provides evidence for an anti-tumour effect of COX-2 inhibitors and implicates COX-2 in the carcinogenic process. For example, NS398 in PC-3 xenografts in nude mice reduced tumour growth by inducing apoptosis and decreasing VEGF and angiogenesis (Liu et al. 2000). Williams et al. (2000) showed that in athymic mice, 1250 mg/kg celecoxib, which equated to 2.3 µM in plasma, a dose which was not toxic in vitro, resulted in reduced tumour volumes. Zweifel et al. (2002) showed that 160 ppm celecoxib and a PGE2 neutralising antibody reduced tumour volume in a head and neck cancer cell xenograft in nude mice, but the COX-1 inhibitor SC-560 did not. Sheng et al. (1997) injected both COX-2 positive and negative COX-2 expressing colorectal cells into athymic nude mice. The COX-2 inhibitor SC-58125, at 5 mg/kg, reduced tumour growth in the COX-2 expressing xenografts only. Gupta et al. (2004) showed that 1500 ppm celecoxib given to TRAMP mice (transgenic adenocarcinoma of the mouse prostate model) over 8-32 weeks reduced tumour incidence and metastasis, reduced COX-2 levels and activity, increased apoptosis (in vivo AV assay), decreased differentiation status of tumours, reduced proliferating cell nuclear antigen (PCNA) and VEGF, caused retention of E-
cadherin and catenins and increased survival. Also, COX-2 protein and activity was higher in TRAMP mice prostates compared to non-transgenic littermates. In support of these findings COX-2 knock out mice have been shown to exhibit reduced tumourigenesis (reviewed by DuBois et al. 1998).

What contribution does COX-2 inhibition have in modulating cell viability due to treatment with COX-2 inhibitors? The review of the above studies suggests that this is a cell line specific effect. It is likely that the answer to this question is dependent on cell type and the particular COX-2 inhibitor used. By inference, how important is COX-2 inhibition in mediating anti-tumour activity? The two questions are subtly, but crucially different. Because COX-2 inhibitors may be mediating an apoptotic or growth inhibitory effect independently of COX-2 in vitro, COX-2 is also expressed in other cell types associated with tumour growth (Chapter 1.2.6) and when administered in vivo, COX-2 inhibitor anti-tumour activity may well be COX-2 dependent. Even though agents such as celecoxib kill cells which do not express COX-2, expression levels of the enzyme itself can mediate a resistant phenotype.

Data from clinical trials also provides evidence for an anti-cancer effect of celecoxib in human malignancy and pre-malignant conditions. In a placebo controlled study to assess the effects of celecoxib, 100 mg or 400 mg twice daily, on colorectal polyps in FAP patients, Steinbach et al. (2000) report a dose dependent reduction in the number of polyps after 6 months with celecoxib.

In summary, this Chapter showed that both transfection and induction of COX-2 into prostate cancer cells mediated a resistance to the cytotoxic effects of etoposide and carboplatin under different conditions and COX-2 over-expression also had the same effect on the activity of celecoxib. We have also reviewed the evidence in the
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literature which suggests that COX-2 inhibitors reduce the viability of cancer cells independently of COX-2. Having established an effect of COX-2 on cell viability, in the next Chapter we try to determine the exact growth inhibitory and cell cycle arrest mechanisms underlying this resistance.
CHAPTER 5

EFFECTS OF COX-2 ON THE CELL CYCLE AND APOPTOSIS
Chapter 5 Effects of COX-2 on the Cell Cycle and Apoptosis

5.1 Aims

As COX-2 was shown to mediate an attenuation of the effects of carboplatin, etoposide and celecoxib under various conditions in stably transfected LNCaP-COX-2 cells, we wished to assess the mechanisms which may be responsible for this. Consequently, we measured changes in cell cycle events and apoptotic markers caused by elevation of COX-2 in LNCaP cells. We concentrated on the resistance observed with carboplatin because it was achieved when cells grew normally in serum (which was not the case for etoposide) and was confirmed by the MTT cell viability assay (which was not the case for celecoxib).

5.2 Introduction

5.2.1 The Cell Cycle and its Regulation

In the context of the cell cycle, the cell has three options (i) remain metabolically active but not undergoing either proliferation or death, i.e. be quiescent or in G0; (ii) undergo DNA synthesis (S), mitosis (M) and divide i.e. proliferate; (iii) undergo apoptosis (reviewed by Denmeade et al. 1996). Cell cycle progression has two main checkpoints; G1S prior to DNA replication and G2M prior to mitosis. In cancer development, the disruption of the cell cycle can be an important event. G1 is the phase at which the main regulatory events for cell proliferation occur. Possible events in tumourigenesis include changes in cyclins and cyclin dependent
kinases (CDKs) during G1 (reviewed by Kyprianou et al. 2000). Cyclin D1 and its catalytic subunits CDK4 and 6 are activated in mid-G1. Cyclin E is a late G1 cyclin, which combined with its catalytic subunit CDK2, phosphorylates the retinoblastoma (Rb) protein. Cyclin E/CDK2 is needed for G1 to S phase transition, whereas Rb regulates or inhibits G1 to S phase transition. Growth arrest correlates with dephosphorylation and activation of Rb, whilst the reverse occurs for progression from G1 to S phase. Rb associates with the E2F family of transcription factors, preventing them from promoting cell proliferation. Over-expression of cyclin E accelerates G1 to S phase progression. Activity of the cyclin E/CDK2 complex is also regulated by Cip/Kip family of CDK inhibitors. CDK inhibitors bind to the CDK/cyclin complex and inhibit their activity. Two classes of inhibitor exist; the p21 family (p21WAF1, p27KIP1, p57KIP2) and the INK4 family (p16, p15, p18, p19) (Cheng et al. 2004).

Loss or decreased expression of p27KIP1 can be a prognostic marker for a number of malignancies (reviewed by Kyprianou et al. 2000). p27KIP1 regulates the G1S checkpoint, as does p21WAF1. Cyclin A/CDK2 is needed for entry into S phase, i.e. for DNA synthesis to begin. Cyclins A and B and cdc2 (also referred as CDK1) are needed for G2M transition. p21WAF1 regulates both G1S and G2M checkpoints (Cheng et al. 2004).
p53 is a transcription factor which regulates cell cycle progression and is considered very important in cancer because of its large number of downstream targets. p53 can either instigate DNA repair in response to damage, inhibiting cell cycle entry or induce apoptosis. Thus, it is a multi-faceted tumour suppressor and plays a key role in the defence against cancer. p53 is also plays a key role in stress induced apoptosis and can induce the expression of genes involved in the mitochondrial pathway of apoptosis.

Figure 5.1 The Cell Cycle and its Regulation. The phases of the cell cycle, the various cyclins and CDKs which drive it, and some regulatory aspects. See text for details.
apoptosis (e.g. Bax, apoptotic protease activating factor-1 [Apaf-1]) or the death receptor pathway (e.g. CD95/ Fas, FADD, DR4, DR5) (see section below) (reviewed by Igney & Krammer 2002). Other p53 responsive genes include p21\textsuperscript{WAF1}, which mediates a G\textsubscript{i} arrest as described above, and it down-regulates Bcl-2, an anti-apoptotic protein (reviewed by Kyprianou \textit{et al.} 2000), PTEN, WIP1 and IGF-BP3 (reviewed by Vousden & Lu 2002).

Most cells are programmed to stop proliferating or to kill themselves, by apoptosing, if survival signals are not regularly received from their environment. Also, if there is DNA damage, molecules such as the tumour suppressor p53 are activated by phosphorylation, which regulates its DNA binding ability and therefore transcriptional activity, leading to cell cycle arrest or apoptosis. Thus, in response to DNA damage, a cell can initiate repair processes, arrest growth or undergo apoptosis and p53 is central to this (reviewed by Tapia-Vieyra & Mas-Olivia 2001). Various stress signals (loss of survival signalling, DNA damage, hypoxia or microtubule inhibitors) induce p53 by stabilising the protein, increasing its levels.

We have discussed previously (Chapter 1.2.4) that COX-2 transcription is negatively regulated by p53 in some cell types. Cells negative for or expressing mutant p53 could allow increased COX-2 induction by oncogenes, growth factors or cytokines. Also, p53 decreases synthesis of VEGF and inhibits tumour cell induced angiogenesis. Repression of COX-2 transcription could be important for p53 mediated apoptosis, as over-expression of COX-2 results in resistance to apoptosis following diverse cytotoxic insults (Subbaramaiah \textit{et al.} 1999 and Chapter 1.2.5.2).

Because COX-2 has been reported to enhance cellular proliferation (Chapter 1.2.5.1), and to modulate cell cycle parameters (Chapter 1.2.5.6), and our data showed that
Chapter 5 Effects of COX-2 on the Cell Cycle and Apoptosis

over-expression of the enzyme was concomitant with resistance to carboplatin, celecoxib and etoposide (Chapter 4), we wished to explore further the mechanisms which may be responsible for this resistance in COX-2 over-expressing LNCaP cells.

5.2.2 Apoptosis

Apoptosis is a form of programmed cell death, distinct from necrosis. In apoptosis the cell plays an active part in its own demise, whereas it is passive in necrosis. In necrosis, pathological changes result in increased plasma membrane permeability, cellular oedema, whereas in apoptosis the cell death is orderly, energy dependent and signal specific (Chan et al. 1998).

Apoptosis is a widespread phenomenon, occurring in processes such as morphogenesis, growth and development and normal turnover in adult tissue. It can be initiated by endogenous tissue specific agents such as hormones or cytokines or exogenous cell damaging agents such as radiation, virus, toxins or chemicals (reviewed by Earnshaw et al. 1999).

It is a complex process involving many molecules which may act in distinct ways depending upon the cell type. Most cells are programmed to die if survival signals are not received regularly from their environment. Apoptosis occurs frequently when cytokines or growth factors are removed in several cell types. As inappropriate apoptosis may be involved in a number of diseases, apoptosis is an important potential target for therapeutic intervention (reviewed by Riss 2001).
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5.2.2.1 Characteristics of Apoptosis

The process of apoptosis has distinct morphological and biochemical characteristics and can be divided into 4 stages: (i) reception of either external or internal stimuli (ii) transduction of these stimuli (iii) an effector phase where nuclear, biochemical and cytoskeletal changes occur (iv) a post-mortem phase which involves phagocytosis of the apoptosed cell. Characteristics of apoptosis include DNA fragmentation and loss of mitochondrial function which has three apoptotic effects: an alteration of electron transport and ATP production and general disruption of cellular redox potential, release of apoptotic molecules, and activation of caspases, the protease effectors of this phenomenon (reviewed by Tapia-Vieyra & Mas-Oliva 2001). Morphological characteristics of apoptosis include chromatin aggregation, nuclear and cytoplasmic condensation and fragmentation of cells into apoptotic bodies, which are membrane bound segments containing cellular organelles. These bodies are readily phagocytosed. The fragmentation of genomic DNA commits the cell to die and occurs before changes in membrane permeability (reviewed by Denmeade et al. 1996). The biochemical characteristics of apoptosis include phosphatidylserine (PS) externalisation, translocation of molecules between cellular locations and cleavage of specific cellular polypeptides, mainly by the caspases (reviewed by Earnshaw et al. 1999).

5.2.2.2 Apoptotic Pathways

It is accepted that at least two apoptotic pathways exist, the receptor or extrinsic pathway and the intrinsic or mitochondrial pathway. The two pathways can function separately or interact together to execute the apoptotic program.
Death Receptor Pathway

The receptor pathway can be activated by one of three endogenous ligands upon binding of their receptors; Fas or CD95, Tumour necrosis factor related apoptosis inducing ligand (TRAIL) and TNF-α.

Fas or CD95 is a glycosylated cell surface molecule which can be up-regulated by cytokines such as interferon-γ and TNF-α. Fas belongs to the TNFR family of receptors. Fas mediated apoptosis is triggered by Fas ligand (FasL or CD95L) which can exist as a transmembrane molecule as well as a cytosolic form. There are two different pathways downstream of Fas: either caspase-8 is activated at the receptor-ligand complex or the mitochondria amplifies the cascade signal if the complex of proteins do not form with Fas (reviewed by Igney & Krammer 2002).

The DISC (death inducing signalling complex) is an assembly of adapter molecules which form at the ligated receptor as part of the signalling process. The DISC comprises activated Fas, the Fas associated death domain (FADD or MORT1) and the initiator caspases-8 and -10. These adaptor molecules allow higher oligomerisation of receptor molecules and better recruitment of zymogens to the complex (reviewed by Earnshaw et al. 1999). Then activated caspase-8 leaves the DISC and activates effector caspases. Caspase-8 also cleaves pro-apoptotic Bid, which induces Cytochrome c (cyt c) release; thus Bid provides a link between the receptor and mitochondrial pathways. However, in some cell types Fas mediated apoptosis is mitochondrial independent as Bcl-2 does not protect against Fas killing i.e. the DISC is sufficient to activate downstream caspases to cause apoptosis (Gewies et al. 2000b).

C-FLIP (FLICE inhibitory protein) is a caspase-8 homologue which lacks the catalytic site and inhibits apoptosis at the DISC by preventing caspase-8 activation. It has two
forms: c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub> (55 and 26 kDa respectively). This is another mechanism to regulate caspase activity. In some cells high c-FLIP expression correlates with a TRAIL resistant phenotype in cancer cells (Kelly et al. 2002).

Some studies report that PC-3, LNCaP and DU145 all express the Fas receptor (Rokhlin et al. 1997). However, Takeuchi et al. (1996) did not detect Fas in both PC-3 and LNCaP. As will be discussed later, these reported differences in expression of apoptosis inducing agents or their receptors in prostate cancer cells does not always account for the reported resistance or sensitivity of these cells to either Fas, TRAIL or TNF-α.

TRAIL (or Apo-2 ligand) is a member of the TNF super family of cytokines. TRAIL has homology with FasL and TNF-α (Rokhlin et al. 2002). Four receptors for TRAIL have been identified; TR1 or DR4 and TR2 or DR5 which induce apoptosis as they contain cytoplasmic death domains, whereas TR3 and TR4 do not. These ‘decoy’ receptors also bind TRAIL but have truncated death domains which cannot transduce the death signal (Kelly et al. 2002).

TRAIL appears to be far less toxic than Fas. Fas has been shown to cause hepatocyte degeneration, necrosis, haemorrhage and death (Griffith et al. 2000). Moreover, TRAIL mRNA is constitutively expressed in a number of cells but FasL is more tightly regulated, due to its toxicity (Griffith et al. 2000). TRAIL appears to differentiate between tumour and normal cells, unlike Fas and TNF-α. But a large number of tumour cells are still resistant to TRAIL (Sah et al. 2003). TRAIL and its receptors are ubiquitously expressed in tissues, suggesting that mechanisms exist which protect normal tissues from TRAIL induced apoptosis (Nesterov et al. 2001).
The TRAIL DISC involves recruitment of FADD and caspase-8 also and thus is similar to Fas signalling in this respect (Rokhlin et al. 2001).

TNF-α mediated apoptosis occurs via binding to its receptor, TNFR1. TNF-α apoptotic signalling is similar to Fas mediated apoptosis as both involve intracellular domains which contain death domains; FADD/MORT1 for Fas, TRADD for TNFR1.

One pathway activated after chemotherapy is the SAPK or JNK stress activated pathway (see Chapter 1.2.4). SAPK regulates the activity of the AP-1 transcription factors, whose targets include FasL and TNFα (reviewed by Igney & Krammer 2002).

**Mitochondrial Pathway**

The role of Bid as the link between death receptor activation and the mitochondrial pathway was briefly described above. Bid causes mitochondrial membrane depolarisation to release apoptotic factors such as cyt c and SMAC. This leads to formation of the apoptosome, a term for the second initiator complex. This consists of mitochondrial factors (cyt c, second mitochondria derived activator of caspase (SMAC) and direct IAP binding protein with low pl (DIABLO)) and cytoplasmic factors Apaf-1 and pro-caspase-9). IAPs are inhibited by SMAC/ DIABLO (reviewed by Igney & Krammer 2002). Apaf-1 binds cyt c, and then binds procaspase-9, activating it, although mechanism of cyt c mitochondrial release is unknown (Earnshaw et al. 1999). Once caspase-9 is activated it is able to activate downstream effector caspases.

In non-apoptotic cells SMAC/ DIABLO is located on the inner mitochondrial membrane space and is released along with cyt c during apoptosis, where it binds
XIAP and other IAPs, preventing their action (reviewed by Salvesen & Duckett 2002).

5.2.2.3 Pro- and Anti-apoptotic factors

Pro-apoptotic Factors

Proteases play a crucial role in apoptosis, particularly the caspases (the cysteine dependent aspartate directed proteases). Proteins cleaved during apoptosis include (i) cytoplasmic and cytoskeletal (ii) nuclear (iii) DNA repair, metabolism and structure e.g. PARP (iv) protein kinases e.g. PKC, ERK, Akt (v) signal transduction e.g. interleukins and PLC (vi) cell cycle and proliferation regulation (vii) apoptotic regulators, both survival and pro-apoptotic. Some substrates are cleaved later and less completely, some are cleaved in some cells but not others and some are cleaved in different locations depending upon cell type (reviewed by Earnshaw et al. 1999).

Three levels of caspase regulation exist; (i) gene transcription (ii) anti-apoptotic members of Bcl-2 family that inhibit proximity induced activation of some procaspases and (iii) cellular inhibitors which bind to caspases, the IAPs. Caspases can efficiently activate other caspases, for example caspase-8 cleaves pro-caspases-3, -4, -7 and -9 \textit{in vitro} and caspase-10 cleaves pro-caspases-3, -7 and -8 \textit{in vitro}. Caspase-9 also activates caspase-3 (reviewed by Earnshaw et al. 1999). Because all caspases have similar cleavage specificities, an activated caspase can activate a number of pro-caspases (reviewed by Tapia-Vieyra & Mas-Oliva 2001).

Effector caspases include -3, -6, -7 and -9 (reviewed by Kyprianou et al. 2000). Caspase-3 exists as a 32 kDa inactive precursor and is activated by cleavage during apoptosis into 20 and 10 kDa active products (Yang et al. 1997). Nicholson et al.
(1995) showed that caspase-3 cleaves poly[adenosine diphosphate-ribose]polymerase (PARP), a DNA repair enzyme, cleavage of which commits cells to apoptosis (reviewed by Kyrpianou et al. 2000). Cardone et al. (1998) showed that pro-caspase-9 was inactivated by phosphorylation via Akt. Initiator caspases include -1, -2, -8 and -10.

Pro-apoptotic factors promote cell death. When Bcl-2 associated x-protein (Bax) homodimerizes it enhances apoptosis. The anti-apoptotic effect of Bcl-2 may be due to its ability to inhibit Bax homodimerization (Denmeade et al. 1996). Bax inserts into mitochondrial membrane, induces hyperpolarisation of the proton gradient, triggers cyt c release and generates reactive oxygen species. But Bax can kill independent of Apaf-1 and cyt c by generating reactive oxygen species which damage molecules and membranes (reviewed by Jin & Reed 2002).

Bad is a member of the Bcl-2 family that promotes cell death and is resident in the cytosol. BAD exerts its apoptotic effects by combining with the anti-apoptotic Bcl-xL. Del Peso et al. (1997) demonstrated that Bad can be phosphorylated and inactivated by Akt. Bad phosphorylation by P13K was confirmed by Datta et al (1997). Treatment of cells with an inhibitor of MEK did not significantly block BAD phosphorylation, whereas inhibition of PI3K did. BAD phosphorylation at one or both of its sites (ser-112 or ser-136) prevents its interaction with Bcl-2 or Bcl-xL.

Cyt c is located in the mitochondrial inter-membrane space and its release into the cytosol is necessary for the mitochondrial pathway of apoptosis. Cyt c is released into the cytosol is required for caspase-3 activation. It is synthesised by cytosolic ribosomes as apocytochrome c and then translocated to the mitochondria where covalent attachment of haem group occurs (Yang et al. 1997). Caspase-9 is activated
by cyt c combining with Apaf-1 (apoptotic protease activating factor) – this is the
apoptosome (reviewed by Igney & Krammer 2002).

Ceramide is one example of lipid second messengers involved in signal transduction.
Actions of ceramide include apoptosis, cell proliferation, differentiation and
inflammation and modulating immune system. Apoptotic or mitogenic effects of
ceramide appear to be cell type specific (reviewed by Ballou et al. 1996).

Activating sphingomyelinase leading to ceramide production could play an important
part in apoptosis. Zhou et al. (1998) demonstrated that ceramide inhibited Akt and
therefore decreased activity of a survival pathway. Targets for ceramide include
ceramide activated protein kinase and ceramide activated protein phosphatase, but it is
not known how ceramide decreases Akt activity.

**Anti-apoptotic Factors**

Anti-apoptotic factors include Bcl-2, Bcl-xl, Mcl-1 and the IAPs. They mediate cell
survival. Bcl-2 family members regulate apoptosis at the mitochondrial level. Some
are pro-apoptotic (described above); here some of the anti-apoptotic, oncogenic
members are briefly described.

Bcl-2 is a 26 kDa protein located mainly on the outer mitochondrial membrane. Its
over-expression prevents cells from undergoing apoptosis in response to a variety of
stimuli (reviewed in the Discussion). Many homologues of Bcl-2 have been described.
They include Bcl-2 itself, Bcl-xl and Bax. They form homo- or heterodimers with
each other or with pro-apoptotic Bcl-2 family members (reviewed by Denmeade et al.
1996). Purified recombinant Bcl-2 exhibits pore forming activity. Its induced over-
expression can lead to malignant transformation, but it is unique among the oncogenes
because it decreases apoptosis, rather than increasing proliferation rate. Haldar et al.
Chapter 5 Effects of COX-2 on the Cell Cycle and Apoptosis

(1995) showed that Bcl-2 serine phosphorylation resulted in its inactivation. Mcl-1 is a cell survival factor, induced by a number of growth factors and cytokines. For example, Lin et al. (2001) demonstrated the importance of Mcl-1 in mediating a resistant phenotype in lung adenocarcinoma cells.

Seven members of the IAPs have been identified in human cells: NIAP, cIAP1, cIAP2, XIAP, survivin, apollon and livin. All except NIAP inhibit caspases-3 and -7, cIAP1, cIAP2 and XIAP also inhibit caspase-9 (Mceleny et al. 2001). Caspase inhibition is not the only function of IAPs. They are also involved in cell cycle regulation, protein degradation and caspase independent signal transduction pathways.

Whilst the first level of caspase regulation is by controlling zymogen activation, the second level involves IAPs, which inhibit activated caspases. Survivin transcription is controlled in a cell cycle dependent manner and is induced at the G2M boundary. XIAP, cIAP2 and Bcl-2 are regulated by NFkB (reviewed by Salvesen & Duckett 2002).

Elevated survivin has been reported in many cancers, including prostate (Krajewska et al. 2003). As survivin is associated with poor survival and more aggressive cancers it has been postulated as a potential prognostic marker. For example, in gastric cancer patients who received cisplatin, survival was longer for patients with low survivin (38 months) in comparison to patients with raised survivin (24 months) (Nakamura et al. 2004).
The apoptotic response and some key factors involved in this process is summarised in Figure 5.2. It shows how the death receptor and mitochondrial pathways can operate independently or in concert to effectuate apoptosis.
5.2.2.4 Apoptotic Defects in Cancer

The biochemistry of apoptosis has been briefly outlined above. When cells no longer receive a growth stimulus (androgen ablation for prostate cancer for example) or their DNA is damaged (treatment with carboplatin for example) it is intended that they commit to the apoptotic program. Cancer cells frequently acquire genetic changes which allow them to survive signals which would normally kill them. This can occur either by inactivation of apoptotic factors or over-expression/ activity of anti-apoptotic factors. COX-2 involvement in mediating a resistance to apoptosis was reviewed in Chapter 1.2.5.2. Prostate cancer cells are resistant to apoptosis inducing agents (Chapter 4 and this Chapter), and this could be achieved by redundancy in the systems involved in preventing, repairing or tolerating DNA damage. This means that a cell may have several anti-apoptotic mechanisms and to enhance therapy, targeting of more than one pathway may be required. Here we discuss other reported changes in cancer cells which can mediate an oncogenic, anti-apoptotic phenotype.

Changes implicated in modulating the apoptotic threshold in advanced prostate cancer include p53 mutation, Bcl-2 over-expression and androgen receptor constitutive activity or amplification. Other genes that might influence the apoptotic threshold are retinoblastoma, c-myc, p21WAF1, p16, PTEN and ras (reviewed by Howell et al. 2000). Reported inactivation/ down-regulation of pro-apoptotic factors implicated in carcinogenesis include Bax (which is induced by p53), Apaf-1, caspase-8, Fas and DR4 and DR5 (reviewed by Igney & Krammer 2002). FLIP has been shown to be up-regulated in some tumours as have death receptor decoys. Survivin, the IAP is found in most tumours but not normal tissues (reviewed by Igney & Krammer 2002).

It is thought that androgen ablation in androgen independent cells does not result in sustained increase in intracellular calcium. Experiments show that androgen
independent prostate cancer cells can be induced to apoptose at any stage of the cell cycle i.e. is proliferation independent, by increasing intracellular calcium for a sufficient time (reviewed by Denmeade et al. 1996). Thus, calcium could be a target for therapy for androgen independent cells, in particular the endoplasmic/sarcoplasmic reticulum Ca\(^{2+}\)-ATPase. Treatment of androgen independent prostate cancer cell line with thapsigargin, an inhibitor of ER/SR Ca\(^{2+}\)-ATPase, results in increase in intracellular calcium and eventually apoptosis after characteristic biochemical and morphological changes (reviewed by Tapia-Vieyra & Mas-Oliva 2001).

In cancer, alterations in either the DNA damage sensing machinery or the mechanisms that implement the response become important. One role of p53 is to transduce DNA injury stimuli into the apoptotic or cell cycle arrest programme. p53 is mutated in half of all cancers. The majority of these are point mutations, resulting in amino acid substitutions (reviewed by Vousden & Lu 2002).

CDKN2A is the gene which encodes for ARF, which binds to and inactivates MDM2, which targets p53 for degradation). This mutation is common in many tumours. Sometimes wild type p53 is expressed but it fails to suppress tumour growth and this may be due to lack of ASPP (apoptosis stimulating protein of p53), which enhances the transcriptional activity of p53 (reviewed by Igney & Krammer 2002).

Various studies show Bcl-2 over-expression in cancer cells and tumour samples in comparison to normal, non-cancerous samples. These will be reviewed in greater detail in the discussion. Alterations in the PTEN and PI3K pathways is frequently cited in the literature as causative factors in carcinogenesis. This will be discussed further in Chapter 6. Indeed, PTEN is one of the most frequently mutated genes in human cancer (Yuan & Whang 2002).
The experimental evidence for the involvement of COX-2 in attenuating apoptosis was presented in Chapter 1.2.5.2. In this Chapter we have briefly described events in cell cycle control and apoptosis. We now present our data which demonstrates the role of COX-2 in modulating the cell cycle and apoptosis.

5.3 Results

5.3.1 Effects of COX-2 on the cell cycle

We hypothesised that the resistance mediated by COX-2 in LNCaP cells to carboplatin could be caused by modulation of the cell cycle, apoptosis or both. We assessed the effect of COX-2 on cell cycle events by a variety of mechanisms. These included PI staining by flow cytometry and measuring changes in proteins levels of p53, p27KIP1 and cyclin B1, key molecules involved in the control of the cell cycle. Activation of cyclin B1 has been shown to facilitate the transition from S phase to the G2M phase. These phases of the cell cycle are important when considering the effects of agents like carboplatin on cell cycle blockade.
Figure 5.3 Changes in the cell cycle caused by carboplatin in LNCaP-Neo and LNCaP-COX-2. DNA histograms measured by flow cytometric analysis of PI binding after 24 and 48 hours treatment with 70 μM carboplatin. Cells were harvested, fixed at 4°C for 24 hours, incubated with 0.8 mg/mL ribonuclease A and 10 μg/mL PI and analysed using a Beckman Coulter Epics XL flow cytometer, as described in Chapter 2.9. 48 hour data from a further three experiments is tabulated in the appendix.
Chapter 5 Effects of COX-2 on the Cell Cycle and Apoptosis

Figure 5.3 shows examples of typical data obtained from PI analysis of DNA content. The percentages in each histogram is an arbitrary assignment of the cell cycle phases, G1, S and G2M, respectively, based on DNA content, following 24 or 48 hours carboplatin treatment. These data show that there is a slight differential cell cycle response between LNCaP-Neo and LNCaP-COX-2 following 70 μM carboplatin treatment, in particular an S phase block, which is evident at 24 hours, but becomes more prominent at 48 hours. At 24 hours the increases in the S phase cell population from control is 11% and 5% for LNCaP-Neo and LNCaP-COX-2 respectively and 34% and 26% at 48 hours. The cell cycle data from a further three experiments is tabulated in the appendix.

Carboplatin is more commonly associated with causing a G2M block (Dhanalakshmi et al. 2003) in DU145 cells. It is also reported to not cause apoptosis in LNCaP and PC-3 cells (Yee et al. 1998). In our LNCaP transfected cells the effect of carboplatin is more growth inhibitory and results in the non-classical S phase we observe. Because sufficient statistical analysis of the cell cycle effects of carboplatin was not performed, cell cycle effects have been assessed by other methods also for confirmation of these effects. However, these experiments demonstrated that the resistance of LNCaP-COX-2 to carboplatin, as measured by the MTT cell viability assay (Chapter 4) relates to cell cycle involvement.

Because there is a differential effect of COX-2 in LNCaP on the cell cycle, as assessed by PI binding, we next probed the molecular nature of these events, by looking at basal levels and changes in levels of proteins which have cell cycle regulatory activity, following carboplatin treatment. These included p53, p27KIP1 and cyclin B1.
Figure 5.4 p53 induction following carboplatin treatment is attenuated due to COX-2. Levels of p53 protein in control or carboplatin treated LNCaP-Neo and LNCaP-COX-2 cells. Both attached and floating cells were harvested following 72 hour treatment with 100 μM carboplatin, as described in Materials and Methods. 50 μg protein was electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose membranes and exposed to murine IgG monoclonal p53 antibody (Santa Cruz Biotechnology) at a 1:1000 dilution and a murine IgM β-actin antibody (Oncogene) at a 1:10000 dilution. HRP conjugated antibodies to detect murine IgG (Sigma) and IgM (Calbiochem) were used at a 1:2000 dilution. Bands were detected using ECL (Pierce).
It can be seen from Figure 5.4 that p53 protein is virtually undetected in control LNCaP-Neo and LNCaP-COX-2 cells, but after 100 μM carboplatin treatment for 72 hours, there is greater p53 induction in Neo versus COX-2 cells. These data are in agreement with the IC₅₀ values obtained using carboplatin (Table 4.1). The induction of p53 due to DNA damage recognition seen in Neo cells is greater than in COX-2 cells where presumably less damage is recognised as these cells are more tolerant to the effects of carboplatin. These finding suggest that COX-2 mediated attenuation of p53 induction is in part responsible for the carboplatin resistance seen in LNCaP-COX-2.

The CDK inhibitor p27^KIP1 is a downstream effector of p53. Because COX-2 mediated a reduced p53 response after carboplatin treatment, we next assessed if the enzyme had the same effect on p27^KIP1 transactivation.
Figure 5.5 Transactivation of p27KIP1 following carboplatin treatment is attenuated due to COX-2. Levels of p27KIP1 protein in control or carboplatin treated LNCaP-Neo and LNCaP-COX-2 cells. Both attached and floating cells were harvested following 48 hour treatment with carboplatin, as described in Materials and Methods. (a) control cells (b) 10 µM (c) 20 µM (d) 50 µM (e) 100 µM treated cells. 50 µg protein was electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose membranes and exposed to rabbit IgG polyclonal p27KIP1 antibody (Santa Cruz Biotechnology) at a 1:1000 dilution and a murine IgM β-actin antibody (Oncogene) at a 1:10000 dilution. HRP conjugated antibodies to detect rabbit IgG (Dako) and murine IgM (Calbiochem) were used at a 1:2000 dilution. Bands were detected using ECL (Pierce).
Chapter 5 Effects of COX-2 on the Cell Cycle and Apoptosis

It can be seen from Figure 5.5 that both LNCaP-Neo and LNCaP-COX-2 do not express p27\textsuperscript{kip1} basally. Following 48 hour carboplatin treatment, p27\textsuperscript{kip1} transactivation occurs for both cell lines, but there is reduced induction in LNCaP-COX-2. This is consistent with the data presented in Figure 5.4, which show a reduced p53 response in COX-2 over-expressing LNCaP cells. This may not be surprising as p27\textsuperscript{kip1} is a down-stream transcriptional target of p53. Furthermore, as p27\textsuperscript{kip1} inhibits S phase kinases and we demonstrated a greater S phase block in LNCaP-Neo (Figure 5.3), it is possible that this is due to greater transactivation of p27\textsuperscript{kip1} in LNCaP-Neo.
Figure 5.6 Changes in the cell cycle caused by celecoxib in LNCaP-Neo and LNCaP-COX-2. DNA histograms measured by flow cytometric analysis of PI binding after 24 hours treatment with 20 μM celecoxib. Cells were harvested, fixed at 4°C for 24 hours, incubated with 0.8 mg/mL ribonuclease A and 10 μg/mL PI and analysed using a Beckman Coulter Epics XL flow cytometer, as described in Chapter 2.9. Data shown are representative of repeat experiments (n=3).

It can be seen from Figure 5.6 that celecoxib causes a G₁ arrest in both cell lines but the effect is larger in LNCaP-Neo. Compared to control cells, there are increases of 16% and 9% in the G₁ population for LNCaP-Neo and LNCaP-COX-2, respectively.
Figure 5.7 $p27^{KIP1}$ induction following celecoxib treatment is attenuated due to COX-2. Levels of $p27^{KIP1}$ protein in control or celecoxib treated LNCaP-Neo and LNCaP-COX-2 cells. Both attached and floating cells were harvested following 24 hour treatment with celecoxib, as described in Materials and Methods. (a) control cells (b) 5 μM (c) 10 μM (d) 20 μM (e) 50 μM treated cells. 50 μg protein was electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose membranes and exposed to rabbit IgG polyclonal $p27^{KIP1}$ antibody (Santa Cruz Biotechnology) at a 1:10000 dilution and a murine IgM β-actin antibody (Oncogene) at a 1:10000 dilution. HRP conjugated antibodies to detect rabbit IgG (Dako) and murine IgM (Calbiochem) were used at a 1:2000 dilution. Bands were detected using ECL (Pierce).
Figure 5.7 confirms that constitutively, p27^{KIP1} expression in LNCaP-Neo and LNCaP-COX-2 could not be detected. After celecoxib treatment, however, there is a dose dependent accumulation of p27^{KIP1} in LNCaP-Neo but very little induction in LNCaP-COX-2. This finding, in addition to the Trypan blue data (Chapter 4) provides accumulating evidence of a resistance to celecoxib in LNCaP-COX-2.

Cyclin B1 expression is necessary for cell cycle progression from the S phase to the G_2M phase. Alterations in its levels may affect the ability of a cell to proceed past this cell cycle check point. We wished to investigate further the nature of the resistance to carboplatin in COX-2 over-expressing LNCaP cells, by also assessing the levels of this cell cycle progression protein.
Chapter 5 Effects of COX-2 on the Cell Cycle and Apoptosis

Densitometric scans of Western blot data:

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>10 µM</th>
<th>20 µM</th>
<th>50 µM</th>
<th>100 µM</th>
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<td>1.22</td>
<td>0.81</td>
<td>0.99</td>
</tr>
<tr>
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<td>1.13</td>
<td>1.04</td>
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Figure 5.8 Cyclin B1 levels are attenuated in LNCaP cell lines following carboplatin treatment. Both attached and floating cells were harvested following 48 hour treatment with 10-100 µM carboplatin, as described in Materials and Methods. 50 µg protein was electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose membranes and exposed to rabbit IgG polyclonal cyclin B1 antibody (Santa Cruz) at a 1:200 dilution. HRP conjugated antibodies to detect rabbit IgG (Dako) was used at a 1:2000 dilution. Bands were detected using ECL (Pierce). Figures in table are from densitometric analysis performed with Quantiscan (Biosoftware, Cambridge, UK).
It can be seen from Figure 5.8 that LNCaP-COX-2 has higher basal levels of cyclin B1 than LNCaP-Neo. Following 48 hour carboplatin treatment, there is dose-dependent decrease in cyclin B1 levels in both cells. These levels decrease in LNCaP-COX-2 to a greater extent than in LNCaP-Neo, but the levels are still more elevated than LNCaP-Neo. This suggests that the elevated basal cyclin B1 levels could contribute to maintaining LNCaP-COX-2 in cell cycle progression following carboplatin treatment. The lower levels observed in LNCaP-Neo may render these cells to leave the cell cycle, either by becoming quiescent or arresting, by undergoing apoptosis, or a combination of these effects. Furthermore, as a greater S phase block is in LNCaP-Neo (Figure 5.3), this could be due to the decreased cyclin B1 levels seen in these cells.
Chapter 5 Effects of COX-2 on the Cell Cycle and Apoptosis

Figure 5.9 Levels of cyclin B1 protein in control or celecoxib treated LNCaP-Neo and LNCaP-COX-2 cells. Both attached and floating cells were harvested following 24 hour treatment with 5-50 μM celecoxib, as described in Materials and Methods. 50 μg protein was electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose membranes and exposed to rabbit IgG polyclonal cyclin B1 antibody (Santa Cruz) at a 1:200 dilution. HRP conjugated antibodies to detect rabbit IgG (Dako) was used at a 1:2000 dilution. Bands were detected using ECL (Pierce). Figures in table are from densitometric analysis performed with Quantiscan (Biosoftware, Cambridge, UK).

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<th>20 μM</th>
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<td>1.51</td>
<td>1.64</td>
<td>1.34</td>
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</table>
We demonstrated in Chapter 4 that celecoxib had a differential effect on COX-2 non-expressing and over-expressing LNCaP cells. We postulated that COX-2 mediated a different effect following celecoxib treatment and Figure 5.9 shows that the COX-2 over-expression in LNCaP which mediates a resistance to the effects of the COX-2 inhibitor may also be due to these changes in cyclin B1 expression. It also confirms elevated basal levels of cyclin B1 seen in Figure 5.8. Celecoxib treatment results in a dose-dependent decrease in cyclin B1 protein in both LNCaP-Neo and LNCaP-COX-2 cells, however, the reduction is much greater in LNCaP-Neo. The explanation provided for the resistance to carboplatin of LNCaP-COX-2, in terms of basal cyclin B1 levels, may also be partly responsible for the resistance observed to celecoxib i.e. COX-2 causes increased cyclin B1 levels after celecoxib treatment.

5.3.2 Effect of COX-2 on apoptosis and apoptotic markers

Having established that COX-2 mediates an inhibition of the growth inhibitory effect of carboplatin, as determined by IC50 values calculated with the MTT assay (Table 4.1), we next assessed if over-expression of the enzyme had a similar effect on the apoptotic response of LNCaP cells to carboplatin. We initially utilised the Annexin V-FITC apoptosis flow cytometric detection assay, following the manufacturer’s instructions (Oncogene Research Products). This assay uses two fluorescent probes, PI and AV conjugated to FITC, to distinguish between viable, early apoptotic, late apoptotic and necrotic cells. AV binds to a membrane phospholipid, phosphatidyl serine (PS), when it is externalised during the apoptotic process. In viable cells, this phospholipid is located on the inner surface of the cell membrane and consequently a low fluorescent signal is detected.
Figure 5.10 LNCaP-COX-2 cells are resistant to carboplatin-induced apoptosis. LNCaP-Neo and LNCaP-COX-2 cells were treated with 100 μM carboplatin for 48 hours and both floating and attached cells were harvested and prepared for apoptotic AV/PI analysis as described in section 2.10, Materials and Methods. Percentage of cells in late stage apoptosis are (a) LNCaP-Neo control 6.9% (b) LNCaP-COX-2 control 6.1% (c) LNCaP-Neo carboplatin 23.3% (d) LNCaP-COX-2 carboplatin 10.3%.
Sample cytograms are from the AV/PI apoptotic assay are shown in Figure 5.10 for control LNCaP-Neo and LNCaP-COX-2 cells (a) and (b), and cells treated with carboplatin for 48 hours, (c) and (d). These cytograms show the range of intensity of the two fluorescent probes amongst the cells, and data from the upper right quadrant, late stage apoptotic cells, is presented and analysed here. A low percentage of apoptosis is detected in both control cells, around 6.0% (Figure 5.10a and 5.10b). However, greater apoptosis is detected in LNCaP-Neo (23.3%) compared to LNCaP-COX-2 (10.3%) after carboplatin treatment. Thus, these data thus confirm the carboplatin resistance of LNCaP-COX-2 that was apparent when we used these cells in the MTT assay (Chapter 4). As a result of demonstrating reduced apoptosis in COX-2 over-expressing LNCaP cells, we next attempted to clarify if this was due to alteration in proteins which modulate the apoptotic response. This was important because statistical analysis was also not performed using this flow cytometric analysis of apoptosis.
Figure 5.11 Anti-apoptotic proteins are elevated in LNCaP-COX-2 compared to LNCaP-Neo cells. Near confluent cells grown in serum were harvested as described in Materials and Methods. 50 μg protein was electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose membranes and exposed to murine IgG monoclonal Bcl-2 and Bcl-xL antibodies and a rabbit IgG polyclonal rabbit survivin antibody (all from Santa Cruz) at 1:1000, 1:100 and 1:200 dilution, respectively. A murine IgM β-actin antibody (Oncogene) was used at a 1:10000 dilution. HRP conjugated antibodies to detect murine IgG (Sigma), rabbit IgG (Dako) and IgM (Calbiochem) were used at a 1:2000 dilution. Bands were detected using ECL (Pierce).
Chapter 5 Effects of COX-2 on the Cell Cycle and Apoptosis

The Western blot analyses of basal cells in Figure 5.11 demonstrates that three anti-apoptotic proteins, Bcl-2, Bcl-xL, and survivin are expressed at a greater level in LNCaP-COX-2 in comparison to control transfected LNCaP-Neo cells. Although individually, the elevation of these three proteins in LNCaP-COX-2 is not massively increased on an individual basis when compared with LNCaP-Neo, the elevation of the proteins combined could be a contributing factor towards the resistance to carboplatin in COX-2 expressing cells. We also assessed if transient transfection of COX-2 into PC-3 cells had the same effect as in LNCaP. However, no significant elevation of anti-apoptotic proteins was observed in PC-3-COX-2 transient transfectant cells compared to PC-3-Neo (data not shown).

### 5.3.3 Effect of COX-2 on TRAIL and Fas

Having established that anti-apoptotic proteins are relatively increased in LNCaP-COX-2 versus LNCaP-Neo and that there is reduced apoptosis in these cells, we wanted to assess if COX-2 could affect the sensitivity to the endogenous apoptosis inducing ligands Fas and TRAIL. Because the effect these agents have on these cells often appears contradictory in the literature (Chapter 5.2.2.2), we measured cell viability using more than one technique.

Because the transfection of PC-3 was transient only, we performed Fas and TRAIL experiments in LNCaP only. We used the HeLa cell line as a positive control, which is known to be sensitive to both Fas and TRAIL.
Figure 5.12 COX-2 over-expression in LNCaP cells does not confer resistance to TRAIL. LNCaP-Neo and LNCaP-COX-2 are resistant to 5-200 ng/mL TRAIL, whilst HeLa are sensitive to TRAIL, in the absence of any sensitising agent. HeLa and LNCaP transfectants were plated at 3x10^4/mL in 96 well plates and grown for 24 hours, followed by 24 hour TRAIL exposure. No difference in sensitivity to TRAIL between LNCaP-Neo and LNCaP-COX-2 is observed. Percentage growth determined by the MTT assay. Each data point represents the average of 3 independent experiments ± S.D.
Figure 5.12 shows that up to 200 ng/mL human recombinant TRAIL did not cause any significant reduction in cell viability of either LNCaP-Neo or LNCaP-COX-2 for 48 hours, whereas HeLa were sensitive to TRAIL in a dose-dependent manner, as assessed by the MTT assay. There was no significant difference in sensitivity to TRAIL between the LNCaP COX-2 negative and positive cells. When resistance to Fas or TRAIL is observed, some cells can be sensitised to these agents with the use of cycloheximide (CHX), or PI3K or MAPK inhibitors. Sah et al. (2003) showed that CHX activates the stress activated protein kinase (SAPK or JNK) and p38 MAPK pathways. CHX inhibits protein synthesis and could prevent the synthesis of proteins which mediate TRAIL or Fas resistance. However, we did not observe significant sensitisation to TRAIL in either LNCaP-Neo or LNCaP-COX-2 when the cells were concomitantly treated with either CHX or the PI3K inhibitor wortmannin (data not shown). Thus, in LNCaP cells, COX-2 over-expression does not cause any further resistance to the effects of TRAIL, and this may be because mock transfected, empty vector cells are inherently resistant to TRAIL.
Figure 5.13 COX-2 over-expression in LNCaP cells does not confer resistance to Fas. LNCaP-Neo and LNCaP-COX-2 are resistant to Fas, whilst HeLa. HeLa and LNCaP transfectants were plated at 3x10^5/mL in 6 well plates and grown for 24 hours. Then 200 ng/mL Fas or 1 μg/mL CHX, alone or combined, or medium alone was added and the cells monitored visually under the microscope for evidence of cell death. (a) HeLa control; (b) HeLa+ CHX+ Fas; (c) LNCaP-Neo control; (d) LNCaP-Neo+ CHX+ Fas; (e) LNCaP-COX-2 control; (f) LNCaP-COX-2+ CHX +Fas. Photomicrographs magnification x200.
Chapter 5 Effects of COX-2 on the Cell Cycle and Apoptosis

We next performed a non-quantitative test to assess the effects of COX-2 over-expression on CD95 Fas IgM (CH-11 clone) mediated cell death. Again, HeLa cells were used as a positive control. Because both LNCaP mock and COX-2 transfectants were resistant to 0.5μg/mL Fas alone (data not shown), CHX was added at the time of treatment. Many cell types are reported to become sensitive to Fas in the presence of CHX. Although this protein synthesis inhibitor had the ability to hasten the onset of morphological cell death in HeLa it did not significantly sensitise either LNCaP-Neo or LNCaP-COX-2 to Fas (Figure 5.13). Thus, in LNCaP, COX-2 over-expression does not cause resistance to the effects of Fas, as mock transfected cells are inherently resistant to Fas. This finding is similar to the TRAIL results, namely that the LNCaP cell line is largely resistant to these agents and COX-2 does not affect this resistance.

5.4 Discussion

COX-2 and the Cell Cycle

These data presented in this Chapter have shown that COX-2 over-expression in LNCaP cells mediated an attenuation of the cell cycle inhibitory effects of carboplatin and celecoxib, and that COX-2 attenuated apoptotic processes underlie carboplatin-induced cytotoxicity.

We saw subtle differences in cell cycle abrogation following carboplatin treatment between LNCaP-Neo and LNCaP-COX-2 cells. A noticeable difference was seen by the lack of p53 and p27KIP1 induction in carboplatin treated LNCaP-COX-2 cells.

Di Felice et al. (1998) found that in p53 wild type Y79 retinoblastoma cells, both cisplatin and carboplatin caused apoptosis. In cis-resistant lines they developed, they observed reduced p53 induction with cisplatin. These data are in line with our findings, in the sense that a cell line resistant to the effects of carboplatin (LNCaP-
COX-2) also demonstrated reduced p53 induction (Figure 5.4). They assessed the induction of a p53 down-stream target, p21\(^{\text{WAF1}}\), and found that it was detected only in the cisplatin sensitive line after cisplatin treatment. Although we did not assess difference in p21\(^{\text{WAF1}}\) levels between LNCaP-Neo and LNCaP-COX-2, we demonstrate a similar effect with the CDK inhibitor, p27\(^{\text{KIP1}}\), i.e. reduced induction after carboplatin treatment.

Dhanalakshmi et al. (2003) demonstrated an S phase arrest with 20 \(\mu\)M carboplatin and a G\(_2\)M arrest with 2 \(\mu\)M cisplatin, although AV/ PI assessment showed that carboplatin was more effective than cisplatin in inducing apoptosis. Consistent with the G\(_2\)M block, cdc2 (needed for progression from G\(_2\)M) and cyclin B1 (which activates cdc2) were decreased. Thus, carboplatin has been demonstrated to cause apoptosis, as well as a cell cycle arrest in a variety of cancer cell lines and our data shows that COX-2 mediates a resistance to these effects in the LNCaP cell line.

Grimberg et al. (2002) showed that introduction of p53 into PC-3 caused apoptosis in serum free media. In p53 wild type H460 lung carcinoma cells, reducing p53 levels by E6 gene transfection reduced apoptosis. Tang et al. (1998) showed that growth factor withdrawal led to differences in survival between normal human prostate, BPH cells and the metastatic prostate cancer lines LNCaP, PC-3 and DU145. These studies demonstrate the importance of p53 in protecting against tumourigenesis. Although we did not detect a difference in survival between LNCaP-Neo and LNCaP-COX-2 following serum withdrawal (data not shown), COX-2 does attenuate p53 induction following carboplatin treatment.
In agreement with findings of others (reviewed below) we showed that celecoxib mediated a G\textsubscript{i} arrest in both LNCaP cell lines, but a greater G\textsubscript{i} arrest in LNCaP-Neo was detected compared to LNCaP-COX-2.

There have been several reports of the cell cycle arrest properties of celecoxib in various cell lines. Maier et al. (2004) performed COX-2 transfection in a colon carcinoma line and found that 20-100 \mu M celecoxib had a stronger apoptotic effect in the COX-2 expressing clone, but there were no differences between COX-2 positive or negative cells for levels of cyclins A and B1 in p21\textsuperscript{WAF1} and p27\textsuperscript{KIP1} protein or cell cycle effects. This suggests that celecoxib caused COX-2 dependent apoptosis, but COX-2 independent G\textsubscript{i} cell cycle arrest and that the pro-apoptotic effects of celecoxib here are associated with COX-2 inhibition. They found concentration dependent decreases in cyclins A and B1 and increases in p21\textsuperscript{WAF1} and p27\textsuperscript{KIP1}, which was COX-2 independent. These effects were noticeable at levels lower than needed for apoptosis. These findings were similar to those of a Hsu et al. (2000) study which also found that COX-2 expressing cells were more sensitive to celecoxib induced apoptosis. Leng et al. (2003) also suggest that celecoxib causes apoptosis by both COX-2 dependent and independent means.

Patel et al. (2005) showed that 5\mu M celecoxib, but not rofecoxib, caused growth arrest in both PC-3 and LNCaP cells, which were both COX-2 negative, over 96 hours and this was associated with a G\textsubscript{0}/G\textsubscript{i} block and cyclin D1 reduction. In PC-3 mice xenografts, celecoxib caused dose dependent reduction in tumour volume via reduced Ki-67, CD34, microvessel density and cyclin D1.

Cheng et al. (2004) showed that 1 mM of the COX-2 inhibitor etodolac caused growth inhibition in two hepatocellular carcinoma cell lines, in COX-2 positive but not COX-2 negative fibroblasts, although PGE\textsubscript{2} addition did not protect against this growth
inhibition, suggesting that etodolac effects were COX-2 independent. There was PCNA inhibition, induction of p21\textsuperscript{WAF1} and p27\textsuperscript{KIP1}, inhibition of CDK2 and 4, CDC2, cyclins A and B1.

The review of the data above in the literature confirms that COX-2 inhibitors, and celecoxib in particular, have cell cycle arrest properties. As will be discussed below, celecoxib also causes apoptosis in cancer cell lines, although typically, higher concentrations are needed. Our data is in general agreement with these studies, because we also demonstrate a G\textsubscript{1} arrest, reduction of cyclin B1 levels and induction of p27\textsuperscript{KIP1} following celecoxib treatment of LNCaP cells. However, the main hypothesis of this study was to identify mechanisms responsible for COX-2 attenuation of cell cycle arrest and, our data shows that COX-2 has the effect of reducing p53 and p27\textsuperscript{KIP1} and also elevating cyclin B1. However, we did not assess if this activity is PGE\textsubscript{2} dependent. We know that resistance to carboplatin is PGE\textsubscript{2} or enzyme activity independent, but this may not be the case for celecoxib.

**COX-2 and Apoptosis**

A second flow cytometric assay, the AV/PI apoptotic assay, provided evidence that COX-2 reduced the levels of apoptosis following carboplatin treatment, in addition to modulating cell cycle parameters (Figure 5.10). It was found that Bcl-2, Bcl-x\textsubscript{L} and survivin were elevated in LNCaP-COX-2 compared to LNCaP-Neo (Figure 5.11). However, COX-2 over-expression did not modulate the response of LNCaP cells to TRAIL (Figure 5.12) or Fas (Figure 5.13).

Other groups have demonstrated anti-apoptotic effects of COX-2, although not in prostate cancer cells (Tsuji & DuBois 1995, Lin \textit{et al.} 2001, Nzeako \textit{et al.} 2002). Frequently, up-regulation of Bcl-2 or other anti-apoptotic proteins is reported when
COX-2 is elevated, which also agrees with our findings (Figure 5.11). Lin et al. (2001) transfected COX-2 into lung adenocarcinoma cells and showed resistance to vinblastine induced apoptosis via elevated Mcl-1 protein. Tsuji & Dubois (1995) showed that in rat intestinal epithelial cells, COX-2 transfection caused altered extracellular matrix adhesion and resistance to butyrate induced apoptosis, increased Bcl-2 levels and decreased expression of E-cadherin. Sulindac sulphide was able to reverse the inhibition of apoptosis and the increased cellular adhesion. Sun et al. (2002) transfected colon carcinoma HCT-115 cells with COX-2 cDNA and found it reduced apoptosis induced by NS398, sulindac sulphide and 5-fluorouridine. Bcl-2 mRNA levels were increased and there was reduced caspase activation. 40 and 80 μM NS398 reduced PGE2 levels but it did not reverse COX-2 mediated apoptotic resistance.

Krysan et al. (2004) showed that COX-2 transfection and 10 μg/mL in NSCLC cells enhanced survivin protein levels. Survivin levels were reduced with COX-2 depletion. They found that survivin ubiquitination was reduced in COX-2 cells, stabilising survivin levels. We too report elevated survivin in COX-2 transfected LNCaP cells, although we have not confirmed if this is due to survivin stabilisation or enhanced transcription of the survivin gene.

The importance of Bcl-2 over-expression in prostate cancer has been highlighted in several studies, and this over-expression has been shown to mediate a resistant phenotype. It is interesting to note the variations in basal Bcl-2 levels reported in LNCaP in particular. We observed Bcl-2 protein expression in LNCaP, however some groups do not detect Bcl-2 and have to perform transfection. For example Fujita et al. (2002) transfected COX-2 into LNCaP cells but did not detect elevation of Bcl-2 mRNA, whereas we observed elevation of Bcl-2 protein in COX-2 expressing LNCaP cells (Figure 5.11). Takeuchi et al. (1996) however did not detect Bcl-2 protein in
both parental LNCaP and PC-3 cells, which highlights again differences in characteristics of supposedly identical cell lines between different research laboratories. We consistently detected Bcl-2 via Western blotting in both LNCaP and PC-3 cells. Tang et al. (1998) agree with our findings, that PC-3, LNCaP and DU145 all express Bcl-2 and Bcl-xL.

Marcelli et al. (1999) showed that Bcl-2 transfection into LNCaP cells prevented breakdown of the mitochondrial membrane potential in response to staurosporine and transfection of caspase-7 restored apoptosis even in the Bcl-2 transfectant. Rokhlin et al. (2001) used PC-3 cells transfected with Bcl-2 and showed that this mediated a resistance to TRAIL. Raffo et al. (1995) used a stable LNCaP-Bcl-2 transfectant in vivo to show that Bcl-2 caused a resistance to apoptosis after androgen depletion. Furthermore, only the Bcl-2 transfected cells formed tumours when injected into castrated, male nude mice. Kajiwara et al. (1999) showed that Bcl-2 transfection into LNCaP caused resistance to the growth suppressing effects of androgen and serum deprivation. Xenografts in nude mice showed higher tumorigenicity, faster initial growth rate and less tumour volume decrease following castration in the Bcl-2 transfected cells. Berchem et al. (1995) showed that DHT induced Bcl-2 protein in LNCaP and this resulted in a resistance to etoposide induced apoptosis. Anti-sense Bcl-2 abrogated this protective effect.

In contrast, some reports also suggest that Bcl-2 and Bcl-xL do not cause a resistance to apoptosis. Jendrossek et al. (2003) showed that in Jurkat cells celecoxib induced apoptosis via mitochondria, but neither Bcl-2 or Bcl-xL could rescue the cells. Hence the mechanism of cell kill was cyt c dependent but Bcl-2 family independent.

However, overall, the literature supports a role for Bcl-2 in mediating a resistance to apoptosis. In the majority of these experiments Bcl-2 can be identified as the major
mediator of this resistance because this protein was specifically elevated in these experiments by transfection. However, we cannot be certain about how important Bcl-2 is in mediating resistance in LNCaP-COX-2 to carboplatin, celecoxib or etoposide. Bcl-2 is only one of three anti-apoptotic proteins known to be raised in this model, and we have not attempted to assess the relative importance of each.

Studies also highlight the impact of elevated Bcl-xL in prostate cancer cells. In a manner similar to Bcl-2, elevation of Bcl-xL produces a resistant phenotype. Yang et al. (2003) and Li et al. (2001) showed that elevated Bcl-xL in PC-3 cells compared to LNCaP prevented PC-3 from apoptosing when treated with the PI3K inhibitor LY294002. Transfection of Bcl-xL into LNCaP caused resistance to apoptosis, and down-regulation of Bcl-xL in PC-3, made PC-3 sensitive to LY294002. Lebedeva et al. (2000) showed that Bcl-xL transfection into PC-3 and LNCaP desensitised these cells to mitoxantrone, paclitaxel, etoposide and carboplatin. Various strategies to down-regulate Bcl-xL had the opposite effect. Vilenchik et al. (2002) showed that Bcl-xL down-regulation in LNCaP and DU145 reduced cellular growth, but an increase in other anti-apoptotic proteins, PKCa, XIAP and cIAP1 was observed. Bcl-xL down-regulation was associated with resistance to carboplatin, etoposide, mitoxantrone, paclitaxel and vinblastine. Re-introduction of Bcl-xL restored cell sensitivity and growth rate. This suggests that at low concentrations, Bcl-xL affected growth rate, rather than being anti-apoptotic.

We also reported elevation of the IAP survivin in LNCaP-COX-2 cells. Several other studies demonstrate IAP elevation in cancer cells. Krajewska et al. (2003) demonstrated elevated levels of cIAP1, cIAP2, XIAP and survivin in BPH, localised prostate cancer samples and T1-T4 metastatic samples and in the TRAMP mouse. Mceleny et al. (2001) showed that NIAP, survivin and cIAP2 were elevated in PC-3
and DU145 compared to LNCaP and this was associated with resistance to etoposide. Nakamura et al. (2004) found no relationship between survivin expression and tumour histology, invasion depth, or lymph node metastasis or stage. But patients with lower survivin had higher survival. Patients who received cisplatin, survival was longer for patients with low survivin (38 versus 24 months). They hypothesised that high survivin was mediating resistance to cisplatin therapy, shortening overall survival.

**Fas and TRAIL**

Even though anti-apoptotic Bcl-2, Bcl-xL and survivin were elevated in LNCaP-COX-2 compared to LNCaP-Neo, we were not able to demonstrate a differential sensitivity to either Fas or TRAIL. It should be noted that the parental LNCaP cells had an inherent resistant phenotype to these two agents (Figures 5.12 and 5.13). As will be discussed below, the literature differs in the reported sensitivities of prostate cancer cell lines to Fas and TRAIL. Often the same clone of antibody or recombinant protein is not used and different clones are likely to have different potencies. Cell viability or apoptosis is reported in different ways. We assessed the effects of Fas by photomicrography and of TRAIL by MTT assay and reported a resistance of LNCaP cells to these two agents. Had we performed specific apoptotic assays, such as measuring DNA fragmentation, caspase activation or cyt c release, could sensitivity have been potentially reported? Even though there was no evidence of morphological cell death with Fas by photomicrography or with TRAIL by the MTT assay, other assays may detect small amounts of cell kill or growth inhibition.

There have been no reports in the literature on the effects of COX-2 on the sensitivity of prostate cancer cells to agents such as Fas and TRAIL. In other cancer cells, COX-
2 has been shown to cause resistance to Fas and TRAIL. For example, Nzeako et al. (2002) showed that in cholangiocarcinoma cells, either transfecting or inducing COX-2 inhibited Fas (but not TNF-α or TRAIL) mediated apoptosis. Mcl-1 was up-regulated. Tang et al. (2002) transfected COX-2 into colorectal cells and assessed the effect on TRAIL induced apoptosis. COX-2 cells had lower DR5 mRNA and elevated Bcl-2 mRNA with less caspase-3, -8 and -9 activation. Sun et al. (2002) performed COX-2 transfection in colon cancer cells and found that it caused repression of DR5 transcription.

Rokhlin et al. (1997) showed that PC-3 and DU-145 needed CHX to be made Fas-sensitive, whereas LNCaP remained resistant even with CHX co-treatment. Takeuchi et al. (1996) report that PC-3 and LNCaP do not express the Fas receptor and consequently are resistant to Fas. Transfection of the FasR into PC-3 and LNCaP converted PC-3 but not LNCaP to a Fas sensitive phenotype. In nude mice xenografts, growth of the Fas receptor transfectants was retarded compared to control cells. Hedlund et al. (1998) correlated the sensitivity of prostate cancer cells to the clinical stage of the parental tumours. They showed that metastatic prostate cancer lines were resistant to Fas, but cell lines derived from primary tumours were Fas sensitive. But this resistance did not correlate to pathological grade or androgen sensitivity. Jiang et al. (2002) showed that there was elevated levels of Fas and Fas ligand in prostate adenocarcinoma and PIN samples versus benign prostate tissue obtained from radical prostatectomy. They suggest that up-regulation of Fas signalling in malignancy may allow cells to escape immune surveillance by lymphocytes and natural killer cells. But the malignant cells themselves do not undergo apoptosis.
Reports in the literature of prostate cancer cell lines' sensitivity to TRAIL is quite variable. Our data that LNCaP cells are resistant to TRAIL is in agreement with many other groups (Munshi et al. 2002, Kelly et al. 2002, Eid et al. 2002). Nimmanapalli et al. (2001) however report that LNCaP cells are sensitive to TRAIL. In addition, PI3K inhibition has been shown to reverse LNCaP resistance to TRAIL (Nesterov et al. 2001, Rokhlin et al. 2002, Chen et al. 2001). Whilst there appears to be some consensus that DU145 cells are sensitive to TRAIL (Nesterov et al. 2001, Yu et al. 2000, Chen et al. 2001, Nimmanapalli et al. 2001), PC-3 cells have been reported to be both sensitive (Nesterov et al. 2001, Rokhlin et al. 2002, Griffith et al. 2000, Yu et al. 2000, Nimmanapalli et al. 2001) and resistant (Munshi et al. 2002, Sah et al. 2003, Eid et al. 2002) to TRAIL.

In this chapter we have presented data which shows that the resistance observed to carboplatin and celecoxib in LNCaP-COX-2 versus LNCaP-Neo is due to both a reduced cell cycle block and lower levels of apoptosis. p53 and p27^Kip1 induction was greater in LNCaP-Neo following cytotoxic carboplatin and celecoxib treatment. Basal cyclin B1 was elevated in LNCaP-COX-2. Elevated anti-apoptotic markers Bcl-2, Bcl-xl and survivin may be associated with this resistance to carboplatin in LNCaP-COX-2. However, we did not observe an effect of COX-2 on the sensitivity of LNCaP cells to Fas and TRAIL.
CHAPTER 6

EFFECT OF COX-2 ON PI3K/ AKT SIGNALLING
Chapter 6 Effects of COX-2 on PI3K/ Akt Signalling

6.1 Aims

It was shown in Chapter 4 that COX-2 inhibition could not reverse the carboplatin resistance observed in LNCaP-COX-2. Therefore, other mechanisms were sought to reverse this resistance in order to clarify the mechanisms responsible. We tried to sensitisie these LNCaP cells via PI3K/ Akt signalling inhibition because they contain a mutation in their PTEN gene which renders the protein dysfunctional, and this PTEN inactivity may mediate a resistance which could mask some effects of COX-2. We also wished to assess if the changes in cellular apoptotic protein levels following COX-2 inhibitor and PI3K inhibitor treatment to help explain the differential effects of celecoxib and wortmannin, respectively, on reversing the resistance in LNCaP-COX-2 to carboplatin.

6.2 Introduction

Survival of cells in multicellular organisms requires continuous stimulation from the extracellular environment, in the form of growth factors. These growth factors activate intracellular signalling pathways to promote survival and cell growth. PI3K/ Akt is a major cell survival signalling pathway.

6.2.1 PI3K/ Akt signalling

PI3K is activated by a number of receptors and is involved in cell survival signalling. It phosphorylates inositol (PI) lipids, the phosphoinositides, that act as membrane
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restricted 2nd messengers for several targets, including Akt. PI3K transfers a phosphate group from ATP to the 3' position of the inositol ring. Growth factors, such as PDGF and IGF-1, can promote cell survival by activating PI3K and its downstream target Akt. There are multiple mammalian forms of PI3K, and they can phosphorylate PIP2 to PIP3. Indeed, the primary consequence of PI3K activity is generation of PIP3 in the membrane (reviewed by Vivanco & Sawyers 2002).

PI3Ks are heterodimers of regulatory and catalytic units. The p85 regulatory unit is a phosphoprotein substrate of receptor and cytosolic tyrosine kinases via its SH domain. p110 is the catalytic unit. Activation of PI3Ks follows phosphorylation of the receptor tyrosine kinase tail. Ras can also bind and activate the p110 unit. PIP3 serves as a ligand for recruiting PH domain containing proteins to the cell membrane, most important of which is Akt.

6.2.2 Akt

Akt, also known as PKB, was originally described as an oncogene, activated by a number of growth factors through a PI3K dependent pathway (del Peso et al. 1997). Akt can be directly activated by PI3K by binding of PIP2 to its PKB domain. Three mammalian members of the Akt family exist; Akt1, Akt2 and Akt3. It is a 57 kDa serine/threonine kinase, and is located primarily in the cytosol. Its N-terminus contains a pleckstrin homology (PH) domain; its C-terminus has a regulatory region similar to PKA and PKC and there is a central catalytic domain.

Akt mediates growth factor induced cell survival and can suppress apoptotic effects of a variety of stimuli, including growth factor withdrawal, loss of cell adhesion and DNA damage (Datta et al. 1997). The major cancerous effects of Akt activation are survival, proliferation and growth.
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Akt has been shown to be activated by PDGF, EGF, bFGF and insulin and this activation could be paralleled by phosphorylation of the kinase itself. In vitro, phosphatases abolish Akt kinase ability (Burgering & Coffer 1995). Other activating stimuli include haemopoietic cytokines (IL-2, -3, -4, -5), heat shock, hypoxia and nerve growth factor.

Although PI3K always results in Akt activation, the reverse does not hold; Akt can be activated in a PI3K independent manner by phosphoinositide dependent kinase 1 (PDK1) and cAMP. At the plasma membrane another serine/threonine kinase with a PH domain, (PDK1), phosphorylates Akt at thr308, which is sufficient for its activation. Akt can be further activated by PDK2 at ser473. Akt activation requires both translocation to the membrane and phosphorylation at these 2 residues for maximal activation. No specific Akt phosphatases have been identified, and therefore, its down-regulation is not understood.

It is known that PI3K inhibition, with agents such as wortmannin and LY294002, block Akt activation by growth factors and constitutively activated PI3K increases Akt activity independent of growth factor stimulation. The observation that PI3K and Akt are involved in cell survival could explain why so many oncoproteins, growth factors and cell survival factors can activate PI3K.

6.2.3 Akt Targets

Akt has a number of cellular targets. It activates some targets and inactivates others, in a manner consistent with its anti-apoptotic, pro-survival function.

Akt activation can rescue cells from p53 mediated apoptosis. It phosphorylates MDM2, the protein which targets p53 for degradation, enhancing its activity, and thus promoting p53 degradation (reviewed by Vousden & Lu 2002).
In LNCaP cells, PI3K and androgen receptor pathways can compensate for one another, as androgens can rescue these cells from apoptosis as a result of PI3K inhibitors (Carson et al. 1999). Also, PI3K activation can rescue neuronal cells from serum or androgen deprivation (Franke et al. 1997). Akt has been shown to phosphorylate the androgen receptor residues at ser210 and ser790. Lin et al. (2003) showed that IGF-1 could phosphorylate the receptor at residue 38, and this effect was blocked with LY294002. However, some reports state that this does not occur in LNCaP cells and LY294002 treatment does not change the phosphorylation status of the androgen receptor.

Other cell cycle regulatory targets of Akt include E2F (Chapter 5.2.1), a family of transcription factors needed for G1 to S phase progression. Akt allows cyclin D1 levels to accumulate by preventing its degradation by phosphorylation from GSK3β. Akt also decreases levels of p21^{WAF1} and p27^{KIP1}.

Apoptotic targets of Akt include caspase-9, pro-apoptotic Bad and the kinase which degrades the inhibitory unit of the anti-apoptotic transcription factor NF-κB, IKK. Bad phosphorylation at 2 serine residues is associated with cell survival, and Akt is thought to phosphorylate one residue – any agents that do this could provide a survival signal.
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Figure 6.1 The PI3K/ Akt signalling pathway. The mechanism of PI3K dependent activation of Akt. Upon activation by PDK1 and PDK2 mediated phosphorylation, Akt phosphorylates numerous cellular targets, promoting a pro-survival, anti-apoptotic response. FKHR = Forkhead transcription factors, IRS = insulin receptor substrate, RAC = Rho family member, SGK = serum glucocorticoid kinase. Other abbreviations are referenced elsewhere (Taken from Vivanco & Sawyers 2002).

The PI3K/ Akt signalling pathway is represented diagrammatically in Figure 6.1. Some key targets and functions of this pathway are shown. Because of the number of cellular targets Akt has, dysregulation of Akt signalling can lead a cell onto the path of oncogenesis. This is discussed below.
6.2.4 PTEN and PI3K/ Akt Dysregulation in Cancer

Amplification of the PI3K/ Akt pathway is observed frequently in many cancers (Graff et al. 2000, Nesterov et al. 2001). This can be due to up-regulation or over-activity of factors which contribute to signalling, or down-regulation or reduced activity of factors which negatively regulate the pathway.

The phosphatase and tensin homologue (PTEN) is a negative regulator of the PI3K/ Akt pathway (Persad et al. 2000, Davies et al. 2002). It functions as both a lipid and protein phosphatase, but it is its lipid phosphatase activity which is important in its anti-tumourigenic function (Figure 6.1). Whereas SHIPS (SH2 containing inositol 5’ phosphatase) 1 and 2 remove the phosphates from the 5’ position of the inositol ring, PTEN removes them from the 3’ position i.e. PTEN converts PIP_3 back to PIP_2. In unstimulated cells PIP_3 is barely detected due to the regulation of PI3K activity by PTEN.

PTEN mutation or losses have been reported in many cancers (Carter et al. 1990, Trotman et al. 2003). 10q23, the chromosomal location of the gene encoding for PTEN, is lost in many cancers, including prostate. Absence of PTEN has been shown to correlate with higher Gleason score, which raises the possibility that PTEN status may be useful as a prognostic marker (Malik et al. 2002).

In LNCaP cells, PTEN protein is inactivated due to a frameshift mutation in its gene; hence the PI3K/ Akt pathway is constitutively activated, and the cells exhibit resistance to apoptosis (Davies et al. 1999, Chen et al. 2001). In these cells there can be an escape from growth factor survival dependence. However apoptosis may be induced in LNCaP cells with PI3K inhibitors (Carson et al. 1999).
In cancer, the PI3K catalytic unit is sometimes over-amplified, with the mutant catalytic unit causing constitutive activation of PI3K (reviewed by Franke et al. 1997). Certain murine strains that have constitutive activation of PI3K/ Akt signalling all develop cancer (reviewed by Vivanco & Sawyers 2002).

We have described above the impact of PI3K/ Akt signalling dysregulation, and how this is relevant to carcinogenesis. Because of the role of PI3K/ Akt signalling in modulating growth and apoptotic responses, and our data which demonstrated a modulatory effect of COX-2 on apoptosis and the cell cycle (Chapter 5), in this Chapter we provide evidence that COX-2 mediates these effects via activation of the PI3K/ Akt pathway in LNCaP cells.

6.3 Results

6.3.1 PI3K inhibition partially sensitises LNCaP-COX-2 to carboplatin

Our observations indicated that COX-2 inhibition using celecoxib could not sensitise LNCaP-COX-2 cells to carboplatin (Coley HM, personal communication). Therefore, other mechanisms were sought to reverse this resistance. We next attempted pharmacological inhibition of the PI3K/ Akt pathway, as COX-2 has been reported to activate this pathway (Leng et al. 2003). The results are shown in Figure 6.2, which shows that 500 nM wortmannin administered concomitantly with cytotoxic doses of carboplatin over a 72 hour period does not significantly alter the IC_{50} for LNCaP-Neo, but there is a significant decrease for LNCaP-COX-2 (p=0.03), although complete reversal with PI3K inhibition was not observed.
Figure 6.2 PI3K inhibition with wortmannin partially reverses the resistance of LNCaP-COX-2 to carboplatin. Graphs show IC\textsubscript{50} values (\(\mu\)M), obtained from MTT assays for LNCaP-Neo and LNCaP-COX-2 treated with carboplatin for 72 hours or co-treated with 500 nM wortmannin. LNCaP-COX-2 is resistant to carboplatin in comparison to LNCaP-Neo (**p=0.006). Wortmannin significantly reduces the IC\textsubscript{50} of LNCaP-COX-2 to carboplatin (++p=0.03).

The data in Figure 6.2 suggests that PI3K inhibition, unlike COX-2 inhibition, can partially sensitise COX-2 expressing LNCaP cells to carboplatin. However, it should be noted that complete reversal of resistance was not achieved.
6.3.2 P-Akt\textsuperscript{ser473} is elevated in LNCaP-COX-2

The fact that PI3K inhibition partially reversed the resistance of LNCaP-COX-2 cells to carboplatin (Figure 6.2) suggested that the over-expression of COX-2 may be activating the PI3K pathway. To test this hypothesis, we assessed the constitutive levels of P-Akt\textsuperscript{ser473}, the downstream effector of PI3K, in LNCaP-Neo and LNCaP-COX-2 cells.

Figure 6.3 P-Akt\textsuperscript{ser473} levels are higher in LNCaP-COX-2 compared to LNCaP-Neo. Near confluent cells were lysed as described in Materials and Methods. 50 µg protein was electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose membranes and exposed to rabbit IgG polyclonal P-Akt\textsuperscript{ser473} antibody (BioSource) at a 1:1000 dilution and a total Akt antibody (BioSource) also at a 1:1000 dilution. HRP conjugated antibodies to detect rabbit IgG (Dako) were used at a 1:2000 dilution. Bands were detected using ECL (Pierce).
Figure 6.3 shows that, as hypothesised, P-Akt\textsuperscript{ser473} levels are higher in LNCaP-COX-2 than LNCaP-Neo cells. This suggests that COX-2 transfection in LNCaP, by an unconfirmed mechanism, activates above basal levels the PI3K pathway. This will be discussed further in the Discussion. It should be noted that parental LNCaP cells (and hence LNCaP-Neo cells) already express P-Akt\textsuperscript{ser473} constitutively due to the PTEN mutation – COX-2 transfection elevates these levels further.

6.3.3 Effect of PI3K inhibition and celecoxib on P-Akt levels

Because celecoxib has also been reported to inactivate the PI3K pathway in prostate cancer cells (Hsu \textit{et al.} (2000), Johnson \textit{et al.} (2001), Zhu \textit{et al.} (2002), Song \textit{et al.} (2002)), we hypothesised that celecoxib did not have the ability to inactivate PI3K signalling in our LNCaP cells, as it was unable to reverse or partially reverse LNCaP-COX-2 resistance to carboplatin.
Figure 6.4 P-Akt\text{ser473} is reduced following both PI3K and COX-2 inhibition. Levels of P-Akt protein in LNCaP-COX-2 cells following cell growth for 24 hours, then treatment with 500 nM wortmannin or 20 \mu M celecoxib for the times indicated. Cells were harvested as described in Materials and Methods. 50 \mu g protein was electrophoresed on a 10\% polyacrylamide gel, transferred to nitrocellulose membranes and exposed to rabbit IgG polyclonal P-Akt\text{ser473} antibody (BioSource) at a 1:1000 dilution and a total Akt antibody (BioSource) also at a 1:1000 dilution. HRP conjugated antibodies to detect rabbit IgG (Dako) were used at a 1:2000 dilution. Bands were detected using ECL (Pierce).
However, it can be seen from Figure 6.4 that celecoxib, like wortmannin and LY294002 (data not shown), had the ability to inhibit PI3K signalling in LNCaP-COX-2, as P-Akt levels were reduced in a time dependent fashion with 20 μM celecoxib up to 12 hours. Interestingly, however, by 24 hours basal levels of P-Akt were restored. Further, 2-50 μM celecoxib exposure for 24 hours did not result in a reduction of P-Akt levels in LNCaP-COX-2 at that time point (data not shown), confirming that at these sub-cytotoxic doses celecoxib only inhibited PI3K in LNCaP-COX-2 within 24 hours. Wortmannin inhibited PI3K over a similar time window to celecoxib, up to 24 hours. These effects were also observed in LNCaP-Neo cells (data not shown). Thus, both COX-2 inhibition and PI3K inhibition can attenuate or inhibit PI3K signalling.

6.3.4 Effect of PI3K inhibition and celecoxib on COX-2 levels

To further examine the differences in ability of COX-2 and PI3K inhibition to sensitize LNCaP-COX-2 to carboplatin, we asked whether wortmannin, LY294002 and celecoxib had differential effects on COX-2 levels. We hypothesised that PI3K inhibition may reduce COX-2 levels, whereas COX-2 inhibition may not.
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Figure 6.5 PI3K inhibition results in COX-2 reduction. Levels of COX-2 protein in LNCaP-COX-2 cells following 48 hour treatment with PI3K inhibitors wortmannin and LY294002. Cells were harvested as described in Materials and Methods. 50 μg protein was electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose membranes and exposed to murine IgG monoclonal COX-2 antibody (Cayman Chemical) at a 1:1000 dilution and a murine IgM β-actin antibody (Oncogene) at a 1:10000 dilution. HRP conjugated antibodies to detect murine IgG (Sigma) and IgM (Calbiochem) were used at a 1:2000 dilution. Bands were detected using ECL (Pierce).
It can be seen from Figure 6.5 that for both wortmannin and LY294002, at doses which inhibit P-Akt levels, but at an extended time point (48 hours) reduce COX-2 protein levels in LNCaP-COX-2.

**Figure 6.6 Celecoxib causes an increase in COX-2 protein.** Levels of COX-2 protein in LNCaP-COX-2 cells following 72 hour treatment with celecoxib at the doses indicated. Cells were harvested as described in Materials and Methods. 50 μg protein was electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose membranes and exposed to murine IgG monoclonal COX-2 antibody (Cayman Chemical) at a 1:1000 dilution and a murine IgM β-actin antibody (Oncogene) at a 1:10000 dilution. HRP conjugated antibodies to detect murine IgG (Sigma) and IgM (Calbiochem) were used at a 1:2000 dilution. Bands were detected using ECL (Pierce).
In contrast to PI3K inhibition, COX-2 inhibition has the opposite effect on cellular COX-2 levels i.e. over 72 hours there is a dose dependent increase in COX-2 levels (Figure 6.6).

### 6.3.5 Effect of PI3K inhibition and celecoxib on Bcl-2 levels

As some anti-apoptotic proteins were found to be evaluated in LNCaP-COX-2 compared to LNCaP-Neo (Figure 5.11), we wished to assess if down-regulation of these proteins was involved in the partial reversal of resistance of LNCaP-COX-2 to carboplatin by PI3K inhibition.
Figure 6.7 PI3K inhibition results in Bcl-2 reduction. Levels of Bcl-2 protein in LNCaP-Neo and LNCaP-COX-2 cells following 24 hour treatment with wortmannin and LY294002. Cells were harvested as described in Materials and Methods. 50 μg protein was electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose membranes and exposed to murine IgG monoclonal Bcl-2 antibody (Santa Cruz) at a 1:100 dilution and a murine IgM β-actin antibody (Oncogene) at a 1:10000 dilution. HRP conjugated antibodies to detect murine IgG (Dako) and IgM (Calbiochem) were used at a 1:2000 dilution. Bands were detected using ECL (Pierce).
Chapter 6 Effect of COX-2 on PI3K/ Akt signalling

Figure 6.7 demonstrates that PI3K inhibition with both wortmannin and LY294002 reduced cellular Bcl-2 levels after 24 hour treatment in LNCaP-COX-2. PI3K inhibition also caused a reduction in Bcl-2 protein in LNCaP-Neo cells but the reduction occurred to a smaller extent. This may be a reflection of the lower Bcl-2 levels in LNCaP-Neo.

![Western blot image]

Control   2 hours   12 hours   24 hours  

20 μM celecoxib

| Densitometric scans of Western blot data, normalised for β-actin: |
|---|---|---|---|
| Control | 2 hours | 12 hours | 24 hours |
| 1.00 | 1.40 | 1.80 | 1.10 |

Figure 6.8 Celecoxib does not decrease Bcl-2 levels. Levels of Bcl-2 protein in LNCaP-COX-2 cells following treatment with celecoxib at the times indicated. Cells were harvested as described in Materials and Methods. 50 μg protein was electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose membranes and exposed to murine IgG monoclonal Bcl-2 antibody (Santa Cruz) at a 1:100 dilution and a murine IgM β-actin antibody (Oncogene) at a 1:10000 dilution. HRP conjugated antibodies to detect murine IgG (Dako) and IgM (Calbiochem) were used at a 1:2000 dilution. Bands were detected using ECL (Pierce). Figures in table are from densitometric analysis performed with Quantiscan (Biosoftware, Cambridge, UK).
Celecoxib did not mediate a reduction in Bcl-2 protein in both LNCaP-COX-2 (Figure 6.8) and LNCaP-Neo (data not shown), when normalised to β-actin, unlike the reduction that was observed for PI3K inhibition (Figure 6.7). There was an initial rise before Bcl-2 levels declined effectively to control levels. Thus, this could be an additional explanation for the inability of celecoxib to sensitise LNCaP-COX-2 to carboplatin – it does not down-regulate anti-apoptotic Bcl-2 to the same extent as PI3K inhibition.

6.4 Discussion

We have demonstrated in this Chapter that PI3K inhibitors partially, but not completely, sensitize LNCaP-COX-2 cells to carboplatin (Figure 6.2). This sensitisation can be partly explained by the elevated P-Akt levels observed in LNCaP-COX-2, in comparison to LNCaP-Neo (Figure 6.3). Because both PI3K inhibitors and celecoxib can inhibit PI3K, as evidenced by decreases in P-Akt (Figure 6.4), but only PI3K inhibitors can sensitize LNCaP-COX-2 cells, this suggests that the two types of agents modulate different signalling pathways. Whereas PI3K inhibition reduces both cellular COX-2 (Figure 6.5) and Bcl-2 levels (Figure 6.6) in LNCaP-COX-2, celecoxib does not modulate Bcl-2 levels and it results in an increase in COX-2 protein.

Partial sensitisation of LNCaP-COX-2 to carboplatin by PI3K inhibition

The fact that the PI3K inhibitor wortmannin does not completely sensitize LNCaP-COX-2 cells to carboplatin suggests that COX-2 transfection may be impacting other signalling pathways in addition to PI3K/ Akt. If PI3K/ Akt was the sole pathway that COX-2 modulated, then pharmacological inhibition should have sensitised LNCaP-
Chapter 6 Effect of COX-2 on PI3K/ Akt signalling

COX-2 cells completely. We did not investigate further which factors COX-2 may be modulating in addition. We have reviewed in Chapter 1.2.5 some of the oncogenic properties and functions of COX-2. Other potential mechanisms that COX-2 could be involved in these LNCaP cells include inducing c-fos and c-jun transcription factors which promote cell proliferation (Chen & Hughes-Fulford 2000), elevating the EGFR (Kinoshita et al. 1999) and activation of the MAPK ERK pathway.

We have shown here that COX-2 activates PI3K/ Akt signalling even more in LNCaP cells. We found that PI3K inhibition could partially reverse this resistance. Many studies highlight the importance of the PI3K/ Akt pathway in LNCaP prostate cancer cells in mediating a resistant phenotype.

Beresford et al. (2001) showed that PI3K inhibition with wortmannin, LY294002 or PTEN transfection sensitised LNCaP but not PC-3 cells to TRAIL and TNF-α. Yuan & Whang (2002) showed that PTEN transfection sensitised LNCaP cells to Fas, TRAIL and TNF, staurosporine and mitoxantrone. Pfeil et al. (2004) showed that basally P-Akt could only be detected in PC-3 and LNCaP but not DU145. DU145 P-Akt phosphorylation status could be modulated by EGF and IGF-1 but not PC-3 and LNCaP. Furthermore, two LNCaP androgen independent sublines were resistant to LY294002 induced apoptosis, unlike parental LNCaP cells. Etoposide produced greater apoptosis in parental LNCaP cells compared to the sublines, but LY294002 abolished this resistance. Chen et al. (2001) showed that LNCaP had higher constitutive Akt activity than PC-3 and this was associated with resistance to TRAIL, which could be overcome with either wortmannin, LY294002 or CHX, via reduced P-Akt levels. Transfection with PTEN and DN-Akt also had the same effect.
Chapter 6 Effect of COX-2 on PI3K/Akt signalling

P-Akt<sup>ser473</sup> is elevated in LNCaP-COX-2

We demonstrated that COX-2 transfection caused an elevation in P-Akt<sup>ser473</sup> levels in LNCaP cells. Other studies report a similar effect, that is enhanced PI3K signalling, in non-prostate cancer cell lines. For example, Leng et al. (2003) showed that either COX-2 transfection or PGE<sub>2</sub> treatment caused elevation in P-Akt<sup>thr308</sup> levels in hepatocellular carcinoma cell lines resulting in increased cell growth. LY294002 partially reversed these effects. Lin et al. (2001) transfected COX-2 into lung adenocarcinoma cells and detected increased P-Akt<sup>ser473</sup> and increases in the anti-apoptotic factor Mcl-1 in COX-2 expressing cells. This resulted in resistance to apoptosis following treatment with chemotherapeutic agents. We too demonstrated elevated anti-apoptotic proteins in COX-2 expressing LNCaP cells (Figure 5.11), although we did not assess Mcl-1 levels. However, Bcl-2 elevation in our LNCaP cells is partly PI3K dependent but COX-2 enzymatically independent, which differs from the Lin et al. (2001) above study, which showed Mcl-1 to be linked to PI3K signalling via COX-2.

What effect could this elevated P-Akt in LNCaP-COX-2 cells be having? We know the effect is slight because parental LNCaP cells, being PTEN mutant, have elevated P-Akt levels anyway. MDM2, the protein which targets p53 for degradation, is a downstream effector of PI3K signalling (Figure 6.1). Because p53 and p27<sup>KIP1</sup> induction is reduced in LNCaP-COX-2 cells which have elevated P-Akt (Figures 5.6 and 5.7, respectively), the possibility exists that P-Akt is directly responsible for this. Other possible explanations for the COX-2 and P-Akt mediated resistance to...
carboplatin by LNCaP-COX-2 include reduced caspase-9 processing, reduced Bad activity and reduced induction of p27Kip1 via the forkhead transcription factors.

Both PI3K and COX-2 inhibition reduce P-Akt levels

We showed in Chapter 4 that celecoxib was not able to sensitise LNCaP-COX-2 to carboplatin. However, both celecoxib and wortmannin exposure inhibited PI3K signalling, as evidenced by reduction in P-AktSer473 levels. This suggests that COX-2 inhibition and PI3K inhibition mediate a reduction in P-Akt via different mechanisms and that celecoxib is working also in a PIP3 or PI3K independent manner. Some potential targets of celecoxib will be discussed below. More importantly, because PI3K inhibition via celecoxib does not sensitise LNCaP-COX-2 cells, COX-2 must be involved in other pathways in addition to PI3K/ Akt signalling.

Numerous studies demonstrate the utility of PI3K inhibitors in either directly causing apoptosis in prostate cancer cells (Lin et al. 1999, Chinni & Sarkar 2002) or to sensitise cells to other agents.

Several studies also demonstrate an inhibitory effect of celecoxib on PI3K signalling and they typically show that reduced or inhibition of P-Akt levels is evidence for this effect. Other COX-2 inhibitors do not possess this ability to prevent Akt activation.

The relative importance of COX-2-dependent and -independent activity of celecoxib however is still unclear. Unlike wortmannin or LY294002, the target of celecoxib is not thought to be PI3K itself. Rather, PDK1 is the enzyme which is cited often as having its activity reduced following celecoxib treatment in cancer cells.

Hsu et al. (2000), Johnson et al. (2001), Zhu et al. (2002) all showed the ability of celecoxib to inhibit P-Akt production in prostate cancer cells and in addition Kulp et al. (2004) demonstrated this was due to inhibition of PDK1 activity. P-Akt and PDK1
inhibition with celecoxib occurs in other cancer cells also, for example colorectal
(Yamazaki et al. (2002), Wu et al. (2003)) and hepatocarcinoma cells (Leng et al. (2003)). Song et al. (2002) showed that celecoxib caused dephosphorylation of ERK2 in addition to Akt.

Bearing in mind that reduced P-Akt is the cited molecular mechanism for celecoxib induced apoptosis in the above studies, another potential target of celecoxib could be integrin linked kinase (ILK). This kinase has been reported to phosphorylate Akt on the ser473 residue, not thr308 in LNCaP cells (Persad et al. (2000), Graff et al. (2000)). Thus, it acts in a manner analogous to PDK2. This could allow us to explain the differential ability of PI3K inhibition (with wortmannin and LY294002) and COX-2 inhibition (with celecoxib) to sensitize LNCaP-COX-2 to carboplatin. Whereas PI3K inhibition results in complete inhibition of the PI3K pathway, celecoxib appears to prevent generation of P-Akt$^{ser473}$ only. It does not prevent generation of the active Akt$^{thr308}$ phospho-molecule (Figure 6.1).

**PI3K, but not COX-2, inhibition reduces COX-2 protein**

Our data showed that PI3K inhibition resulted in down-regulation of COX-2 protein. Other studies also report a similar effect. This should not be surprising, as PI3K activation is known to induce COX-2 in many cell types (Chapter 1.2.4). It should be noted that these studies demonstrate reduction in COX-2 via transcriptional mechanisms. COX-2 protein synthesis in our transfected cells is not under normal physiological control – its synthesis is driven by a viral promoter and therefore factors which affect COX-2 transcription, stability and degradation, both mRNA and protein may not be the same.
Tang et al. (2001) demonstrated significant reduction, but not complete inhibition, of COX-2 protein, with LY294002, following ultraviolet B radiation induced COX-2 induction in human keratinocytes. Kulkarni et al. (2001) showed that PI3K inhibition with LY294002 reduced COX-2 protein induced by EGF in cervical carcinoma cells. Bradbury et al. (2004) showed that both PI3K and p38 MAPK inhibition, but not MEK inhibition caused reduced COX-2 activity but not COX-2 levels. A mechanism was not proposed. Thus, because PI3K signalling can induce COX-2, PI3K inhibition can lead to a reduction in COX-2 levels.

Whereas we found that celecoxib treatment increased COX-2 protein levels, other groups report either no changes or a reduction in COX-2 levels following celecoxib treatment in various tumour models. The significance of these differences is unclear. Lai et al. (2003) showed that COX-2 levels were unaffected by celecoxib treatment in cholangiocarcinoma cells, and Arico et al. (2002) also found no change in levels following celecoxib treatment of colon cancer cells. In contrast, El-Rayes et al. (2004) demonstrated a reduction in COX-2 protein and mRNA in pancreatic cancer cells after celecoxib treatment combined with gemcitabine. Importantly, Gupta et al. (2004) showed that COX-2 protein, mRNA and activity was reduced in the TRAMP mouse model following dietary celecoxib over 8 to 24 weeks.

**PI3K, but not COX-2, inhibition reduces anti-apoptotic proteins**

Our data also showed that PI3K inhibition with wortmannin or LY294002 reduced Bcl-2 protein, but not survivin or Bcl-xL (data not shown) in LNCaP-COX-2. In contrast, celecoxib at sub-cytotoxic doses did not reduce Bcl-2 protein. These same effects were also observed in Bcl-2 expressing LNCaP-Neo cells.
Davies et al. (1999) inhibited PI3K signalling in LNCaP cells by performing PTEN transfection and assessing its effects on Bcl-2 transcription and p53 expression. PTEN suppressed P-Akt levels, as did wortmannin, but it did not affect Bcl-2 levels. Further, Bcl-2 transfection did not affect ability of PTEN to reduce P-Akt. Lin et al. (1999) demonstrated apoptosis with wortmannin and LY294002 in LNCaP, but not with inhibitors for pp70S6K, ERK or MEK. Apoptosis was attenuated by DHT and EGF ligands, suggesting that the survival signal was originating from cell surface or nuclear receptors. Chinni & Sarkar (2002) showed that indole-3-carbinol induced apoptosis in PC-3 by preventing Akt phosphorylation and EGF was unable to phosphorylate PI3K after indole-3-carbinol pre-incubation. There was also down-regulation of Bad and Bcl-xL, downstream targets of Akt. Carson et al. (1999) showed that serum withdrawal did not cause apoptosis in LNCaP cells and that Akt remained in its active, phosphorylated state. LY294002, however, did cause apoptosis in these cells. In contrast, DU145 cells, which contain wild type PTEN, phosphorylation only occurred in presence of exogenous IGF-1 or EGF. Davies et al. (2002) assessed the effects of PTEN transfection on proliferation, apoptosis and metastasis in PC-3 cells. In vivo PTEN had no effect on apoptosis in PC-3 cells injected into mice prostate, but proliferation was reduced. PTEN did not prevent tumour formation, however tumour growth was reduced and metastasis was significantly prevented.

Thus, both experimental agents (wortmannin and LY294002) and natural compounds (e.g. indole-3-carbinol) which inhibit PI3K signalling can have anti-tumour activity, in both in vitro and in vivo cancer models, including prostate cancer. PI3K inhibition can cause down-regulation in anti-apoptotic proteins such as Bcl-2 and Bcl-xL and this could be important in mediating these effects.
Although we did not observe a strong anti-proliferative effect of wortmannin in either LNCaP-Neo or LNCaP-COX-2 (data not shown) in the presence of serum, PI3K inhibition has been shown to cause apoptosis in LNCaP cells. The reduction in cell viability was greater in LNCaP-Neo, although not significantly, as assessed by the MTT assay, which may be a reflection of the elevated PI3K activity measured in LNCaP-COX-2.

Although celecoxib has been shown to be apoptotic in various cell types (Chapter 4), here we are more concerned with its ability or inability to modulate anti-apoptotic proteins at sub-cytotoxic, sensitising doses. Other groups generally report no reduction in Bcl-2 in particular following celecoxib treatment, at apoptotic doses.

Hsu et al. (2000) showed no involvement of Bcl-2 in celecoxib induced apoptosis in PC-3 and LNCaP cells, as enforced expression of Bcl-2 did not affect the viability of these prostate cancer cells to celecoxib. Johnson et al. (2001) obtained similar results in PC-3 cells with celecoxib, demonstrating Bcl-2 independent apoptosis. Song et al. (2002) depleted COX-2 protein in PC-3 cells using a tetracycline induced anti-sense system and assessed the effects of celecoxib, rofecoxib, DuP-697 and NS398 mediated apoptosis. They found that COX-2 expression mirrored PGE2 levels. However, reduction of COX-2 had no effect on Bcl-2. COX-2 inhibitors induced apoptosis whereas COX-2 ablation did not. Yamazaki et al. (2002) assessed the effect of 6 different COX-2 inhibitors on COX-2 positive and negative colon adenocarcinoma lines by assessing cell proliferation and apoptosis. Only celecoxib induced apoptosis, although all 6 drugs inhibited PGE2 production, whilst Bcl-2 levels remained unchanged. Arico et al. (2002) also showed that celecoxib induced apoptosis in colon adenocarcinoma cells but Bcl-2 levels were not affected. Wu et al.
(2003) showed that celecoxib caused apoptosis in cholangiocarcinoma cells, independent of Bcl-2 and Bax. PGE$_2$ partially inhibited this but COX-2 depletion by anti-sense transfection did not result in decrease in P-Akt suggesting both COX-2 dependent and independent mechanisms for celecoxib's mechanism of action. Leng et al. (2003) demonstrated that Bcl-2 and Bax levels were unaffected by celecoxib treatment of hepatocarcinoma cell lines. In contrast, Liu et al. (1998) showed that NS398 induced apoptosis in LNCaP cells, by up-regulating the inactive phosphorylated forms of Bcl-2 and down-regulating the active, unphosphorylated form.

What is the clinical significance of the findings from the data presented in this Chapter? The potential utility of COX-2 inhibitors, in particular celecoxib, as chemopreventive agents or as adjuvants to sensitisise tumours to existing therapy was discussed in Chapter 4. There, it was emphasised that these agents could cause a reduction in cell viability in cell lines or tumours regardless of their COX-2 status, but at doses which are at the upper limit of acceptable toxicity. At sub-cytotoxic doses celecoxib could not sensitisise resistant COX-2 expressing LNCaP cells to carboplatin. PI3K inhibition thus appears to be a more attractive target than COX-2 inhibition. This is especially true since we demonstrated an enzyme independent effect of COX-2 on apoptotic resistance. It was also stated that whilst agents such as celecoxib may induce apoptosis in epithelial cancer cells via predominantly COX-2 independent mechanisms, in vivo their effect on other tumourigenic processes such as angiogenesis or metastasis may be COX-2 dependent.
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To summarise, we have reported in this Chapter that stable COX-2 transfection in LNCaP activates the PI3K/ Akt cell survival pathway, as evidenced by elevated P-Akt$^{\text{ser473}}$ levels in LNCaP-COX-2 and other pharmacological effects seen from the data within this part of the thesis.
CHAPTER 7

GENE MICROARRAY ANALYSIS
Chapter 7 Gene Microarray Analysis

7.1 Aims

The purpose of performing gene microarray analysis of LNCaP-Neo and LNCaP-COX-2 cells was to assess which genes, especially those concerned with the cell cycle and apoptosis, are differentially expressed in LNCaP cells as a result of stable COX-2 transfection, using a set of 3000 oligos for cancer related genes.

7.2 Introduction

Gene microarray is sometimes referred to as DNA microarray or gene array. The terms all refer to a technique in which the principle is nucleic acid base pairing of a ‘probe’ which is attached to a substrate, and a ‘target’ whose nucleic acid identity or abundance is being measured. Various platforms can be utilised to measure the relative mRNA amounts in a sample. We used spotted cDNA microarrays on a glass slide which was hybridised with both the labelled test sample and a labelled reference sample.

The technique provides different information from Western blotting, which detects protein levels, and offers greater efficiency and diversity than mRNA analysis by either RT-PCR or Northern blot analysis. The same information is derived from RT-PCR mRNA analysis, however gene microarrays allow analysis of thousands of genes, whereas separate experiments would be required for RT-PCR, which work on a ‘one gene for one experiment’ basis. Just as important, using the microarray approach, genes which would not ordinarily be considered in a carcinogenesis model may be identified and validated. In addition, gene microarray can allow gene function
interaction to be better understood, although we have not attempted to do this in this project.

It would be expected that gene microarray experiments be performed first to identify differentially expressed genes between LNCaP-Neo and LNCaP-COX-2 cells. Then, based on the data that these experiments generate, the significance of the identified genes would be explored further. However, it should be noted that we performed gene microarray experiments at the end of the project, rather than using gene microarray data as a basis to form hypotheses. Therefore, we have not been able to further investigate the data presented in this Chapter.

7.3 Results

Using a '3000 cancer oligo' set from Qiagen Inc., a number of genes were identified as being expressed significantly differently between LNCaP-Neo and LNCaP-COX-2 cells. Not all of these genes have cell cycle or apoptosis related functions.
Figure 7.1 Dendogram of clustering analysis of gene expression variations in the prostate cell lines, LNCaP-Neo and LNCaP-COX-2. The condition tree was performed using GeneSpring using an unsupervised Spearman correlation, as described in Chapter 2.11. The colours representing the gene expression vary from green (reduced), yellow (same) and red (increased expression relative to Universal Human Reference).
The data obtained from this analysis indicating key differentially expressed genes are listed in Tables 7.1 (up-regulated in LNCaP-COX-2) and Table 7.2 (down-regulated in LNCaP-COX-2).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>p-value</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-tubulin</td>
<td>NM_006082</td>
<td>0.026</td>
<td>Cytoskeletal component</td>
</tr>
<tr>
<td>ADP-ribosylation</td>
<td>NM_001667</td>
<td>0.0171</td>
<td>GTP binding RAS protein</td>
</tr>
<tr>
<td>factor-like 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin I</td>
<td>NM_006835</td>
<td>0.00992</td>
<td>Cell cycle progression</td>
</tr>
<tr>
<td>Cytochrome c-1</td>
<td>NM_001916</td>
<td>0.00842</td>
<td>Respiratory electron transport chain; apoptosis</td>
</tr>
<tr>
<td>Fatty acid synthase</td>
<td>NM_004104</td>
<td>0.00829</td>
<td>Fatty acid synthesis</td>
</tr>
<tr>
<td>PKC-like 1</td>
<td>NM_002741</td>
<td>0.0123</td>
<td>Rho- and PDK1 signalling</td>
</tr>
<tr>
<td>Protein tyrosine kinase β</td>
<td>NM_004103</td>
<td>0.0262</td>
<td>MAPK signalling activation</td>
</tr>
<tr>
<td>Signal recognition</td>
<td>NM_006947</td>
<td>0.0113</td>
<td>Polypeptide membrane translocation</td>
</tr>
<tr>
<td>particle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivin</td>
<td>NM_001168</td>
<td>0.000607</td>
<td>IAP, anti-apoptotic</td>
</tr>
</tbody>
</table>

Table 7.1 Some genes that are up-regulated as a result of COX-2 transfection in LNCaP cells. Some genes identified as being significantly differentially expressed between LNCaP-Neo and LNCaP-COX-2 are tabulated along with the p-value. The arrays were analysed using BlueFuse software and the data was exported into GeneSpring for the statistical analysis.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>p-value</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST-π</td>
<td>NM_000852</td>
<td>0.0127</td>
<td>Detoxification enzyme, anti-inflammatory</td>
</tr>
<tr>
<td>Protein kinase</td>
<td>H200008636</td>
<td>0.0418</td>
<td>Tumour suppressor gene</td>
</tr>
<tr>
<td>cAMP dependent</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>regulatory type 1α</td>
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<td></td>
</tr>
<tr>
<td>Protein tyrosine</td>
<td>NM_151460</td>
<td>0.0258</td>
<td>T- and B-lymphocyte activation regulation</td>
</tr>
<tr>
<td>phosphatase receptor type C</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7.2 Some genes that are down-regulated as a result of COX-2 transfection in LNCaP cells. Some genes identified as being significantly differentially expressed between LNCaP-Neo and LNCaP-COX-2 are tabulated along with the p-value. The arrays were analysed using Blue Fuse software and the data was exported into GeneSpring for the statistical analysis.

Table 7.1 and Table 7.2 shows that gene microarray analysis identified a mixture of significantly differentially expressed genes, not all of which have direct cell cycle or apoptosis involvement.

There are a number of functional classes of genes represented in Tables 7.1 and Table 7.2. These include anti-oxidants (GST-π), cell cycle progression (Cyclin I, ARF-2, PKC-like 1, protein tyrosine kinase β) and the anti-apoptotic survivin. Other genes differentially expressed between LNCaP-Neo and LNCaP-COX-2 which may contribute to tumourigenesis include fatty acid synthase and α-tubulin.
7.4 Discussion

Survivin function and protein levels in our transfected LNCaP cells were discussed in Chapter 5. Gene microarray data from Table 7.1 would suggest that the elevated survivin protein levels were due to the elevated mRNA levels. However, Krysan et al. (2004) demonstrated that in NSCLC, COX-2 transfection resulted in increased survivin protein via reduced survivin ubiquitination and degradation. It is possible that this could also represent a mechanism for COX-2 mediated survivin elevation. Gene microarray experiments did not confirm the elevated Bcl-2 and Bcl-\(x_L\) protein detected in LNCaP-COX-2 reported in Chapter 5 due to technical difficulties. Thus, these two anti-apoptotic markers could have increased protein but not mRNA levels due to COX-2 mediated increased protein stability or reduced protein degradation. However, it is also possible that LNCaP-COX-2 does have elevated Bcl-2 and Bcl-\(x_L\) mRNA compared to LNCaP-Neo but experimental artefacts prevented the biological difference to be detected.

GST-\(\pi\) belongs to the GST family of enzymes which are involved in detoxification reactions (Chapter 1.2). It conjugates many hydrophobic and electrophilic compounds with reduced glutathione. GST-\(\pi\) is a recognised cancer susceptibility gene (reviewed by Nelson et al. 2001). We found that GST-\(\pi\) mRNA levels were lower in LNCaP-COX-2 cells compared to LNCaP-Neo, suggesting that COX-2, by an unknown mechanism, lowers the levels of this anti-inflammatory enzyme. We can speculate that COX-2, being pro-inflammatory itself (Chapter 1.2.5.4), may negatively regulate GST-\(\pi\), which has an opposing anti-apoptotic function. It has been proposed that inactivation of GST family members may serve as an initiating lesion for prostate cancer (Chapter 1.1.2). Our data show that COX-2 may be involved in reducing
cellular GST-\(\pi\) levels. Furthermore, GST-\(\pi\) levels have been reported to be lower in HGPIN and prostate cancer compared to normal epithelia, and GST-\(\pi\) gene silencing by hypermethylation has been reported (reviewed by Pathak et al. 2005).

The function of cyclin I is not fully understood, but it does show the highest similarity with cyclin G. We found that cyclin I mRNA was higher in LNCaP-COX-2 than LNCaP-Neo. Nakamura et al. (1995) first isolated cyclin I from a human brain derived cDNA library and they showed that it was expressed independently of the cell cycle, suggesting that it may not play a typical cyclin role. Landberg et al. (2005) found a strong correlation between cyclin I, VEGF and the VEGF receptor, KDR in human breast tumours, although no correlation between cyclin I and P-Rb, cyclin E, tumour grade or survival was noted. Bolduc et al. (2004) showed that in adipose tissue of male mice, DHT caused up-regulation of a number of genes involved in energy metabolism, such as fatty acid synthase and putative cell cycle regulators such as cyclin I.

Protein tyrosine kinase \(\beta\), also known as PYK2 or Focal adhesion kinase 2 (FAK2) is thought to play an important role in neuronal activity, but its involvement in prostate physiology is not yet established. Van der Horst et al. (2005) demonstrated the importance of this kinase in mediating hereregulin mediated invasion of glioma cells as a result of HER2 and HER3 heterodimerization. Further, MAPK was activated following protein tyrosine kinase phosphorylation by HER3. We found that the mRNA for this gene was elevated in LNCaP-COX-2 cells (Table 7.1). As COX-2 has been implicated in invasion and metastasis (Chapter 1.2.5.5) it is possible that this could in part be due to elevation of protein tyrosine kinase \(\beta\). Avraham et al. (2003) also demonstrated the involvement of this family of proteins in mediating VEGF induced angiogenesis in human brain endothelial cells. In particular, VEGF
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phosphorylated protein tyrosine kinase \( \beta \) which permitted migration of endothelial cells.

The protein encoded by the PKC-like 1 gene belongs to the PKC super family. This kinase is activated by Rho family of small G proteins and mediates the Rho-dependent signalling pathway. PDK1 is reported to phosphorylate this kinase also. PKC-like 1 mRNA was found to be elevated in LNCaP-COX-2 compared to LNCaP-Neo. Prostate tumours have been reported to over-express this protein also. Metzeger et al. (2003) showed that PKC-like 1 could activate the androgen receptor, independent of ligand in prostate cancer cells. This protein thus could be providing an additional survival signal, when over-expressed even further via COX-2, in LNCaP cells.

\( \alpha \)-tubulin, upon polymerisation, forms part of the cytoskeletal system. Its mRNA levels were found to be increased in LNCaP-COX-2 cells and has also been reported to be increased in paclitaxel resistant breast cancer cells (Banerjee 2002). However, the significance of our findings is not known. Similarly, the 72 kDa signal recognition particle, one component of the ribo-protein complex which targets proteins to membranes, was also up-regulated in LNCaP-COX-2. The relevance of this is not known at this stage.

The gene which encodes for fatty acid synthase produces a multifunctional enzyme whose main function is to synthesise palmitate from acetyl-CoA and malonyl-CoA into long chain fatty acids. The mRNA for fatty acid synthase was found to be elevated in LNCaP-COX-2 (Table 7.1). As Van de Sande et al. (2002) showed that PI3K inhibition with both LY294002 and PTEN transfection resulted in decreased fatty acid synthase mRNA, we can hypothesise that elevated PI3K activity in LNCaP-COX-2 may be responsible for this elevated fatty acid synthase mRNA. Further,
androgens mediate increased fatty acid synthase gene expression (reviewed by Swinnen & Verhoeven 1998).

Protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1) is a regulatory subunit of protein kinase A. It is reported to have tumour suppressive function. We found that this gene was down-regulated in LNCaP-COX-2. In neuroblastoma cells, Constantinesan et al. (2004) demonstrated that this protein inhibited MAPK signalling via B-raf. Veuglers et al. (2004) showed that in mice haplosufficient for this protein, tumourigenesis was more prevalent. They also demonstrated 65% of mutations in this gene in humans with Carney complex, a multiple neoplasia syndrome. We do not know at this stage how important reduced expression of this protein in LNCaP-COX-2 is in mediating a resistant phenotype in these cells.

Cyt c is a multi-subunit enzyme involved in oxidative phosphorylation. Some subunits are encoded for by the mitochondrial genome, the others by nuclear DNA. Hermann et al. (2003) showed that the ratio of nuclear to mitochondrial cyt c subunits was greater in prostate cancer samples compared to normal and pre-malignant samples. There was also altered oxidative phosphorylation between normal and prostate tissue and they proposed that this could offer a survival advantage in tumourigenesis. Although their findings did not refer to the cyt c subunit 1, we found that this subunit was elevated in LNCaP-COX-2 (Table 7.1). Subunit 1 is encoded for by mitochondrial DNA. How and why COX-2 mediates an elevation of this gene could be explored further. The change in cyt c expression levels could relate to an alteration in mitochondrial integrity which in turn could influence the mitochondrial pathway of apoptosis.
Chapter 7 Gene Microarray Analysis

The function of the ADP-ribosylation factor-like (ARL) group of genes has not been fully elucidated. Like the ADP-ribosylation factor (ARF) gene products, they are GTP-binding proteins of the RAS super family. These proteins hydrolyse GTP to perform a wide range of cellular functions. Bhamipidati et al. (2000) showed that ARL2, whose transcript we have found to be elevated in LNCaP-COX-2 (Table 7.1) prevented destruction of tubulin and microtubules. This however is unlikely to explain why we also found α-tubulin levels to be elevated in LNCaP-COX-2 (Table 7.1) as ARL2 mediated preservation of microtubules is mediated through protein-protein interaction and not transcriptional events. The relevance of ARL2 up-regulation in COX-2 expressing LNCaP cells is therefore not understood yet.

To summarise, the data in this Chapter highlighted some key differences in gene expression between COX-2 non- and over-expressing LNCaP cells as detected by gene microarray analysis. This microarray data presented some opportunities for further investigation. This was because genes potentially involved in tumourigenesis were identified with this technique. These included genes implicated in inflammation, cell cycle progression and apoptosis inhibition. It confirmed some data presented in previous Chapters, which used techniques such as Western blotting and flow cytometry. For example, elevation of survivin mRNA by microarray analysis corroborated with elevated survivin protein (Chapter 5). However, further experiments via RT-PCR and Western blotting need to be performed because we cannot exclude the possibility that the findings in these gene microarray experiments represent false positives.
Chapter 8

OVERALL CONCLUSIONS AND FUTURE WORK
Chapter 8 Overall Conclusions and Future Work

This study showed that COX-2 transfection in the LNCaP cell line mediated a resistance to the effects of carboplatin in serum and to etoposide in the absence of serum, as measured with the MTT assay, and to celecoxib, as measured with the Trypan blue assay. The resistance to carboplatin was due to a number of factors. We found an attenuation of its cell cycle effects, and there was reduced p53 and p27KIP1 induction and elevation of cyclin B1. We also detected reduced apoptosis, as COX-2 produced an elevation of anti-apoptotic Bcl-2, Bcl-xL, and survivin proteins. However, COX-2 could not mediate a resistance to the endogenous apoptosis inducers Fas and TRAIL. Surprisingly, celecoxib was not able to sensitisze LNCaP-COX-2 to carboplatin and PGE2 could not increase the resistance of LNCaP-Neo to this chemotherapeutic agent. This suggested that COX-2 was having a protein (COX-2) dependent, but catalytic (PGE2) independent effect on LNCaP cells’ sensitivity to carboplatin. COX-2 over-expressing LNCaP cells had elevated P-Akt^{ser473} levels and PI3K inhibitors were able to partially sensitisze these cells to carboplatin, suggesting that COX-2 activated not only PI3K/Akt but also other pro-survival or pro-proliferation pathways.

Gene microarray work confirmed the elevated survivin levels in LNCaP-COX-2. It also highlighted a number of other genes which are differentially expressed between COX-2 expressing and COX-2 negative cells. These included reduced GST-π in LNCaP-COX-2, and elevated α-tubulin, fatty acid synthase and PKC-like-1 mRNA in LNCaP-COX-2. These COX-2 mediated changes may enhance the tumourigenic potential of LNCaP cells by a variety of mechanisms including resistance to apoptosis, cell cycle progression or promoting inflammation.
We had to develop strategies and models to assess the effect of COX-2 on cell viability following cytotoxic treatment because our three prostate cancer cell lines, PC-3, DU145 and LNCaP did not express COX-2 protein. Even if a variation of COX-2 levels was detected between the cells, it would not have been possible to accurately correlate their sensitivity to various cytotoxic agents to their COX-2 levels. The reason for this is that these cell lines are genetically very different and their sensitivity to cytotoxic agents would be reflected not just by COX-2 but by a number of other genetic factors. This was the reason that PMA induction of COX-2 in PC-3, which mediated a resistance to etoposide was not pursued further. PMA may have been inducing other genes or stabilising other proteins, in addition to COX-2, and could therefore not be considered specific for COX-2.

We concentrated our experiments on the LNCaP stable transfectant. This was because COX-2 levels in this model remained consistently elevated and COX-2 mediated resistance was observed in a number of scenarios. A stable transfectant of other cell lines, for example PC-3 and DU145 could have been attempted. This would be useful for several reasons. Firstly, PC-3 and LNCaP have different characteristics to LNCaP – they are androgen insensitive, whereas LNCaP can be either androgen dependent or androgen sensitive. Both PC-3 and DU145 are also p53 defective, unlike LNCaP which is wild-type for p53. PC-3 does not express p53 at all, whereas DU145 expresses mutant, non-functional p53. Secondly, no effect of COX-2 was demonstrated in the transient PC-3 transfectant, and this may have been due to the lower COX-2 levels, or the fact that it is p53 negative.

The differential effects of celecoxib on LNCaP-Neo and LNCaP-COX-2 cells could be explored further. Initial experiments utilising the MTT assay showed that the two
cell lines had similar IC$_{50}$ values in a 72 hour cytotoxicity assay. However, Trypan blue data provided evidence that COX-2 over-expression caused greater cell kill in LNCaP-Neo at 80-100 μM, doses which exceed the IC$_{50}$. To confirm if COX-2 anti-apoptotic effects are catalysis- or prostaglandin-independent, we could have transfected prostate cancer cells with a gene encoding a mutant COX-2 protein, deficient in catalysis.

We did not measure the amount of DNA damage carboplatin induced in LNCaP-Neo or LNCaP-COX-2. We cannot exclude the possibility that COX-2 is affecting components of the DNA damage sense machinery in addition to p53. We did not assess if DNA repair enzymes were implicated in anyway. This could be looked at in the future.

Although both LNCaP-Neo and LNCaP-COX-2 cells did not detect p27$^{kip}$ basally, LNCaP-COX-2 did express elevated levels of cyclin B1. We did not assess if this cyclin B1 elevation was a direct result of PI3K activity. This could have been achieved by measuring the levels of cyclin B1 following wortmannin or LY294002 treatment of LNCaP-COX-2 cells.

We demonstrated up-regulation of three anti-apoptotic proteins due to COX-2 transfection, Bcl-2, Bcl-$x_L$ and survivin. We do not know if this was this due to increased transcription of their genes or due to increased mRNA or protein stability. Gene transcription could be assessed by inserting promoter regions of each of the genes, linked to β-galactosidase for example, and measuring activity for both LNCaP-Neo and LNCaP-COX-2. We could also have checked if celecoxib or PGE$_2$ can modulate these gene transcriptional activities; we suspect not because neither celecoxib nor PGE$_2$ could modulate the sensitivity of LNCaP-COX-2 and LNCaP-
Neo respectively to carboplatin. Krysan et al. (2004) showed that COX-2 caused survivin stabilisation via reduced ubiquitination in NSCLC cells. We do not know if COX-2 in LNCaP is involved in enhanced protein stability or degradation of a variety of cellular proteins. This could be explored further.

We could have transfected either Bcl-2, Bcl-xL or survivin into LNCaP-Neo or parental LNCaP cells to verify the importance of each in mediating the resistance to apoptosis. Conversely, we could attempted to down-regulate Bcl-2, Bcl-xL or survivin in LNCaP-COX-2, using an anti-sense strategy to assess how significant each is in mediating resistances to carboplatin, etoposide and celecoxib.

We did not assess caspase activities in LNCaP-Neo or LNCaP-COX-2 cells following either carboplatin, etoposide or celecoxib treatment. This would be interesting to explore because there is elevated survivin in LNCaP-COX-2 and this should reduce caspase-3 and caspase-7 activity. Similarly, as LNCaP cells are known to be resistant to TRAIL at the mitochondrial level (Chapter 6), and factors which prevent mitochondrial membrane potential breakdown (Bcl-2 and Bcl-xL) are elevated in COX-2 expressing cells, we could have assessed if COX-2 affects the mitochondrial pathway, for example by preventing mitochondrial membrane potential breakdown or cyt c release.

It is known that NF-κB is anti-apoptotic, with target genes such as cIAP1, cIAP2, xIAP, FLICE and Bcl-xL. Therefore, it could be investigated if COX-2 is involved in NF-κB signalling in some way; for example, activating it by promoting degradation of its inhibitory subunit, IκB.

It would also be possible in the future to generate a xenograft model of COX-2 over-expressing LNCaP cells, for example in athymic, nude mice to confirm if the
resistance observed in cell lines to carboplatin, etoposide and celecoxib can be reproduced \textit{in vivo}.

Because celecoxib, in a manner similar to PI3K inhibition, causes decreases in P-Akt$^{\text{ser473}}$, but it is not thought to inhibit PI3K itself, we could have confirmed that celecoxib does not attenuate PI3K activity but wortmannin and LY294002 do with a specific assay in LNCaP cells. For example, Arico \textit{et al.} (2002) did have performed specific immunoprecipitation kinase assays of PDK1 and Akt.

We demonstrated that COX-2 elevates P-Akt$^{\text{ser473}}$ in LNCaP. We could have assessed if COX-2 depletion, for example with an anti-sense approach, will also lead to reduced P-Akt$^{\text{ser473}}$ levels. This would confirm if COX-2 catalytic activity or COX-2 binding is important in activating Akt. Wu \textit{et al.} (2003) showed in cholangiocarcinoma cells that it did not, but this may not the case in LNCaP prostate cancer cells.

MDM2, the protein which targets p53 for degradation, is a downstream effector of PI3K signalling (Figure 6.1). Because p53 and \textsuperscript{p27}KIP1 induction is reduced in LNCaP-COX-2 cells which have elevated P-Akt, the possibility exists that P-Akt is directly responsible for this. We could have confirmed if reduced p53 induction in COX-2 expressing cells was due to elevated activity of MDM2 by assessing its phosphorylation status.

The partial reversal of resistance of LNCaP-COX-2 to carboplatin with PI3K inhibitors could have been confirmed with the use of a dominant negative form of Akt in LNCaP-COX-2.

Although no difference in sensitivity between LNCaP-Neo and LNCaP-COX-2 to TRAIL and Fas was observed, we could have observed if COX-2 was mediating any
changes in the levels of molecules involved in these signalling pathways. For example, death receptors DR4, DR5 and Fas, decoy receptors DR1 and DR2, caspase-8 or cFLIP. We can suspect not, but it is another area which could be investigated further.

It is known that LY294002 treatment of PC-3 and LNCaP cells causes reduction of pro-apoptotic P-Bad (Yang et al, 2003). This is one example of other potential effects of wortmannin or LY294002 we could have potentially explored, particularly in LNCaP-COX-2. We showed PI3K inhibition with both wortmannin and LY294002 caused reduction in COX-2 and Bcl-2 protein. However we did not assess if PI3K inhibition could alter cellular levels of the other elevated anti-apoptotic and pro-proliferative markers such as Bcl-xl, survivin and cyclin B1. The JNK MAPK pathway phosphorylates Bcl-xl, Bcl-2, Bad and p53 to promote cell cycle arrest and apoptosis (Sah et al. 2003). What else does PI3K inhibition do particularly in LNCaP-COX-2 but also in LNCaP-Neo? This question could be addressed in the future.

ILK has been shown to phosphorylate Akt on the ser473 residue (Chapter 6). Does COX-2 increase ILK levels or activity? We could have inhibited ILK pharmacologically and determine if this has the same effect as PI3K inhibition on sensitising LNCaP-COX-2 to carboplatin.

Because COX-2 has been shown to increase EGFR levels and EGFR activity (Chapter 1.2.5.1) and that the EGFR can activate the PI3K/ Akt pathway, we could have assessed if the elevated P-Akt levels detected in LNCaP cells transfected with COX-2 were associated with elevated EGFR levels also. This could have been confirmed by Western blot or immunohistochemistry.
Celecoxib and PGE$_2$ were shown in Chapter 4 not to be able to modulate the sensitivity of LNCaP cells to carboplatin. This was explained in Chapter 6 where it was shown that celecoxib could not reduce Bcl-2 or COX-2 levels, whereas PI3K could. In follow up experiments we could treat LNCaP-Neo with PGE$_2$ and LNCaP-COX-2 with celecoxib and perform gene microarray experiments to assess which genes are modulated by COX-2 enzymatic activity. For example, is the elevated survivin and Bcl-2 reported in LNCaP-COX-2 due to COX-2 enzymatic activity or not. Whilst Western blotting and RT-PCR allow changes in protein and mRNA levels respectively to be determined, gene microarray analysis would allow many differences in gene expression between LNCaP-Neo and LNCaP-COX-2 to be identified and investigated further.
REFERENCES


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241
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Appendix

The tables in this appendix provide data from individual experiments which form the basis of the results in the Figures and Tables below. Experimental details are described in the main body of the thesis.

Table 4.1 and Figure 5.12

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p values: 0.006916213 LNCaP-Neo vs LNCaP-COX-2
2-tail, paired: 0.032276216 LNCaP-COX-2 control vs wortmannin

Table 4.2

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**Figure 4.2**

**VP-16, 3 day, serum free**

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**p-value** 0.106628
2 tail, paired
Figure 4.3

VP-16, 6 day, serum free

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Table 4.3

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<td>76.019734</td>
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<td>233.75</td>
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</table>
Figure 4.4

### Celecoxib trypan blue data

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<th>Average</th>
<th>Individual values</th>
<th>SEM</th>
<th>Average</th>
<th>Individual values</th>
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<th>p-value</th>
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<td>99.8, 100, 99.4</td>
<td>0.176383</td>
<td>0.829749</td>
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<td>100, 100, 100</td>
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<td>96.1</td>
<td>95.5, 96.7, 96.1</td>
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<td>0.135443</td>
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<td>95.0, 98.9, 95.2</td>
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Table 4.4

### Zero FCS control IC_{50} data (µM)

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<th>SD</th>
<th>Individual values</th>
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<tbody>
<tr>
<td>Celecoxib</td>
<td>31.825</td>
<td>3.888766</td>
<td>33, 31, 27, 36.3</td>
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<tr>
<td>Sulindac</td>
<td>128.575</td>
<td>62.60928</td>
<td>112, 126, 213, 63</td>
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<tr>
<td>Etoposide</td>
<td>44.975</td>
<td>14.87781</td>
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### 100 nM PMA data (µM)

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<td>1.284199</td>
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<td>Sulindac</td>
<td>128.95</td>
<td>57.71201</td>
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<tr>
<td>Etoposide</td>
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Figure 4.5

<table>
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<th>5.0</th>
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<td>103.4</td>
<td>114.7</td>
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<td>100.9</td>
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<td>107.4</td>
<td>103.767</td>
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### Figure 4.6

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<td>106.4</td>
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<tr>
<td>95.1</td>
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### Figure 5.3

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<th>S</th>
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<td>LNCaP-Neo</td>
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</tr>
<tr>
<td>Control</td>
<td>51.8 (3.3)</td>
<td>26.6 (2.4)</td>
<td>21.6 (3.9)</td>
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<tr>
<td>Carboplatin</td>
<td>36.9 (6.5)</td>
<td>53.9 (5.5)</td>
<td>9.2 (4.4)</td>
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<tr>
<td>LNCaP-COX-2</td>
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<tr>
<td>Control</td>
<td>61.5 (7.7)</td>
<td>23.4 (3.9)</td>
<td>14.1 (3.0)</td>
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<tr>
<td>Carboplatin</td>
<td>45.6 (10.2)</td>
<td>39.0 (8.8)</td>
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