Validation of cytochrome P450 1B1 as a target for the enzyme-prodrug therapy of cancer

by
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Gray Cancer Institute
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This thesis describes research conducted at the Gray Cancer Institute whilst registered at the School of Pharmacy, University of London between 2001 and 2004 under the principal supervision of Dr. Steven A. Everett with support from Prof. Laurence H. Patterson and Dr. Andy Wilderspin. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.

Mhairi Greer
Signature

6.9.05
Date
Abstract

This study is comprised of three parts including: (1) clinical validation of cytochrome P450 CYP1B1 in clinical head and neck squamous cell carcinoma (HNSCC) and associated pre-malignant tissue, (2) the identification of a human tumour xenograft model which exhibits either constitutive or inducible expression of CYP1B1 for the evaluation of novel prodrug therapies in vivo, and (3) the identification of stable prodrugs targeted at CYP1-family enzymes designed to deliver nitric oxide to tumours.

CYP1B1 protein was detected in HNSCC by immunohistochemistry (IHC) with a selective monoclonal antibody and was over-expressed to a high frequency in carcinoma (94 %, n = 70) and associated pre-malignant, hyperplastic (100 %, n = 14), and dysplastic tissues (100 %, n = 22). Quantitative analysis of CYP1B1 staining intensity by spectral imaging microscopy facilitated correlations with other clinico-pathological parameters including tumour grade, stage and anatomical site. CYP1B1 is mainly localised in the cancer tissue and is not present in adjacent stromal tissue, suggesting it may be a possible target for the enzyme prodrug therapy of cancer.

A primary human tumour xenograft model was identified that constitutively expresses CYP1B1. Target enzyme protein expression was also inducible in a time- and dose-dependent manner in vivo as detected by western blotting, IHC and spectral imaging microscopy. Parallel studies also demonstrated CYP1A1 induction in the MCF-7 breast tumour xenograft model. This work has helped validate two potential human tumour xenograft models for the in vivo evaluation of both CYP1A1- and CYP1B1-activated prodrugs for cancer therapy.

In order to exploit the cytotoxic and hypoxic cell radiosensitising properties of nitric oxide (NO*), 10 novel benzo- or pyrido-fused indole oximes designed as prodrugs for NO* were screened for their selectivity for CYP1-family enzymes. GCI 510 was identified as a selective prodrug for CYP1A1, which generated NO* efficiently. This study has contributed to an understanding of the structural features of these prodrugs which confer selectivity for individual CYP1-family enzymes. Future work will seek to identify CYP1B1-selective prodrugs for NO* based on data from this study.
Acknowledgements

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Firstly, thank you to my supervisors Dr. Steven Everett (Gray Cancer Institute, GCI), Dr. Andy Wilderspin and Prof. Laurence Patterson (School of Pharmacy) for their leadership, vision and for making this Ph.D. project possible.

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## Abbreviations

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<thead>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>5F 203</td>
<td>2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (Phortress™)</td>
</tr>
<tr>
<td>5-FC</td>
<td>5-fluorocytosine</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>ABC</td>
<td>avidin biotin complex</td>
</tr>
<tr>
<td>ADEPT</td>
<td>antibody directed enzyme prodrug therapy</td>
</tr>
<tr>
<td>Ah</td>
<td>aryl hydrocarbon</td>
</tr>
<tr>
<td>AhR</td>
<td>aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium per sulphate</td>
</tr>
<tr>
<td>ARNT</td>
<td>aryl hydrocarbon nuclear translocator</td>
</tr>
<tr>
<td>BE</td>
<td>bystander effect</td>
</tr>
<tr>
<td>CHART</td>
<td>continuous hyperfractionated accelerated radiotherapy</td>
</tr>
<tr>
<td>CPA</td>
<td>cyclophosphamide</td>
</tr>
<tr>
<td>CYP/CYP450</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>CYP-R</td>
<td>Cytochrome P450 reductase</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3-diaminobenzidine</td>
</tr>
<tr>
<td>DEA/NO</td>
<td>2-(N, N-diethylamino)-diazenolate 2-oxide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>oestrogen response element</td>
</tr>
<tr>
<td>EROD</td>
<td>ethoxyresorufin O-dealkylase</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>GC</td>
<td>guanylate cyclase</td>
</tr>
<tr>
<td>GCV</td>
<td>gancyclovir</td>
</tr>
<tr>
<td>GDEPT</td>
<td>gene directed enzyme prodrug therapy</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>glucose transporter-1</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>H₄B</td>
<td>tetrahydrobiopterin</td>
</tr>
<tr>
<td>HIF</td>
<td>hypoxia-inducible factor</td>
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HNSCC  head and neck squamous cell carcinoma
HPLC  high performance liquid chromatography
HRP  horseradish peroxidase
HSP-90  heat shock protein-90
HSV  herpes simplex virus type-1
IFA  ifosphamide
IHC  immunohistochemistry
i.p.  intraperitoneally
LI  labelling index
Log P  log partition coefficient
MMDX  3’deamino-3’-(2(s)-methoxy-4-morpholinyl
MNA  mean normalised absorbance
NFI  nuclear factor 1
NO*  nitric oxide
NO₂⁻  nitrite ions
NO₃⁻  nitrate ions
NOS  nitric oxide synthase
PAH  polycyclic aromatic hydrocarbons
PAPA/NO  3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine
PBS  phosphate-buffered saline
ROS  reactive oxygen species
RNS  reactive nitrogen species
SAR  structure-activity relationship
SDS  sodium dodecyl sulphate
SDS-PAGE  sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TEMED  N, N', N''-tetramethylethlenediamine
TBS  tris-buffered saline
TCDD  2,3,7,8-tetrachlorodibenzo-p-dioxin
TK  thymidine kinase
Tpot  potential doubling time
TRX  thioredoxin
Ts  duration of S phase
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>UKCCR</td>
<td>United Kingdom Coordinating Committee for Cancer Research</td>
</tr>
<tr>
<td>VDEPT</td>
<td>virus directed enzyme prodrug therapy</td>
</tr>
<tr>
<td>XRE/DRE</td>
<td>xenobiotic response element/dioxin response element</td>
</tr>
</tbody>
</table>
"...for there to be selective tumour cell kill the prodrug must be a good substrate for the enzyme under physiological conditions and the enzyme should be present ideally uniquely in the tumour cells..." (Connors 1986)

Tom A. Connors, 1934-2002
Chapter 1

Introduction
1.1 The Cytochrome P450/P450 reductase system

The Cytochrome P450 mono-oxygenases are a large family of constitutive and inducible iso-enzymes, which have evolved as an adaptive response to environmental chemicals (Nebert & Gonzalez 1987). They are named as such, due to their haem-thiolate containing characteristic, with an absorption spectrum at 450 nm in carbon monoxide (Estabrook et al., 1963), with the P standing for pigment (Estabrook 1999). Since their discovery in 1958, (Garfinkel 1958; Klingenberg 1958), much of the research around the cytochrome P450s has focused around their role in metabolism and toxicology. In mammals, CYP P450s are membrane bound in the endoplasmic reticulum and are found in most organs in the body, including the intestine and cortex of the brain. The concentration in the liver exceeds that of other haemproteins, with the total percentage of CYPs representing greater than 1 % in rat liver protein (Estabrook 1999).

In the year 2000, there were 1200 identified P450 genes, which are classically arranged into gene families, based on amino acid sequence (Lewis 2001). Currently there are 18 families of CYPs with 57 members (Nelson 2003; Rooney et al., 2004). The P450 nomenclature is shown with this example; the protein CYP1A1 designates that this enzyme is in the CYP 1 gene family, the subfamily is A and the specific gene is 1. Amino acid sequences of above 40 % homology between isoforms, designate that the proteins belong in the same family, and above 55 % indicates that the proteins belong in the same subfamily. P450s are italicised if the gene is being referred to and mouse P450s are usually written in lower case.

CYP P450s main function is to catalyse natural reactions in the body, such as steroid metabolism and fatty acid biosynthesis/metabolism (Lewis 2001). In some cases the primary role of some CYP isoforms is the oxidative metabolism of xenobiotics (foreign substances) (Murray 1999). Typically, this is carried out by the CYP1-4 family members, which includes the CYP1A1, CYP1A2, CYP2D6, CYP3A4 and CYP1B1 isoforms (Guengerich et al., 1994; Shimada et al., 1997; Miyazaki et al., 2005). CYPs can carry out a variety of oxidation reactions, including hydroxylations (Crewe et al., 1997; Wilson & Reed 2001) and epoxidations (Guengerich 2003). This is achieved by the incorporation of molecular oxygen into the organic substrate, with the production of water and a mono-oxygenated metabolite (Lewis 2001). This is shown in Figure 1.1.
The first stage in the catalytic cycle of CYP P450 is the addition of the substrate to the haem-containing cytochrome (1) and this occurs when the haem is in its ferric ($^{3+}$) state. The substrate-enzyme complex is then modified by the reduction of the iron atom from a ferric to ferrous ($^{2+}$) state. Cytochrome P450 reductase (CYP-R) functions synergistically with CYP mono-oxygenase to donate electrons to the enzyme complex. These electrons come from the co-factor NADPH (2). CYP undergoes re-arrangement when molecular oxygen is added at stage 3. CYP-R donates a second electron at stage 4 resulting in water production at (5) (Lewis 2001). The substrate becomes hydroxylated via the hydroxyl radical (produced in the reaction) and may further be metabolised by other detoxifying enzymes, such as glutathione transferases (GSTs) (Sheehan et al., 2001).

Different CYP isoforms are known to carry out very individual functions as summarised in Table 1.1.
Table 1.1 The CYP isoforms responsible for a range of metabolic functions

<table>
<thead>
<tr>
<th>CYP</th>
<th>Functionality or species</th>
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<tbody>
<tr>
<td>1</td>
<td>Metabolism of foreign compounds</td>
</tr>
<tr>
<td>2</td>
<td>Metabolism of foreign compounds</td>
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<tr>
<td>3</td>
<td>Metabolism of foreign compounds</td>
</tr>
<tr>
<td>4</td>
<td>Metabolism of foreign compounds</td>
</tr>
<tr>
<td>5</td>
<td>Thromboxane synthesis</td>
</tr>
<tr>
<td>6</td>
<td>Insect forms</td>
</tr>
<tr>
<td>7</td>
<td>Steriod metabolism</td>
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</tr>
<tr>
<td>21</td>
<td>Steriod biosynthesis</td>
</tr>
<tr>
<td>24</td>
<td>Vitamin D metabolism</td>
</tr>
<tr>
<td>26</td>
<td>Retinoid metabolism</td>
</tr>
<tr>
<td>27</td>
<td>Bile acid biosynthesis</td>
</tr>
<tr>
<td>51-70</td>
<td>Fungal forms</td>
</tr>
<tr>
<td>71-100</td>
<td>Plant forms</td>
</tr>
<tr>
<td>101-140</td>
<td>Bacterial forms</td>
</tr>
</tbody>
</table>

Even within one gene family there are a range of isoforms that have their own metabolic identity. This is illustrated by the CYP1 gene family where the 3 isoforms within this group carry out very different metabolic functions. CYP1A1 hydroxylates benzo(a)pyrene (Schmalix et al., 1993) (a carcinogen found in cigarette smoke), as well as O-dealkylating 7-ethoxyresorufin (Burke et al., 1994). CYP1A2 can N-hydroxylate 2-acetylaminofluorene (Battula et al., 1991), as well as O-deethylating phenacetin (Venkatakrishnan et al., 1998). CYP1B1 is known to metabolise oestradiol (Spink et al., 1994) as well as having the capacity to oxidise an array of xenobiotics (Shimada et al., 1997).

1.1.1 Cytochrome P450 reductase

The flavoprotein P450 Reductase (P450R) is also located in the endoplasmic reticulum and catalyses the reactions of P450, by reducing NADP and generating electrons, which are donated to the substrate (Wada et al., 1968; Murataliev et al.,
There is only one gene coding for one CYP-R enzyme, and it is responsible for electron transfer to all of the CYP mono-oxygenase isoforms (Porter & Kasper 1985). Electrons are transferred one at a time from NADPH to the CYP haemprotein, via bound co-factors, namely FAD (Flavin Adenine Dinucleotide) and FMN (Flavin MonoNucleotide) (Wada et al., 1968).

Later in this chapter, the therapeutic potential of CYP mono-oxygenases will be discussed. However, there are strategies currently in place to exploit the CYP-R component for cancer therapy. Bioreductive drugs are effective in treating hypoxic diseases, and are activated by CYP-R in tumour reducing conditions such as hypoxia. There are 3 main classes of bioreductive prodrugs; the N-oxides, the nitroaromatics and the quinones (Jaffar et al., 2001). Tirapazamine (1) is bioactivated by CYP-R in a one electron reduction reaction (Lloyd et al., 1991). The toxic nitroxide radical generated in the reaction, which causes single and double strand DNA breaks, is quenched by molecular oxygen in oxic conditions (Brown 1993), hence tirapazamine’s acceptance as a hypoxia-selective drug. Another example of a bioreductive CYP-R activated prodrug is menadione (2). This has been shown to be effective in a variety of tumour cell lines including those of the liver, colon, kidney stomach and prostate (Nutter et al., 1991). This drug also undergoes one electron reduction with the toxic species being a semi-quinone radical (Winterbourn et al., 1979). The indolequinone compound EO9 (3) is also metabolised by CYP-R but has failed to show clinical efficacy, therefore, compounds which are structurally similar and exert better anti-tumour activity are currently under investigation (Loadman et al., 2002; Phillips et al., 2004). AQ4N (4) is unusual in the field of cancer prodrugs as it is metabolised by CYP-R as well as CYP mono-oxygenase in hypoxic/anoxic conditions (Patterson et al., 1999; Patterson 2002). Both pathways will result in the production of the active metabolite AQ4, which is a potent topoisomerase II inhibitor, as well as a radiation enhancer (Chen & Waxman 2002).
1.1.2 Compounds metabolised by cytochrome P450

CYPs can activate an array of toxic compounds and this is emphasised by their high abundance in the liver. The aim of most cancer therapeutic small
molecules is to utilise intra-tumoural expression of CYPs, allowing the bioactivation of drugs, resulting in direct toxicity (Connors 1986). The lack of expression of specific CYPs in cancer can be circumvented with gene therapy (see section 1.1.4). Tamoxifen is hydroxylated by CYPs 2D6, 2C9 and 3A4 to an active metabolite (4-hydroxy-tamoxifen) that is effective as an oestrogen receptor antagonist (Crewe et al., 1997). This can occur in the liver by CYP2B6, CYP2C9 and CYP2D6 (Chang et al., 1993; Coller et al., 2002) or directly in the tumour by CYP2B6 (Bieche et al., 2004). Cyclophosphamide (CPA, 5) (first synthesised in 1958), is an oxazaphosphorine prodrug which undergoes hydroxylation by CYP2B6 and CYP3A4 (Chang et al., 1993). It is not known if this metabolism can occur in tumours, but toxicity has been demonstrated in vitro, upon incubation with certain CYP isoform microsomes and also with mouse fibroblasts and macrophages (Gut et al., 2000). Additionally, gene transfer of CYP2B6 and 3A4 in gliosarcoma cells illustrated that cell death can be achieved upon treatment with CPA (Jounaidi et al., 1998). The anti-tumour effects of CPA are due to the production of the toxic metabolite acrolein. In these initial studies CPA metabolism occurred upon incubation with liver microsomes, 4 hydroxy CPA (shown in red, 5) was generated, which undergoes subsequent β-elimination to produce the aforementioned acrolein (Alarcon et al., 1972). Phosphoramide mustard (the non membrane-permeable DNA alkylator) is also generated in CPA metabolism, and this can contribute to this compound’s toxicity (Plowchalk & Mattison 1991). As with many cancer drugs, non-specific toxicity is elicited, and the disadvantages of CPA relate to bladder toxicity (Philips et al., 1961). Ifosfamide (IFA), an isomer of CPA, is also metabolised by the same CYPs as CPA (Chang et al., 1993) and exerts the same toxic effects by acting as an alkylating agent (Della Morte et al., 1986; Jounaidi et al., 1998). Studies have shown that injection of CYP expressing cells (CYP2B1) are successful in the bioactivation of oxazaphosphorine based compounds, where the toxic metabolites exert localised toxicity in experimental animals (Karle et al., 1998). Doxorubicin (6) is a clinically established drug which is activated by CYP2B1 (Goepart et al., 1993).
3’deamino-3’-(2(s)-methoxy-4-morpholinyl (MMDX) is a derivative of doxorubicin and will inhibit topoisomerase I and II, is more lipophilic and 80 times more potent than the original compound (Ripamonti et al., 1992). Doxorubicin, thioTEPA and tegafur (prodrug of 5-flourouracil) are metabolised by CYPs, namely CYP3A4, 2B6 and 2A6 (Ikeda et al., 2000), respectively, and are also used clinically. There is an array of other anti-cancer drugs including flutamide (an anti-androgen) and decarbazine (a DNA methylating agent) which are metabolised by different CYP isoforms, namely CYP1A2 (for both drugs) and CYP2E1 (for flutamide) (Shet et al., 1997; Reid et al., 1999). Their metabolism and effectiveness can be affected by the expression of those isoforms within the body. Therefore, increased expression of a CYP that activates the prodrug gives increased potency, whilst increased expression of a deactivating CYP could decrease potency. Acetaminophen (paracetamol) is
metabolised by CYP1A2, amongst other CYP isoforms (Patten et al., 1993) and has also been proposed in gene therapy approaches (Thatcher et al., 2000)( See 1.1.4)

1.1.3 Detoxification (inactivation) of compounds via CYP P450 metabolism

Some anti-cancer drugs are metabolised by CYP isoforms, giving reduced bioavailability and pharmacological action. This is illustrated by paclitaxel and docetaxyl, which are inactivated by CYP3A4 and CYP2C8 in the gastrointestinal tract (Schellens et al., 2000). A group of anti-cancer compounds isolated from the periwinkle plant, the vinca alkaloids (Johnson et al., 1963), exhibit anti-tumour properties by binding to tubulin whilst disrupting the chromosomal apparatus (Singer & Himes 1992). Most of the attention in this group for compounds has focused upon vincristine and vinblastine. Drug inactivation of these compounds occurs with members of the CYP3A family (Yao et al., 2000), and specific toxicity has been shown towards an array of solid tumours (Jordan & Wilson 2004). Intra-tumoural drug de-activation of commonly used anti-cancer drugs can be achieved with the CYP1B1 isoform (Rochat et al., 2001).

1.1.4 Gene directed enzyme prodrug therapy (GDEPT)

GDEPT, also known as suicide gene therapy, involves the delivery of a gene coding for an activating enzyme, contained in a vector, to tumour cells (reviewed in (Greco & Dachs 2001)). Choice of vector, as well as therapeutic gene, is also of importance and can include liposomes (Fife et al., 1998) and viruses (retro- and adeno - and adeno -associated virus (El-Anreed 2004)). In some cases, the vector can be more harmful than the desired therapy itself, and can cause immunogenic responses (Bessis et al., 2004). Conventional cancer therapy agents are often metabolised before they can reach the desired site, and can have low bioavailability due to enzyme action in the gastrointestinal tract and liver (Connors 1986; Schellens et al., 2000). Therefore, it is proposed that gene therapy could eliminate these problems.

Prodrugs, first described by Albert, are pharmacologically inert prior to metabolic activation (Albert 1958), and can be separated into 2 distinct classes; 1)
prodrugs designed to increase bioavailability of anti-tumour agents and, 2) prodrugs designed to locally deliver anti-tumour agents, by way of toxic metabolites (Rooseboom et al., 2004). Prodrugs should ideally offer site specific metabolic activation and toxicity by specific enzymes, thus bypassing toxicity found in normal tissues and organs (Connors 1986).

In addition to CYP gene therapy (section 1.1.5), there are a number of other enzymes used with a variety of prodrugs. These include herpes simplex virus thymidine kinase (HSV/TK), with the prodrug gancyclovir (GCV, 7) (Moolten 1986; Caruso et al., 1993), nitroreductase with the prodrug CB1954 (8) (Bridgewater et al., 1995) and the bacterial *Eschericia coli* cytosine deaminase with the anti-fungal prodrug 5-fluorocytosine (5-FC, 9) (Mullen et al., 1992).

HSV/TK with GCV has been the most widely studied, and has entered into phase III clinical trials in the USA (McCormick 2001). The phosphorylated toxic metabolite of GCV, produced upon metabolic activation, incorporates itself into DNA and blocks any further DNA synthesis (Greco & Dachs 2001).

AQ4N is also metabolised by CYP mono-oxygenase in hypoxic/anoxic conditions as well as being reduced by CYP-R component (Patterson 2002), and has been proposed in gene therapy.
1.1.5 Cytochrome P450 in gene therapy

Gene therapy has encountered problems associated with organ toxicity as some CYPs, (CYP3A4, for example) which are exploited in GDEPT (McCarthy et al., 2003) are also constitutively expressed in the liver (Luo et al., 2004). Many approaches have been investigated, using different CYP isoforms with different combinations of prodrugs (Patterson et al., 1999; Waxman et al., 1999; Baldwin et al., 2003; McFadyen et al., 2004a). As illustrated previously, cytochrome P450 mono-oxygenase requires CYP-R in order to function, therefore, it is important to co-express the CYP-R polypeptide component to ensure catalytic activity (Chiocca & Waxman 2004).

One example of CYP activated drugs include a naturally occurring compound found in red wine called resveratrol. This compound is currently under investigation as a new prodrug for CYP cancer gene therapy (McFadyen et al., 2004a), as it is metabolised, in vitro, by CYP1B1 (Potter et al., 2002) to piceatannol, which is known to have anti-leukaemic effects (Wieder et al., 2001). Another naturally occurring compound, 4-ipomeanol (1-(3-furyl)-4-hydroxy-1-pentanone) (isolated from a fungus on sweet potatoes), is metabolised by rabbit CYP4B1 to a potent alkylating species (Rooseboom et al., 2004). The rabbit P450 gene is ideal for this approach, as human CYP4B1 only has 1 % of the activity of the rabbit isoform (Rainov et al., 1998). Gene therapy with CYPs is an ever growing field and with the recent discovery of a tumour related CYP, CYP1B1 (Murray et al., 1997), there is potential for new prodrug combinations. The common anti-cancer prodrug, CPA and its isomer IFA have now been postulated as ideal prodrugs in gene therapy systems (Wei et al., 1994; Karle et al., 1998; Jounaidi & Waxman 2000; Hunt 2001; Jounaidi & Waxman 2001). Systemic application of IFA and CPA resulted in activation in the liver with associated bladder toxicity, due to expression of CYP isoforms in non-tumour/normal tissue (Philips et al., 1961). IFA combined with CYP2B6 and CYP3A4 has been successful using a CYP/gene therapy approach (Jounaidi et al., 1998). In addition, the co-expression of CYP2B6 with CYP-R produced enhanced toxicity in transfected cells treated with tirapazamine in combination with CPA (Jounaidi & Waxman 2000).
An aminophenyl-benzothiazole drug, Phortress™ (10) is metabolised by CYP1A1 (Chua et al., 2000), however, it also induces this CYP enzyme by acting as a potent agonist of the aryl-hydrocarbon (Ah) receptor (see section 1.2.1). The prodrug Phortress, 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203), elicits anti-tumour activity in mice, and DNA adducts in breast cancer cell lines (Leong et al., 2003). This prodrug is now being evaluated in combination with gene therapy approaches.

![Chemical structure of Phortress](image)

### 1.1.5.1 Bystander effect

The bystander effect (BE), first described by Moolten in 1986 (using a gene therapy strategy with HSV/TK/GCV), describes how toxic species generated in one cell can spread to neighbouring cells, which are not expressing the trans-gene, inducing cell death (Moolten 1986). This is advantageous in cancer gene therapy as only a small percentage of cells are transduced with the transgene of interest (Greco & Dachs 2001). The toxic species can 1: travel via gap junctions (Bi et al., 1993; Mesnil & Yamasaki 2000), 2: travel via apoptotic vesicles (Freeman et al., 1993) or 3: diffuse, as in the case of gases such as NO⁺ (Lancaster 1997). These phenomena are illustrated in Figure 1.2.
The bystander effect in suicide gene therapy. Neighbouring cells can also be affected by the toxic metabolites. This can occur via gap junctions and/or diffusion. It is anticipated that in GDEPT strategies only 10% of the cells are transfected with the gene, hence the advantage of the bystander effect in cancer therapy.

The importance of gap junctions on the BE was illustrated experimentally with neoplastic murine cells, in vitro, as cells needed to be in direct contact for this effect to occur (Moolten 1986), due to the inability of the nucleoside phosphates to diffuse across cell membranes (Freeman et al., 1993). These junctions can alter bystander toxicity and the BE can be enhanced by increasing the capacity of gap junctions. This can be achieved in different ways (Mesnil & Yamasaki 2000) including treatment with flavonoids (Chaumontet et al., 1994) or connexin gene transfer (Tanaka et al., 2001). In contrast, other prodrugs do not pose this problem, as their toxic metabolites can readily diffuse across cell membranes (e.g. 4-hydroxy-CPA), and do not require cell-to-cell contact (Chen & Waxman 2002). Time scales for these events to happen vary from hours to days, with CPA treatment taking days in CYP expressing cells (Schwartz et al., 2002).

1.2 Regulation of the CYPl gene family

The regulation of the CYPl gene family, and in particular CYPlB1, has been sought to ascertain the specific roles of this protein (Kress & Greenlee 1997; Eltom et al., 1999; Shehin et al., 2000; Bandiera et al., 2004). This section reviews some of the literature centred on this family of isoenzymes.
1.2.1 Constitutive expression and the role of the Ah receptor

The aryl hydrocarbon receptor, (AhR), is so called because it recognizes aromatic hydrocarbons. First discovered in 1976 (Poland et al., 1976), this 95-126 kDa sized protein belongs to a family of the basic, helix-loop-helix/per-ARNT-sim proteins (bHLH/PAS) (Swanson & Bradfield 1993). It is localised to the cytoplasm where it remains inactive. Two other proteins are bound to the AhR in the cytoplasm, heat shock protein 90 (HSP 90) and XAP2 (Petrulis & Perdew 2002) (which binds to the C terminal of HSP 90 (Meyer & Perdew 1999)). The AhR is critical in the constitutive expression of the CYPl isoforms (Roblin et al., 2004). In vitro, fibroblast cells that lacked the gene for the AhR, were also found to be deficient in CYP1B1 expression (Zhang et al., 1998). A schematic diagram of the interplay of receptor interaction and critical proteins is shown in Figure 1.3.

The multifaceted roles of CYPs will have an effect on their expression levels, in specific organs and sites, whether that is in the role of drug metabolism (Murray 1999; Lewis 2003), fatty acid oxidation (Wada et al., 1968) or hormone biosynthesis (Pikuleva & Waterman 1999). The CYPl gene families are regulated in a specific way which will play a role in where, when and how they are expressed in terms of the specific transcription factors involved in CYPlB1 gene expression, whether this is in the liver, brain or cancer tissue. This summary will concentrate on the CYPl isoforms, namely, CYPlA1, CYPlA2, and CYPlB1.

1.2.2 Inducible expression via the aryl hydrocarbon (Ah) receptor

CYPl gene expression can be triggered upon chemical stimulation which is due to chemicals entering the cell and binding to the AhR. Typically, these chemicals are planar, aromatic structures e.g. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, 11) and benzo (a) pyrene (12), a carcinogen commonly found in cigarette smoke.
Binding of such ligands to this receptor will cause dissociation of the aforementioned bound proteins, translocation to the nucleus and heterodimerisation to one of the other bHLH/PAS proteins, the aryl hydrocarbon nuclear translocator (ARNT) (Reyes et al., 1992). Once inside the nucleus, the AhR/ARNT complex will bind to specific sequences within double stranded DNA (Denison et al., 1988, 1989) and these recognition sequences are within a region termed the xenobiotic response element (XRE), also known as the dioxin response element (DRE). There are eight
known XREs in the 5' flanking region of the CYP1B1 gene (Tang et al., 1996). This binding will promote gene expression of target genes (Sogawa & Fujii-Kuriyama 1997). Once in a dimer the AhR-ARNT complex will bind other transcription factors including nuclear factor 1 (NF1), which is important for CYP1A1 expression and binding of Sp1 (Morel & Barouki 1998). There are many genes that will be ‘switched on’ in response to this sequence of events (Mimura & Fujii-Kuriyama 2003). These include not only the CYP1 gene family members but also the recently discovered CYP isoform, CYP2S1 (Rivera et al., 2002).

Cell specific expression and induction patterns of CYP1B1 vary from cell line to cell line (Murray et al., 2000). Some cell lines, for example the kidney tumour cell line (ACHN), will express CYP1B1 on treatment with TCDD (protein and mRNA), whilst others, such as the hepatoma cell line (HepG2) will not (Spink et al., 1994). Both lines express a functional Ah receptor (which is important in constitutive expression), but it appears that some kind of post transcriptional protein modification could be occurring in certain cell lines (Morel & Barouki 1998).

The literature suggests that genes responsive to TCDD, especially CYP1A1, usually contain multiple XRE copies, and inducible expression via the Ah receptor is less effective for CYP1B1 compared with CYP1A1 (Bhattacharyya et al., 1995).

In addition to its role in CYP1 regulation, ARNT can also function to regulate genes in response to hypoxia (Nie et al., 2001). This is achieved by binding to the hypoxia inducible factor 1 (HIF-1α), whereby HIF-1α undergoes conformational change (Kallio et al., 1997). Under normoxic conditions the HIF1α protein is localised to the cytosol, (in the same way as the AhR) and upon stimulation under hypoxic conditions or by transition metals, which elicit the same response as hypoxia, the HIF1α protein is translocated to the nucleus and heterodimerises with ARNT (Jiang et al., 1996). As previously described, the AhR/ARNT complex recognises specific DNA recognition sequences; this is the same for the HIF1α/ARNT complex, but instead this enhancer region is called the hypoxia response element (HRE) (Semenza et al., 1991). Target genes are up-regulated in response to hypoxia in the same way as the CYP1 gene family via planar aromatic molecules interacting with the AhR, because of the overlapping regulatory proteins. These genes include vascular endothelial growth factor (VEGF)
(Shweiki et al., 1992) and glucose transporter-1 (Glut-1) (Clerici & Matthay 2000). There is very close cross-talk between the hypoxia pathway and the TCDD regulated pathway involving ARNT (Nie et al., 2001), consequently stimulation of one pathway (namely the TCDD/AhR or HIF1α) potentially results in the sequestration of ARNT, which has effects on other pathways involving this protein (Nie et al., 2001; Prasch et al., 2004).

1.2.3 CYP1 gene expression.

Oxidative stress has been implicated as playing a role in expression of many different genes (Morel & Barouki 1999), as well as being implicated as having a role in CYP gene expression (Morel & Barouki 1998). During oxidative stress conditions, free radical moieties, including reactive oxygen species (ROS) and reactive nitrogen species (RNS), are generated, that can harm cells and trigger 'suicide' events (Halliwell & Gutteridge 1985). Intracellular oxidative stress (which results in DNA damage (Navasumrit et al., 2001) can arise from many different pathological states, including inflammation (Ohshima et al., 2003) and ethanol metabolism (Halliwell & Gutteridge 1985). The fate of the free radicals or oxidants generated from oxidative stress will be evaluated here with respect to CYP1 gene expression.

The XRE is important in the transcription of CYP1A1 and CYP1B1 (Ma 2001; Tsuchiya et al., 2003), and the proximal promoter upstream of the CYP1A1 gene contains a region termed the basic transcriptional element (BTE). Treatment of cells in vitro with H2O2 affects the binding of NF1 to this recognition sequence within the BTE, thus suppressing CYP1A1 transcription (Morel et al., 1999). The heterodimer ARNT and AhR synergise with NF1 (Barouki & Morel 2001), therefore the interaction of all of these proteins will ultimately affect gene transcription.

The target for H2O2 is cysteine-427, which is contained within the transactivation domain of NF1/CTF, and was proved by mutation analysis (Morel & Barouki 2000). During CYP1A1 metabolism, H2O2 is produced which exerts a negative feedback upon CYP1A1 (Morel & Barouki 1998). In terms of CYP1B1, there have been no major studies to look at regulation of the gene and protein production in response to oxidative stress conditions per se. However, using
transfection studies, the redox regulated protein thioredoxin-1 (Trx-1) has been postulated as having an influence on basal CYP1B1 expression in vitro. A reduction in CYP1B1 expression was seen when using a mutant form of the Trx-1 protein (Husbeck & Powis 2002). Oestrogen, which is metabolised by CYP1B1 (Hayes et al., 1996), has been implicated as playing a role in CYP1B1 gene expression. Additionally, CYP1B1 is expressed in oestrogen-dependent tissues (Iscan et al., 2001). Treatment of cells with oestrogen induced CYP1B1 mRNA levels in vitro, therefore, it has been suggested that there is a putative oestrogen response element (ERE) within the promoter region of CYP1B1 (Tsuchiya et al., 2004).

1.3 Cytochrome P450 1B1 (CYP1B1)

CYP1B1 belongs to the dioxin-inducible CYP1 gene family, and was first identified in 1994 in human keratinocyte cells. A 50-fold increase in CYP1B1 mRNA levels was demonstrated after treatment with TCDD (Sutter et al., 1994). This isoform is one of the largest CYPs as it has 543 amino acids and the mRNA is 5.2 kb (Zhang et al., 1998). The CYP1B1 gene maps to human chromosome 2 at 2p21-22 and contains three exons and two introns (Murray et al., 2000). The protein translation start is close to the 5’ end of exon 2 and continues into exon 3, multiple poly-adenylation sites are untranslated in the 3’ region (Tang et al., 1996). CYP1B1 showed approximately 40 % amino acid sequence homology with its two most closely related members of the CYP1 family, CYP1A1 and CYP1A2, but differs in the number of exons it contains (3 versus 7), as well as its chromosome location, 2 versus 15 (Tang et al., 1996).

The elucidation of the physiological function of CYP1B1 has shown that this enzyme plays a major role in the metabolism of 17-β-oestradiol (13), as does CYP1A1 (Hayes et al., 1996). CYP1A1 will catalyse the hydroxylation of oestradiol, forming 2-hydroxyoestradiol (Spink et al., 1992), whereas 4-hydroxylation predominates for CYP1B1 (Spink et al., 1994; Liehr et al., 1995; Hayes et al., 1996). This metabolic pathway is now commonly used for CYP1B1 functional enzyme assays. 4-hydroxyoestradiol (the major metabolite of CYP1B1 activated 17-β-oestradiol hydroxylation) has been implicated as having a role in cancer progression in oestrogen dependent tissues, and where 2-hydroxylation
predominates, tumours fail to develop (Liehr & Ricci 1996). It is of interest to note that this metabolite (2-hydroxyoestradiol) is not mutagenic (Yager & Liehr 1996).

1.3.1 CYP1B1 in cancer

The identification of CYP1B1 in 1994 (Sutter et al., 1994) led many research groups to elucidate the function of this newest member of the CYP1 gene family. In 1997 Murray and colleagues identified that this CYP isoform is over-expressed in cancer (Murray et al., 1997). Table 1.2 provides a summary of cancer tissues which have been shown to express CYP1B1 protein.

The lack of CYP1B1 in stroma surrounding tumour tissue has been seen in many studies (McFadyen et al., 1999; McFadyen et al., 2001b; Gibson et al., 2003; Carnell et al., 2004); suggesting CYP1B1 is a tumour specific marker (Murray et al., 1997), and elevated levels may indicate a malignant phenotype (Spink et al., 1998). Expression was not confined to tumours of specific anatomical sites, as it was identified in cancer of the colon, oesophagus, kidney, lung, ovary, testis and uterus. Furthermore, CYP1B1 has been detected not only in primary ovarian cancer but also in metastatic disease (McFadyen et al., 2001b). Although other CYP isoforms, including CYP1A1 (Murray et al., 1995), CYP2C8/9 (mRNA) (Knupfer et al., 2004) and CYP3A (Huang et al., 1996) are also present in tumours, they are not expressed to the same diversity and frequency as CYP1B1 (Patterson & Murray 2002).
Table 1.2 Protein expression of CYP1B1 in human cancer. * denotes that study by Murray and colleagues (Murray et al., 1997)

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Number (frequency %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>60 (77) (McFadyen et al., 1999)</td>
</tr>
<tr>
<td>Bladder</td>
<td>8 (100)*, 22 (100) (Carnell et al., 2004)</td>
</tr>
<tr>
<td>Brain</td>
<td>12 (92)*</td>
</tr>
<tr>
<td>Colon adenocarcinoma</td>
<td>12 (92)*, 61 (100) (Gibson et al., 2003)</td>
</tr>
<tr>
<td>Connective tissue</td>
<td>9 (88)*</td>
</tr>
<tr>
<td>Oesophagus</td>
<td>8 (100)*</td>
</tr>
<tr>
<td>Kidney</td>
<td>11 (100)*</td>
</tr>
<tr>
<td>Lung</td>
<td>8 (88)*</td>
</tr>
<tr>
<td>Lymph node</td>
<td>9(100)*</td>
</tr>
<tr>
<td>Primary/metastatic ovarian</td>
<td>167 (92)/48 (94) (McFadyen et al., 2001b)</td>
</tr>
<tr>
<td>Prostate</td>
<td>33 (75) (Carnell et al., 2004)</td>
</tr>
<tr>
<td>Skin</td>
<td>6 (100)*</td>
</tr>
<tr>
<td>Stomach</td>
<td>10 (90)*</td>
</tr>
<tr>
<td>Testis</td>
<td>8 (100)*</td>
</tr>
<tr>
<td>Uterus</td>
<td>7 (100)*</td>
</tr>
</tbody>
</table>

As with most enzyme studies, protein expression as determined by IHC is valuable when backed up with functional enzyme studies. This has been shown with a few studies to date. Renal cell carcinoma (the most common cancer of the kidney), expressed functional CYP1B1 and was found to be active in 70% of the tumours examined, and there was no detectable CYP1B1 activity (as determined by ethoxyresorufin O-deethylation ± α-naphthaflavone, a CYP1B1 enzyme inhibitor) in normal tissues (McFadyen et al., 2004b). Using the oestradiol assay (for detecting 4-hydroxyoestradiol), breast cancer tissue was positive for CYP1B1 active protein (Liehr & Ricci 1996). These studies could further validate this enzyme as a target in cancer therapy.
1.3.2 CYP1B1 mRNA in tissues

There have been many studies looking into mRNA expression of CYP1B1 in normal tissues and some studies looking at expression in cancer tissues. These studies are summarised in Table 1.3. All mRNA expression studies to date have detected CYP1B1 mRNA, but no corresponding CYP1B1 protein, using either immuno-blotting or IHC. In liver microsomes, mRNA was present but the corresponding protein was not detectable (Chang et al., 2003b). Differences occur between studies, in terms of sample origin. The mRNA in some cases was identified from fresh tissue samples (McKay et al., 1995; Baron et al., 1998; Dassi et al., 1998; Rieder et al., 1998; Cheung et al., 1999; Piipari et al., 2000; Finnström et al., 2001; Spivack et al., 2001; Chang et al., 2003b; Tuominen et al., 2003; Yengi et al., 2003; Bertrand-Thiebault et al., 2004), from tissue explants (Bofinger et al., 2001) or from cell lines (Hakkola et al., 1997; Spink et al., 1998; Hellman et al., 2001; Malaplate-Armand et al., 2003; Verheyen et al., 2004; Yu et al., 2004).

CYP1B1 mRNA has been seen in tumour tissue from the breast (McKay et al., 1995; Iscan et al., 2001), prostate (Finnström et al., 2001), kidney (Cheung et al., 1999) and lung (Spivack et al., 2001; Spivack et al., 2003). In these studies mRNA was also seen in the corresponding normal tissue; however, Spivack and colleagues identified that there were more subjects displaying mRNA in tumour, compared with non tumour tissue (Spivack et al., 2001).

CYP1B1 mRNA was seen at a basal level of expression in vitro, using an array of different cancer cell lines, including MCF-7 (breast adenocarcinoma) (Spink et al., 1998; Yu et al., 2004), A549 (lung adenocarcinoma), NCI-H596 (lung adenosquamous carcinoma) (Hellman et al., 2001), MOG-G-CCM and U373 MG (astrocytoma cell lines) (Rieder et al., 1998; Malaplate-Armand et al., 2003). Some studies looked at different treatments (including interleukin-1β (IL-1β) (Malaplate-Armand et al., 2003) and TCDD (Walker et al., 1999)) and analysed the change in levels of CYP1B1 mRNA whilst all looked at constitutive mRNA expression, finding mRNA expression in all samples analysed.
Table 1.3 Overview of studies looking into CYP1B1 mRNA in non-tumour tissue

<table>
<thead>
<tr>
<th>Normal human Tissue</th>
<th>Comments</th>
<th>Method of analysis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult liver, lung, lymphocytes bronchoalveolar lavage samples, uterine endometrium</td>
<td>Expression in liver although low levels, present in all other tissues except lung</td>
<td>RT-PCR</td>
<td>(Hakkola et al., 1997)</td>
</tr>
<tr>
<td>Blood monocytes and macrophages</td>
<td>Constitutive expression in both cell types analysed</td>
<td>RT-PCR</td>
<td>(Baron et al., 1998)</td>
</tr>
<tr>
<td>Blood mononuclear cells</td>
<td>No differences between smokers versus non smokers. Inter-individual variability in CYP1B1 expression (1), no gender or age differences, suggestion that allelic variants (polymorphisms) did not have effect on CYP1B1 mRNA in vivo, variability in expression between samples (2)</td>
<td>Quantitative competitive RT-PCR&lt;sup&gt;1&lt;/sup&gt; RT-PCR&lt;sup&gt;2&lt;/sup&gt;</td>
<td>(1) (Dassi et al., 1998) (2) (Tuominen et al., 2003)</td>
</tr>
<tr>
<td>Brain, kidney, breast, prostate, ectocervix, endocervix, uterus, ovary, lymph node, uterine myometrium and liver</td>
<td>Constitutively expressed in tissues analysed except liver. Location: mRNA expressed in cytoplasm of brain cortex, glandular cells of breast, stratified squamous non-keratinising epithelial cells of the ectocervix, epithelial cells of distal kidney cells.</td>
<td>ISH</td>
<td>(Muskhelishvili et al., 2001)</td>
</tr>
<tr>
<td>Breast</td>
<td>Positive in tumour and control tissue</td>
<td>RT-PCR</td>
<td>(Iscan et al., 2001)</td>
</tr>
<tr>
<td>Tissue Type</td>
<td>Description</td>
<td>Method</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>------------------------------------------------------------------------------</td>
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</tr>
<tr>
<td>Bronchioalveolar macrophages</td>
<td>Higher in smokers compared with non-smokers and did not differ between heavy and light smokers</td>
<td>RT-PCR</td>
<td>(Piipari et al., 2000)</td>
</tr>
<tr>
<td>Buccal mucosal cells</td>
<td>Tobacco exposure resulted in ↑ CYP1B1 mRNA. 79% patients expressed CYP1B1 mRNA. Females gender correlated with ↑ CYP1B1 as did levels of plasma nicotine</td>
<td>Real time RT-PCR</td>
<td>(Spivack et al., 2004)</td>
</tr>
<tr>
<td>Endometrium</td>
<td>Looked at effect of TCDD on human explants, ↑ on treatment with TCDD, present in untreated cells at low levels</td>
<td>Northern blotting</td>
<td>(Bofinger et al., 2001)</td>
</tr>
<tr>
<td>Foetal placenta, liver, adrenal gland, lung, brain, kidney</td>
<td>Low level in foetal liver detected in 50% samples, high level in kidney, low levels in the placenta</td>
<td>RT-PCR</td>
<td>(Hakkola et al., 1997)</td>
</tr>
<tr>
<td>Liver</td>
<td>Variability in expression in CYP1B1 mRNA, greater in smokers compared to non smokers, present in all samples analysed</td>
<td>Real time RT-PCR</td>
<td>(Chang et al., 2003b)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>Looked at TCDD exposure in vitro, resulting in ↑ in CYP1B1 mRNA. Untreated cells had variable CYP1B1 levels</td>
<td>Quantitative RT-PCR</td>
<td>(Spencer et al., 1999)</td>
</tr>
<tr>
<td>Prostate</td>
<td>2-6 fold ↑ CYP1B1 expr^n in peripheral zone compared to transition zone. Variability in expr^n</td>
<td>Real time RT-PCR</td>
<td>(Ragavan et al., 2004)</td>
</tr>
<tr>
<td>Skin</td>
<td>CYP1B1 expressed in all samples, to different levels</td>
<td>Real time RT-PCR</td>
<td>(Yengi et al., 2003)</td>
</tr>
<tr>
<td>Varicose veins (saphenous veins)</td>
<td>CYP1B1 mRNA levels were higher in varicose veins compared to normal veins</td>
<td>RT-PCR</td>
<td>(Bertrand-Thiebault et al., 2004)</td>
</tr>
</tbody>
</table>
1.3.3 CYP1B1 protein in human tissues

Many groups have focused upon researching specific tissue expression and the intracellular location of this protein. Some groups have identified immunoreactivity localised only to the tumour cytoplasm (McFadyen et al., 1999; McFadyen et al., 2001b; Lin et al., 2003), and others in the nucleus (Muskhelishvili et al., 2001; Ragavan et al., 2004), using IHC. Some groups have not identified any CYP1B1 localised adjacent to normal tissue, whilst others have noted some CYP1B1 protein in some anatomical sites adjacent to tumour tissue. Gibson et al., noted that normal colonic epithelia stained positive for CYP1B1, albeit at low levels, and blood vessels in the colon also stained positive for the CYP1B1 antigen (Gibson et al., 2003). Studies looking at normal tissue expression using IHC are limited; however, some CYP1B1 protein was found in the cytoplasm of smooth muscle cells (100 % positive) and in bronchiolar epithelium (16 % positive), whereas no CYP1B1 was found in pneumocytes (type I and II) (Lin et al., 2003). Reider et al. identified the CYP1B1 protein in the human temporal lobe (Rieder et al., 2000) which could suggest that CYP1B1 is important in brain xenobiotic metabolism and could be crucial in brain toxicity when using CYP1B1 activated cancer therapeutics. Previous studies have identified CYP1B1 in prostate cancer (Carnell et al., 2004), and noted that normal tissues are devoid of CYP1B1 expression. Ragavan et al. identified CYP1B1 protein expression in prostate glandular stromal tissue, as well as noting carcinoma tissue expression (Ragavan et al., 2004). Two other studies have looked at CYP1B1 normal tissue expression more extensively with IHC. In one study, 32 normal tissues were analysed, using a polyclonal CYP1B1 antibody with IHC. Protein expression was strongest in the fallopian tube, breast, uterus (cervix endometrium) and ureter (Maecker et al., 2003). All of these tissues, except the last, are influenced by hormones and CYP1B1 is known to play a role in oestriadiol metabolism (Hayes et al., 1996) and is expressed in a range of hormone-related cancers (Murray et al., 1997; McFadyen et al., 1999). CYP1B1 protein expression in other tissues, such as the kidney, liver and spinal cord amongst others, were negative. All cancers examined in this study, including colon, lung and ovarian, were strongly positive and surrounding stroma was negative. Cancer cell lines also examined in this study were 100 % positive for CYP1B1 (Maecker et al., 2003). In a separate study by Muskhelishvili and colleagues, CYP1B1 was identified, using
IHC, but unlike previous studies, the protein was found in the nucleus, leading the authors of this study to suggest that CYP1B1 could have a nuclear function (Muskhelishvili et al., 2001). The majority of the immuno-stained cells in different tissues were of epithelial origin and displayed strong immunoreactivity.

Protein expression can also be evaluated using western/immuno-blotting. This gives a quantitative answer but does not elucidate the cellular localisation of the protein. This approach has been carried out to investigate CYP1B1 and has been reported in the literature. However, the data reported tends to vary with respect to the size of protein being detected. This may be due to different antibodies used or because of the different nature of CYP1B1 proteins being detected. The size of proteins detected varies from 52 kDa (Baron et al., 1998; McFadyen et al., 1999; Spivack et al., 2001; Lin et al., 2003) to 58 kDa (Rieder et al., 2000), to 60 kDa (McKay et al., 1995; Malaplate-Armand et al., 2003). Some studies do not report the size of the protein but all studies use the same positive control.

Tumour tissues examined (using western blotting) included breast (McKay et al., 1995; Maecker et al., 2003) and lung (Spivack et al., 2001). CYP1B1 protein was also seen in oesophageal, ovarian and rhabdomyosarcoma (Maecker et al., 2003). The correlation of smoking status was examined using western blotting and interestingly, highlighted a correlation between smoking in males and CYP1B1 protein expression in lung tumour versus non-tumour tissue (Spivack et al., 2003). Using a different method of detection, protein was detected in non-tumour tissues with immuno-blotting and there was an obvious differential between tumour and non-tumour tissue (Spivack et al., 2001). In lung cancer, there was presence of CYP1B1 protein in the adjacent ‘normal’ tissue to the tumour as well as being present in normal lung tissue (Lin et al., 2003). In vitro, protein was detected in cell lines of breast cancer origin upon treatment with TCDD (Gibson et al., 2003) and constitutively in an astrocytoma cell line (Rieder et al., 2000).

Further to these studies, normal tissue protein has been studied and protein identified in human endometrium (Bofinger et al., 2001), bronchioalveolar macrophages (Piipari et al., 2000), blood monocytes (Baron et al., 1998) and temporal lobe (Rieder et al., 2000). A study by Tsuchiya and colleagues found that in normal endometrial tissues, CYP1B1 protein is preferentially found in proliferative tissues compared with secretory tissues and was localised to glandular
epithelial and stromal cells. Expression diminished as the cells passed through the secretory phase, and therefore suggests, in this case, that CYP1B1 is oestrous phase dependent (Tsuchiya et al., 2004).

In summary, it is widely accepted that CYP1B1 mRNA is present in a plethora of normal human tissues but the corresponding protein is absent (Rieder et al., 1998; Cheung et al., 1999; Piipari et al., 2000; Finnström et al., 2001; Chang et al., 2003b; Bertrand-Thiebault et al., 2004). In tumour tissue however, mRNA is present, as well as protein. Normal human liver did express mRNA whilst the corresponding protein was not detected (Chang et al., 2003b). Factors which govern expression are now being considered, including the possibility of physiological conditions exclusive to tumours (Murray et al., 2000) and proteasomal mediated degradation in normal tissue (Bandiera et al., 2004).

1.3.4 CYP1B1 in immunotherapy

CYP1B1 is expressed in a large number of solid tumours, making it an attractive target for immunotherapy (Gordan & Vonderheide 2002). Specific epitopes are capable of inducing CYP1B1 specific T-cell responses via the production of cytotoxic t-lymphocytes (Maecker et al., 2003), eliciting immune responses and ensuing cell death. This initial work has prompted further work in this area and therapeutic issues were addressed. CYP1B1 antigens were administered, encoded in a plasmid and tolerability investigated. There was no toxicity in the parameters measured, including food consumption, body weight and urinalysis parameters. Additionally, there was no inflammation seen in any major organs (Luby et al., 2004). These studies are currently in Phase 1/2a open-label clinical trial, in late-stage metastatic cancer, with the product Zyc300, and initial clinical results look promising (Zycos 2004).

1.4 Head and neck squamous cell carcinoma (HNSCC) as a model of disease progression

Head and neck squamous cell carcinoma (HNSCC) is the most common cancer of the upper aerodigestive mucosa (Quon et al., 2001) and comprises of all cancers arising from squamous cells in this region (more detail on the pathology of
HNSCC can be found in chapter 3). HNSCC is known to progress through a series of distinct pathological stages before forming an invasive carcinoma (Crowe et al., 2002). Age is often a risk factor with cancer, but in ¼ of cases of HNSCC in the United States, tobacco and alcohol consumption is thought to play a considerable role (Blot et al., 1988). The squamous mucosa is the inner layer of any hollow organ and consists of the epithelial layer and below which sits the lamina propria (see chapter 3), which lets the epithelial layer move freely.

1.4.1 Genetic alterations in HNSCC

It has long been established that invasive carcinomas will arise from pre-malignant lesions in the mucosal layer (Hittelman 2003) within the head and neck region. Along with the gross microscopic changes, there are many genetic abnormalities which occur simultaneously during malignant progression. These include genetic instability, oncogene amplifications and gene mutations (Roh et al., 2000). Genetic instability is common in HNSCC, and there is a trend for microsatellite instability with progression of this disease, when comparing hyperplastic tissue and invasive carcinoma (5.9 % compared with 33 %) (Ha et al., 2002). Amplifications in the oncogene, CCND1 (gene for cyclin D1), were prevalent in HNSCC and did show variability with anatomical sites (oral cavity versus pharynx versus larynx). In all cases, the frequency of amplification was above 25 % (Freier et al., 2003). Gene mutations occur in tumour suppressor proteins such as p53. This protein is the most frequently mutated protein in human cancer (Greenblatt et al., 1994), and mutation has been linked with an increase in progression of HNSCC (Boyle et al., 1993.).

1.4.2 Environmental risk factors in HNSCC

Cancer can arise from spontaneous genetic events but it can also be caused by chemicals in the environment or exposure to carcinogens encountered in daily life (Stewart & Kleihues 2003). These events will be considered individually, and their impact on cancer of the head and neck will be addressed.
1.4.2.1 Field cancérisation

The phenomenon of field cancérisation has existed since 1953 (Slaughter et al., 1953). The concept is defined as the tissue exposed to carcinogenic insult (carcinogens in tobacco), is at increased risk of progressing through a multistage tumour development process, ultimately forming an invasive cancer which is at risk of metastasising (shown in Figure 1.4).

There are three distinct stages of cancer development: initiation, promotion and progression (Farber 1984). Initially, the tissue is damaged (DNA adducts, mutations and polysomy), then it grows, progresses and forms clones. Premalignancy, in the case of HNSCC (in oral tissues), occurs with leukoplakia (thick white patches), which will often precede malignancy (Hittelman 2003).

1.4.2.2 Tobacco use

There are more than 4000 chemicals in cigarette smoke, including nicotine, polycyclic aromatic hydrocarbons (PAH), N-nitroso compounds, aromatic amines, benzene and heavy metals (Stewart & Kleihues 2003). Metabolic activation results in the toxicity and subsequent carcinogenicity of these compounds (Kaminsky & Spivack 1999) and references therein). Ultimately, the expression level and metabolic activity of these enzymes in the exposed tissue will determine the fate and
toxicity of these compounds. Upon activation by a number of CYP isoforms (CYP1A1, CYP1B1 and CYP3), reactive metabolites of PAH will form DNA adducts (covalent binding to the DNA) (Piipari et al., 2000). CYP P450s are also responsible for the metabolic activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanolone (a compound commonly found in cigarette smoke) to form potent keto-aldehyde and keto-alcohol oxidation products (Smith et al., 1995). It has been demonstrated recently that tobacco smoke induces the CYP1B1 isoform at the mRNA and protein level (Port et al., 2004). Considering this isoform is pivotal in carcinogen metabolism (Shimada et al., 1996; Shimada et al., 1997; Miyazaki et al., 2005), this could have profound effects on cancer initiation and toxicity of tobacco-containing compounds.

The long established link between cigarette use and squamous cell carcinoma has now promoted research at a molecular level. This will ascertain the cellular events that promote cancer progression to provide a better understanding of the disease. As previously mentioned, the incidence of HNSCC is associated with tobacco and alcohol use (Blot et al., 1988). Some studies have investigated trends in smoking status and showed that the risk of cancer of the oral cavity, pharynx and larynx rose for current smokers and declined for ex-smokers and also showed a relationship with duration of smoking and number smoked per day (Choi & Kahyo 1991).

### 1.4.2.3 Alcohol use

Excessive alcohol (ethanol) intake is responsible for many cancers, including cancer of the liver with increased risk of breast and colorectal cancer (Stewart & Kleihues 2003). Heavy alcohol use is associated with cancer of the oral cavity, pharynx, larynx and oesophagus (IARC 1998). There are clear geographical patterns in head and neck cancer where occurrence is at it highest in countries with heavy alcohol consumption (Stewart & Kleihues 2003).

Ethanol intake, on its own, is a major contributing factor in disease pathology but alcohol, in combination with smoking, showed a synergistic effect in cancer causation (Franceschi et al., 1999). Upon metabolic activation, ethanol will yield acetaldehyde, which is thought to play a role in its toxicity (Matsuo et al., 2001). A clear mechanism of toxicity has yet to be established. Alcoholic beverages
contain many other chemicals, such as N-nitrosamines, which are postulated as having a role in carcinogenesis as well as being a risk factor in HNSCC (Stewart & Kleihues 2003). Upon ethanol exposure the oral squamous mucosa becomes more permeable, thus allowing tobacco-containing carcinogens to pass through and exert their effect more readily; ethanol also eliminates the lipid component of the barrier present in oral tissues allowing carcinogens easy access to the squamous tissue (Squier et al., 1986; Figuero Ruiz et al., 2004).

Induction of P450s occurs on treatment of cells with aromatic hydrocarbons. A similar phenomenon exists with the CYP2E1 family with alcohol (Hu et al., 1995), suggesting that induction of cytochrome P450 CYP 2E1 by ethanol may also be important for the metabolism of procarcinogens in tobacco smoke (Guengerich et al., 1994).

1.4.3 Polymorphisms in the \textit{CYP1} gene family in HNSCC

All exogenous and endogenous compounds are metabolised to increase polarity in order to be eliminated from the body. This can happen at many sites but mainly in the liver, which contains a range of enzymes. Polymorphisms in these enzymes can result in increased or decreased activity in drug and carcinogen metabolism.

1.4.3.1 Phases of metabolism

Traditionally drug metabolism can be separated into different phases. Phase I includes oxidation, reduction and hydrolysis reactions (Guengerich & Shimada 1991). The original molecule is altered and a functional group is added. The CYPs are classified within this group. Further metabolism makes the compounds more polar and readily available \textit{in vivo}. Phase II involves conjugation of the metabolically altered molecule, with different groups, which make the compound more polar. Such reactions include sulphation, glucuronidations, glutathione conjugation, methylation and acetylation.

1.4.3.2 Polymorphisms in CYP1 gene family enzymes in HNSCC

There have been various studies to elucidate the metabolic activity and protein expression of phase I enzymes within cancer tissue in the head and neck
region. The metabolism of carcinogenic PAHs (which are present in cigarette smoke) has been shown to be carried out by two of the CYP1 gene family members CYP1A1 and CYP1B1 (Thier et al., 2002; Shimada & Fujii-Kuriyama 2004). Therefore, CYP1 enzyme expression with corresponding functional activity may influence the adverse effect of these chemicals.

The cytochrome P450 enzymes are largely responsible for the metabolic activation of carcinogens in tobacco smoke (Shimada et al., 1997; Thier et al., 2002) and susceptibility to cancer may well depend on the isoform and the enzyme activating or detoxifying the toxic compound. CYP1A1, 1A2, 1B1, 2A6, 2D6, 2E1, 2C9, 2C19, 17, and 19 polymorphisms have been studied with respect to their metabolic capabilities, in tobacco related cancers (Bartsch et al., 2000) There are many polymorphisms identified in CYP1A1. In the context of HNSCC, CYP1A1 had three polymorphisms that affected susceptibility to cancer at this site. The MspI (CYP1A1) variant showed an increased risk of oral SCC (Tanimoto et al., 1999), the CYP1A1 Val-Val variant was identified in one population, especially in those with pharyngeal cancer (Morita et al., 1999) and the Ile-Val variant was higher in non-smokers than smokers (Park et al., 1997).

The CYP1B1 gene is known to have a number of polymorphisms that will impact on metabolic function, the most prevalent being an increase in activity with Val 432 to Leu (Li et al., 2000). In terms of head and neck cancer one study identified this polymorphism, named CYP1B1*3, in HNSCC and identified it as a susceptibility factor in carcinogenesis. This polymorphism was also more prevalent in smokers compared to non-smokers (Ko et al., 2001). Studies are now underway to evaluate the impact on enzyme activity with polymorphisms of CYP1B1 (Hanna et al., 2000; Li et al., 2000; Tanaka et al., 2002; Bandiera et al., 2004).

1.4.4 Biomarkers for HNCCC

A biomarker has been defined as: 'a specific biochemical in the body which has a particular molecular feature that makes it useful for measuring the progress of disease or the effects of treatment' (http://cancerweb.ncl.ac.uk/omd/1998). The use of clinico-pathological data (patient, tumour site and stage and treatment), are useful in the prediction of outcome of therapy (Quon et al., 2001). Gender can have prognostic importance (Daugaard & Sand 1998) as can treatment
time (Hoffstetter et al., 1997), whereas tumour site can impact on patient survival (Fu et al., 1994). These factors are useful to some degree, but the use of biomarkers may be crucial in predicting response to therapy or clinical outcome; whether it is because of the presence (or lack of) an enzyme (McFadyen et al., 2001a), or due to mutation or deletion of various tumour suppressor proteins such as p53 (Gasco & Crook 2003). Currently the major clinical treatment options for HNSCC are surgery and radiotherapy, although this does depend on site and stage of tumour (Stewart & Kleihues 2003). These rationales can be given singly or in combination with each other and also with chemotherapy (Adelstein 1998). Therapies can be modulated to suit each individual and the potential of having biomarkers that can indicate the outcome of therapy is advantageous.

Gasco and Crook (2003) describe that p53 status can be important to the outcome with DNA damaging agents, as p53 function restoration can restore sensitivity to p53 dependent apoptosis. p53 can play an active role in tumourgenesis as well as being a valuable marker in identifying individuals at high risk of developing recurrences and secondary primary tumours (Kim & Shin 1997). Epidermal growth factor receptor (EGFR) is overexpressed in HNSCC and has been found to correlate with poor prognosis (Kim & Shin 1997). Interestingly EGFR has been identified in pre-malignant tissues and expression increased with malignancy (Shirasuna et al., 1991). Glucose transporters have been identified in head and neck cancer (Mellanen et al., 1994) and Glut 1 has been postulated as a biomarker as it is expressed throughout the malignant progression of HNSCC (Reisser et al., 1999). The anti-apoptotic marker bcl-2 has been shown to be indicative of advanced and aggressive disease and tumour progression yet paradoxically, can also be indicative of good outcome (Wilson et al., 2001).

The necessity to combine and analyse biological data with response to treatment is paramount for improving patient therapy. This study will aim to further validate the P450 mono-oxygenase, CYP1B1, in this role.

1.5 Role of nitric oxide in cancer

Nitric oxide (NO*) (which has a short half-life in vivo in the order of 5-10 seconds (Ignarro et al., 1987)) is produced by a family of isoenzymes termed nitric
oxide synthases (NOS). Nitric oxide synthase (NOS) plays an important role in tumour biology, and the NOS protein is over-expressed in many tumours including head and neck cancer (Rosbe et al., 1995). Figure 1.5 shows a cartoon representation of the NOS enzyme. These enzymes are specialised cytochrome P450 monooxygenases and exist as homodimers in the cytosolic fraction of the cell (Marletta 1993). As with CYPs, NOS's have different isoforms, of which there are currently three, originating from different genes (Feelisch & Stamler 1996; Xu et al., 2002b). One isoform is inducible whilst two are constitutive (Knowles & Moncada 1994), the latter include calcium dependent isoforms namely, neuronal NOS (nNOS or NOS-1) and endothelial cell NOS (ecNOS or NOS-3), both of which require calmodulin for activity. The calcium independent and cytokine inducible isoform, (iNOS or NOS-2), does not require calmodulin for iNOS activity (Marletta 1993; Tozer & Everett 1997a). The ‘genetic’ composition of all 3 genes are similar, consisting of between 26-29 exons with a similar number of introns (Alderton et al., 2001). However, all 3 isoforms are located on three different chromosomes (Knowles & Moncada 1994). NOS’s require NADPH and oxygen as co-substrates in the formation of NO\(^*\) and citrulline, from L-arginine (Marletta 1993). The intermediate in the reaction is \(N\)-hydroxy-L-arginine (NOHA) (Stuehr et al., 1991).

In 1987 it was discovered that the vasodilator, endothelium-derived relaxing factor (EDRF), had identical properties to NO\(^*\) (Ignarro et al., 1987; Palmer et al., 1987). Further work identified that vascular endothelial cells could synthesise NO\(^*\), (as well as identifying that L-arginine is the precursor for NO\(^*\) in endothelial cells (Palmer et al., 1988)). Co-factors involved in these reactions include FMN (Flavin MonoNucleotide), FAD (Flavin Adenine Dinucleotide) as well as (6R)-5,6,7,8-tetrahydrobiopterin (H\(_4\)B) (which is considered to be important in the expression of NOS activity (Mayer et al., 1990)). As highlighted previously, calmodulin is associated with NOS activity, and electron flow through the reductase domain requires bound calcium/calmodulin (CaM) (Alderton et al., 2001).
Figure 1.5 Schematic diagram of nitric oxide synthase, showing the interaction between the reductase and oxidative haem domains. Adapted from (Marletta 1993) and (Alderton et al., 2001). In the reaction L-arginine is converted to citrulline, with an intermediate, N-hydroxy-L-arginine (NOHA) formed. Finally nitric oxide is generated. The enzyme biochemistry of NOS’s is the same, in principle, to cytochrome P450 mono-oxygenases. Nitric oxide has been shown to exert feedback inhibition with nNOS (Abu-Soud et al., 1995).

1.5.1 Alternative substrates for nitric oxide

As previously highlighted, the metabolic intermediate in the 2 step oxidation by NOSs of L-arginine to citrulline is N-hydroxy-L-arginine. It has therefore been proposed that compounds bearing the CHN or CNOH function could be exploited for the generation of NO* by recombinant NOS isoforms (Mansuy & Boucher 2004). This NOS catalysed reaction is very similar, mechanistically, to CYPs highlighted in section 1.1 as it involves changes in valency of the iron species of the haem. 4-chlorophenyl-N'-hydroxyguanidine (14) will release NO* and is currently being developed to yield a more specific iNOS substrate (which can be NOS isoform
dependent) (Mansuy & Boucher 2002). Metabolism by CYPs is also documented for these chemicals (Jousserandot et al., 1998).

1.5.2 Roles of nitric oxide

NO\textsuperscript{\cdot} plays important roles in a variety of biological processes, including muscle biology, vascular biology, immunology and tumour biology, due to the range of biochemical reactions it is involved in. NO\textsuperscript{\cdot} is a small, uncharged and diffusible molecule that can travel 100-200\textmu m away from an NO\textsuperscript{\cdot} producing cell, in any direction (Lancaster 1997). It will readily react with superoxide (O\textsubscript{2}\textsuperscript{\cdot}) to produce peroxynitrite (ONOO\textsuperscript{-}). NO\textsuperscript{\cdot} can also react with O\textsubscript{2} to generate nitrogen dioxide (NO\textsubscript{2}). The half life of these species has been estimated at 5-15 sec (Lancaster 1997). NO\textsuperscript{\cdot} as well as the two other redox forms of nitrogen monoxide (nitroxyl anion (NO\textsuperscript{-}) and nitrosium cation (NO\textsuperscript{\cdot\cdot})) will play a role in the biological effects of this molecule (Butler et al., 1995).

1.5.2.1 Nitric oxide in vascular biology

NO\textsuperscript{\cdot} has been described as a potent vasodilator (Palmer et al., 1987) and its clinical use reflects this, as it is used to treat angina pectoris and acute myocardial infarction (Horowitz 1991). Smooth muscles will relax when NO\textsuperscript{\cdot} is produced in endothelial cells (Gruetter et al., 1979). The guanylate cyclase (GC) pathway is pivotal in vasodilation (Ignarro et al., 1987). Activation of GC by NO\textsuperscript{\cdot} produces a secondary messenger, cGMP. This will, in turn, decrease intracellular free calcium, through interactions with the myosin light chain kinase (MLCK). This will deactivate the enzyme (Watanabe et al., 2001). This has been shown by two different methods; the increase in blood flow from administration of NOS inhibitors to animals (Tozer et al., 1997), and ecNOS knockout mice studies (Huang et al., 1995).

1.5.2.2 Nitric oxide in tumour biology

Different NOS isoforms in different locations (tumour and non-tumour associated tissue) have been shown to varying extents (Tozer & Everett 1997a). Evidence of NOS activity was shown by the measurement of citrulline production
complemented with an increase in nitrite and nitrate levels (Thomsen et al., 1995). Interestingly, tumour grade has also been investigated where an increase in tumour grade has resulted in both an increase in NOS enzyme activity and comparable increases in nitrate/nitrite measurement (Thomsen et al., 1995).

The tumour growth promoting effects of NO* have been shown (Thomsen et al., 1994), and growth of experimental tumours can be halted by administering the NOS inhibitor L-N^6-nitroarginine methyl ester (L-NAME) (Kennovin et al., 1994). NO* also works by promoting neo-vascularisation in vivo (Ambs et al., 1998). It is apparent that there is a balance between the cytotoxic effects of NO* and the pro-angiogenic/pro-tumour growth effects (Chinje & Stratford 1997).

Tumour metastasis has also been attributed to NO*; cells stimulated to metastasise (by cytokines) produced NO* (as measured by nitrite levels) whilst showing they had the potential of metastasising (when looking at cell deformability) (Igawa et al., 2004). In addition, when using an NOS inhibitor, (L-NAME) in vivo, the occurrence of lung cancer metastases was reduced (Edwards et al., 1996).

1.5.2.3 Nitric oxide in radiosensitisation

Principally radiotherapy targets tissue via free radicals generated from ionizing radiation. This occurs when radiation exposure generates OH* radicals, from water in the cells. In essence, the radical species of oxygen are 'fixed' onto the radical species generated from DNA (Halliwell & Gutteridge 1985). The peroxynitrite (ONOO⁻) radical can also oxidize and nitrate DNA causing strand breaks (Shafirovich et al., 2001; Dedon & Tannenbaum 2004). It has been established for many years that some cancers can be radioresistant, which affects clinical treatment (Chapman et al., 1974; Gatenby et al., 1988) and patient outcome to therapy. This is thought to be due to tumour hypoxia (and lack of molecular oxygen for radical formation). Physiological hypoxia is defined as having an oxygen pressure of < 2.5 mm Hg. The difference in resistance to radiation can be as much as three-fold (Tozer & Everett 1997b), so new approaches in the introduction of radiosensitisers for the treatment of cancer could be beneficial to patient outcome.

As far back as 1957 experiments were underway in vitro to evaluate NO* as a radiosensitiser (Howard-Flanders 1957). It was a few years later that Gray showed
that anoxic tumour cells were sensitised to radiation to the same degree as oxic tumour cells, by the introduction of NO* (Gray et al., 1958). The fixation ‘model’ of radiation free radical damage for NO* is thought to be the same as that for oxygen. Further work confirmed these theories, when an NO* donor system was employed (Mitchell et al., 1993; Griffin et al., 1996). These studies use this principle in vitro whereas manipulation of this in vivo could prove more complex. Introducing NO* to tissues without the complications associated with blood flow modification proves a challenge. Existing endogenous levels of NO* could give radiosensitisation and damage in non-tumour tissues (if radiation was not targeted) when nitric oxide radicals combine with other cell macromolecule radicals, eliciting toxicity. After radiation intra-tumoural levels of NO* would decrease, causing vasoconstriction and loss of tissue re-oxygenation. Therefore a fractionated radiotherapy rationale was employed (Tozer & Everett 1997b). This allows for the tissue to re-oxygenate and subsequent radiation to cause damage. Clearly the two roles of NO* (vasodilation and radiosensitisation) have to be considered for effective therapy.

1.5.3 Prodrugs for nitric oxide

The role of nitric oxide in cancer biology has been established through the use of diazeniumdiolate compounds, or ‘NONOates’ (Fitzhugh & Keefer 2000; Keefer 2003). These compounds contain the [NO(O)NO] functional group and will release 2 molecules of NO* per X [NO(O)NO] unit, when they undergo hydrolysis in aqueous solution at pH 7.4. The half lives for 2-(N, N-diethylamino)-diazenolate 2-oxide (DEA/NO, 15), and 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine (PAPA/NO, 16) are 2 and 15 min respectively (Mooradian et al., 1995), but other analogues have half lives of up to 20 h (Fitzhugh & Keefer 2000). However, these compounds show release of NO* is non-selective, and occurs spontaneously in a pH dependent fashion. DEA/NO, is mutagenic in bacteria cells although this effect was not pronounced in chinese hamster ovary cells (Donovan et al., 1997). These compounds have shown anti-leukaemic effects in vitro upon metabolism by esterase (Saavedra et al., 2000) and have also been shown to have vasodilatory effects on pulmonary arteries ex vivo (Abuo-Rahma Gel et al., 2005); therefore, the clinical potential of these compounds is wide-ranging from vascular
biology (vasodilation) (Morley & Keefer 1993) to radiosensitisation for cancer therapy (Fitzhugh & Keefer 2000).

These chemicals have shown promise for a plethora of disease states, but the further development of ‘NONOates’ for the treatment of cancer relies upon specificity and selectivity for cancer cells, compared to normal tissues.

1.5.4 Nitric oxide use for cancer therapy

The physiological role of NO* in normal cellular processes has been highlighted. The manipulation and exploitation of free radical chemistry and cellular physiology is crucial in the development of novel nitric oxide therapies as NO* is a potent cytotoxin (Xu et al., 2002b), making this an attractive candidate for cancer therapy. As NO* will diffuse easily, in the region of 100-200 μm, (Lancaster 1997), it is important that it is only generated from within the cancer cells themselves, allowing for minimal toxicity to other non-tumour cells.

Gene therapy strategies have utilised this property of NO* and delivered a NOS gene, i.e. iNOS, to tumour cells for a plethora of responses including modified vascular tone (Worthington et al., 2000), tumour suppression (Xie et al., 1995; Xu et al., 2002a) and alteration in response to radiation (Worthington et al., 2002; Wang et al., 2004). If the aim of the gene therapy protocol is to deliver NO* to cells for radiosensitisation, the combination of radiosensitive promoters such as WAF1 (Worthington et al., 2002) is beneficial as the gene of interest (NOS) will only be expressed with radiation (Scott et al., 2000) whilst still maintaining radiosensitisation. Cellular toxicity of NO* is linked to apoptotic events in the cell. The tumour suppressor gene p53 was expressed in response to NO* production (by NO* donors or cytokine induced NOS), prior to the cells undergoing apoptosis.
Cells are also shown to be cytostatic when NO* mediates damage in a dose dependent manner (Stuehr & Nathan 1989).

The combination of gene therapy using viruses with iNOS transgenes is a new approach which is being utilised by a few research groups. Retroviruses using specific tumour targets such as carcino-embryonic antigen (CEA), single-chain fragment variable (scFv) antibody may prove promising (Khare et al., 2002; Kuroki 2004). Direct injection of genes into the tumour mass has also been investigated, and has proven efficacious even with the low gene transfer efficiency (<10% (Greco & Dachs 2001)). This tumour cell kill was attributed to the bystander effect (Soler et al., 2000). A number of studies have clearly demonstrated that the over-production of NO* in tumour cells through NOS gene transfer inhibits tumour growth (Xie et al., 1995; Xu et al., 2002a). Such approaches will benefit from the bystander effect, due to the diffusion of NO*.

1.6 Aims of the present work

This project aims to validate cytochrome P450 1B1 (CYP1B1) as a target for the enzyme prodrug therapy of cancer. Although the expression of CYP1B1 has been investigated in a range of human cancers, protein expression has not been investigated in the pre-malignant stage of the disease. Head and neck squamous cell carcinoma provides an excellent clinical model of disease progression, as it has been recognised to proceed through a series of pre-malignant stages before forming an invasive carcinoma. Therefore, Chapter 3 will focus upon clinical validation of the enzyme target during malignant progression of HNSCC. Spectral imaging microscopy will be employed to provide quantitative analysis of CYP1B1 protein expression in immunohistochemically stained clinical tissue samples.

Evaluation of CYP1B1-activated prodrugs in vivo requires the use of a human tumour xenograft model that expresses the target enzyme. Therefore, Chapter 4 will aim to identify a suitable tumour model that expresses the target enzyme. In addition the inducibility of CYP1B1 will be investigated with a known CYP1 inducer, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), in tumour cells in vitro and in vivo.
Chapter 5 will focus upon the identification of prodrugs for nitric oxide, specifically activated by the CYP1 family of enzymes. The key issue is to understand structural features which confer selectivity of the prodrug for the target enzyme(s).
Chapter 2
Materials and methods
2.1 Culture of breast carcinoma and primary head and neck tumour cell lines

Chemicals were purchased from Sigma Chemical Company (Poole, Dorset, UK) unless otherwise stated.

2.1.1 Subculture of cells

Human Caucasian breast adenocarcinoma cells (MCF-7) were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The MDA-MB-468 cell line was a gift from De Montfort University (Prof. G.A. Potter, supplied through the American Type Culture Collection). Cells were cultured in Eagles minimum essential medium with Earles salt (EMEM), supplemented with 10 % foetal calf serum (FCS), 1 × non-essential amino acids (NEAA), 2 mM L-glutamine and 0.18 % sodium bicarbonate (Invitrogen, UK). Primary cells from a head and neck squamous cell carcinoma (UT-SCC-14), were obtained from R. Grénman, Turku University Central Hospital, Finland (Hessel et al., 2004), and were maintained in EMEM as above, supplemented with 1 mM sodium pyruvate.

Cells were sub-cultured in sterile conditions in a class II laminar flow hood (Advisory Committee on Dangerous Pathogens, ACDP II) in 75 cm² (T75) flasks (Helena Biosciences, UK), and were maintained in an incubator at 37 °C with 20 % O₂, 5 % CO₂, balanced air. Near confluent cells were treated with trypsin/ethylene diamine tetra-acetic acid (EDTA) (Invitrogen, UK), resuspended in sterile phosphate buffered saline Dulbecco A (1 × PBS-A, Oxoid, UK) and pelleted at 168 ×g in a Denley™ BR40 centrifuge at room temperature for 5 min. Cells were resuspended in complete EMEM, split 1:3 for UT-SCC-14 or 1:5 for MCF-7 and MDA-MB-468 cells.

2.1.2 Freezing/thawing of cell lines

Near confluent cell monolayers, were washed with 1 × PBS-A, trypsinised, resuspended in EMEM, centrifuged at 168 × g (Denley™ BR40 centrifuge) and resuspended in 10 % DMSO/EMEM. Freezing vials (Nalgene, Hereford, UK) were allowed to freeze slowly in a -80°C freezer. After 24 h of slow freezing they were transferred into liquid nitrogen for long term storage.
When thawing cells from liquid N\textsubscript{2} storage, vials were placed at 37°C (to allow rapid thawing). Once thawed, cell suspensions were resuspended in the appropriate growth medium and then centrifuged at 168 \times g as above, to remove the DMSO. The cell pellet was then resuspended in EMEM and transferred to a T75 flask and incubated at 37 °C in the conditions described in 2.1.1.

2.1.3 Mycoplasma testing

To ensure cells were not contaminated with mycoplasma, detection tests were employed on a routine basis (every 2 weeks). A sterile coverslip was placed in a 5 cm diameter Petri dish and covered with 4 ml of antibiotic free medium. Cells (5 \times 10^5) were plated and left to grow to 25-50 % confluency for 48 h. After 48 h cells were washed twice in 1 \times PBS-A and fixed with 70 % ethanol for 30 min. Hoechst 33342 solution (1 mg/ml) was prepared in sterile distilled water. Ethanol was removed, cells were washed twice with 1 \times PBS-A and Hoechst 33342 solution (0.5 \mu g/ml) was incubated for 15 min, in the dark. The coverslip was inverse mounted (in 1 \times PBS-A/glycerol; 1:1) on a glass slide, and viewed at 400 nm on a Nikon™ eclipse TE200 microscope. Bright nuclei, (with no staining in the cytoplasm) indicated mycoplasma free cells.

2.1.4 Lysis of cell cultures

Cells were trypsinised and pelleted at 670 \times g and cell pellets were resuspended in lysis buffer (20 mM Tris, 1 % Tween, pH 7.4 with protease inhibitor tablets, (Roche, Lewes, East Sussex, UK)). 100 \mu l of lysis buffer per near confluent T75 was used for each lysate. Cells were kept on ice and sonicated (Status™ 200 homogeniser) in 20 sec intervals for 1 min 20 sec, storing on ice between intervals. Cell lysates were kept in a -20°C freezer until required.

2.1.5 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) treatment in vitro

TCDD (Promochem, Welwyn Garden City, Herts, UK) was diluted in DMSO and added to complete media. The final DMSO concentration did not exceed 0.6 % and the typical TCDD concentration was 10 nM (Sutter et al., 1994; Spink et al., 2003). Controls were performed using the same concentration of DMSO.
Incubation times varied, depending upon the experiment, but typical treatment times for TCDD treatment was 21 h, as highlighted in the literature (Sutter et al., 1994; Bofinger et al., 2001).

2.1.6 Mouse xenograft preparation

Animal experiments were carried out according to UKCCR guidelines on welfare of animals and experimental animals in neoplasia. UT SCC-14 tumour cells \((2 \times 10^6 \text{ cells per mouse})\) from \textit{in vitro} culture were implanted sub-cutaneously, into the rear dorsum of anaesthetized 8-12 week old SCID mice. Once the tumours reached a geometric mean diameter (GMD) of 6.5 - 7.5 mm\(^3\), treatment was initiated. TCDD was administered in DMSO, intraperitoneally, at doses of 5, 15 and 50 µg/kg. Tumours were excised at stated times post TCDD and fixed in formalin (10 % neutral buffered) for histological examination.

2.2 Western blot analysis of CYP1 proteins

The CYP1B1 antibodies (monoclonal: Prof. G Murray, University of Aberdeen (McFadyen et al., 1999) and polyclonal: Gentest, Woburn, MA, USA) were checked for their reactivity and specificity towards CYP1B1 in a series of validation steps (Figure 2.1 and Figure 2.2), which is in agreement with the validation studies performed previously (detailed in (McFadyen et al., 1999)). The monoclonal antibody raised by McFadyen and co-workers was against a synthetic peptide coupled to a carrier protein as the immunogen (VNQWSVNHDPVKWPN). The antibodies recognised the C-terminal end of the CYP1B1 protein, antibodies were then tested for their immunoreactivity using immunoblotting (McFadyen et al., 1999). The Gentest™ polyclonal antibody used for the \textit{in vitro} and \textit{in vivo} western blots also recognised the C-terminal of CYP1B1 (at a stated size of 60.8 kDa) however the peptide sequence against which it was raised was proprietary information.
Figure 2.1 Western blotting analysis to determine the specificity of the CYP1B1 monoclonal antibody. Panel A shows a dilution series of CYP1B1 Gentest supersomes™ and panel B shows a dilution series of CYP1A1 supersomes™.

Figure 2.2 Western blotting analysis to determine the specificity of the CYP1B1 polyclonal antibody. Panel A shows a dilution series of CYP1B1 supersomes™. Panel B shows the same dilution of CYP1A1 supersomes™.
2.2.1 Preparation of tumour homogenates

Frozen tumours for western blot analysis were removed from -80°C storage, thawed on ice and cut into 1 mm³ cubes and suspended in 4 volumes of tumour lysis buffer (20 mM Tris, 20% glycerol, 1% Tween, pH 7.4 with protease inhibitor tablets). Samples were homogenised in a Potter-Elvehjem homogeniser, (Jencons, Leighton Buzzard, UK) and kept on ice until aliquotted and stored at -80°C until further analysis.

2.2.2 Bradford protein assay

Protein from cell lysates or xenograft homogenates were measured using the Bradford protein assay (Bradford 1976), using a 96 well plate format. A protein standard curve was determined using bovine serum albumin (BSA) (1-20 µg/ml). Samples were diluted accordingly to fall within this range. An equal volume of Sigma™ protein reagent was added to 100 µl diluted sample in the 96 well plate and mixed. Absorbance was recorded at 600 nm using a multiwell plate reader (Labsystems Multiskan® MCC/340). Protein concentration was then determined using polynomial regression analysis (Excel, Microsoft® Corporation).

2.2.3 Western blotting reagents buffers and controls

Details of buffers and reagents used for western blotting are detailed as follows:

- Antibody buffer – for primary antibody incubation: 2.5% milk powder (Waitrose, Bracknell, UK) in 1 x PBS-A with 0.1% Tween-20. For secondary antibody incubation: 5% milk powder with 1 x PBS-A with 0.1% Tween-20
- Blocking buffer - 5% milk powder in 1 x PBS-A with 0.1% Tween-20
- Denaturing buffer -0.125 M Tris/HCl, 4% sodium dodecyl sulphate (SDS), 20% glycerol, 0.5 mg/ml bromophenol blue, 10% mercaptoethanol
- Running buffer -32.5 mM Tris/HCl, 192 mM glycine, 0.1% SDS
- Washing buffer – 1 x PBS-A, 0.2% Tween-20
- Transfer buffer – 32.5 mM Tris HCl, 192 mM glycine, 20% methanol
• Gels – freshly made each time. 3/4 separation gel ¼ stacking gel (10 % acrylamide gels were used and are described as follows)
• Separation gel – protogel™ (National Diagnostics, Hull, UK), final 10 % acrylamide/bis acrylamide, 375 mM Tris/HCl pH 8.8, 0.1 % (w/v) SDS, 0.05 % (w/v) ammonium per-sulphate (APS), 0.05 % (v/v) N, N, N’, N’-tetramethylethlenediamine (TEMED), in 20 ml.
• Stacking gel – 2.66 ml protogel™ (final 4 % acrylamide), 0.123 mM Tris/HCl pH 6.8, 0.098 % (w/v) SDS, 0.147 % APS (w/v), 0.098 % (v/v) TEMED in 20 ml.
• Stripping buffer – 100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris, pH 6.3.
    CYP1A1, CYP1A2 and CYP3A4 controls (pure enzymes from Baculovirus-insect cell expressed supersomes™, Gentest™, Woburn, MA, USA), were used for testing the cross reactivity of antibodies for validation of the western blotting protocol (data not shown for CYP1A2 and CYP3A4). Gentest Supersomes™ samples were incubated with denaturing buffer (sample:buffer, 1:1) and loaded onto the gels (typically 0.5 μg supersomal protein). The antibodies and the dilutions applied are listed as follows:
    • Cytochrome P450 1B1 monoclonal antibody 1:1000 (Graeme Murray, Department of Pathology, Aberdeen, UK)
    • Cytochrome P450 1B1 polyclonal antibody 1:2000 (Gentest™, Woburn, MA, USA)
    • Actin monoclonal antibody 1:1500
    • Actin polyclonal antibody 1:1500
    • Cytochrome P450 1A1 1:2000 (Gentest™, Woburn, MA, USA)
    • Anti mouse secondary horseradish peroxidase HRP and anti goat secondary HRP (DAKO, Ely, Cambridgeshire, UK)

2.2.4 Gel electrophoresis

Western blotting was performed on whole cell lysates (2.1.4) and tumour xenograft homogenates (2.2.1). Protein concentration was determined using the Bradford protein assay (2.2.2). Denatured proteins (~25 μg sample protein) were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-
PAGE). Polyacrylamide gels (10 %) and buffers were prepared fresh prior to electrophoresis. Equal volumes of sample protein and 2 × denaturing buffer were incubated at 95°C for 10 min on a temperature controlled heating block (ThermoStat Plus, Eppendorf, Cambridge UK). Equal concentrations of denatured protein for each sample were loaded onto the gel with protein molecular markers (Amersham Biosciences, Little Chalfont, UK). Proteins were electrophoresed on an SDS gel at a constant voltage of 200 V in running buffer, using the Hoeffer Mighty Small™ gel system (Amersham Biosciences, Little Chalfont, Bucks, UK).

2.2.5 Blotting procedure

After separation, proteins in the polyacrylamide gel were transferred onto a 48 cm² nitrocellulose membrane (Amersham Biosciences, Little Chalfont, Bucks, UK) at a voltage of 100 V for 1 h in transfer buffer. Proteins were visualised to check for transfer with ponceau stain (0.1 % ponceau S in 5 % acetic acid). Antibody incubations were performed on an orbital rotating platform and at room temperature unless otherwise stated. The blot was de-stained with blocking buffer at room temperature for 15 min, after which, the blot was blocked in fresh blocking buffer overnight at 4°C. After blocking, the membrane was rinsed in wash buffer, 3 × 10 min. Primary antibody incubations were performed in 2.5 % (w/v) fat free milk powder (Waitrose, Bracknell, Berkshire, UK) antibody buffer for 1 h on a vigorous shaking platform, after which the membranes were rinsed in wash buffer as previously described. Secondary antibody incubations were performed in 5 % (w/v) milk antibody buffer for 1 h under the same conditions as the primary antibody incubation. Membranes were washed and immunoreactive bands detected with chemiluminescence detection. Blots were stripped and re-probed using stripping buffer and incubated on a shaking platform for 2 h at 37°C.

2.2.6 Development of membrane

Membranes were incubated with 10 ml 1.25 mM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), in 0.1 M Tris, pH 8.5, 100 μl 68 mM p-coumaric acid (4-hydroxycinnamic acid) with 3 μl H₂O₂ (30 %, w/v) for 1 min. Membranes were exposed to chemiluminescent photographic film (Amersham Biosciences,
Little Chalfont, Buckinghamshire, UK) for different exposure times (2-15 min) depending on the primary antibody, developed, fixed (Kodak, Rochester, USA), and washed in running tap water.

2.3 Immunohistochemical procedures and spectral imaging microscopy

DAKO ChemMate reagents were purchased from DAKO Ltd. (DAKO, Ely, Cambridgeshire, UK). Washes were carried out with Tris buffered saline (TBS) - 0.05 M Tris/HCl with 0.15 M NaCl, pH 7.6. The ChemMate™ system was employed for all immunohistochemical procedures. Figure 2.3 illustrates the avidin biotin complex (ABC) method used in this study. Previous reports have performed CYP1B1 immunohistochemistry using signal amplification techniques (Murray et al., 1997). This involves the addition of biotinylated tyramine to the section to ‘darken’ the staining already achieved by conventional avidin biotin complex (ABC) methods (see Figure 2.3). However, using the staining methods described above, no amplification methods were performed as staining was visible in a high percentage of the tumour samples, hence did not require amplification.

**Key**
- = peroxidase labelled streptavidin
  - = biotin
  * = antigen
  = primary antibody
  = secondary antibody

**Figure 2.3** The avidin biotin complex (ABC) method of staining. Schematic diagram of the immunohistochemical method employed to stain the human tissue and xenografts. 3,3-diaminobenzidine (DAB) is added to the tissue sections to detect where the antibodies have bound. The HRP on the secondary enzyme will metabolise the DAB to give a brown colour at the site of immunoreactivity.
2.3.1 Sample preparation

Biopsies were obtained from patients with head and neck squamous cell carcinoma (HNSCC) enrolled on the Continuous, Hyperfractionated Accelerated RadioTherapy (CHART), pilot trial (Dische et al., 1997) prior to any radiation/chemotherapy treatment. CYP1B1 immunohistochemistry was carried out using a human bladder carcinoma section as a positive control. Sections (3 µm) were cut on a microtome and mounted on poly-L-lysine coated slides (Surgipath, Peterborough, UK). Sections were stained using a DAKO™ autostainer. Histopathological examination was carried out using haematoxylin and eosin (H & E) staining to ascertain the tumour distribution and pathological stage of the tissue (i.e. pre-malignant or malignant) by an independent observer (Dr P. I. Richman, Mount Vernon Hospital).

2.3.2 Immunohistochemical staining

Sections were de-paraffinised in xylene twice × 5 min, re-hydrated through a graded alcohol series (100 %, 90 %, 70 % (v/v)) and rinsed in water for 5 min followed by TBS for 3 min. Sections were subjected to heat mediated antigen retrieval by microwaving (800 W) for 15 min in 300 ml citrate buffer (0.1 M, pH 6), for 3 cycles of 5 min, (topping up with distilled water in between microwave irradiations). Samples were left to stand for 20 min to cool before rinsing in tap water for 5 min.

2.3.3 DAKO™ autostaining method for immuno-histochemical detection of CYP1B1

The following staining incubations were carried out on a DAKO™ autostainer. After washing with TBS/Tween, endogenous peroxidase activity was blocked with DAKO™ peroxidase block solution for 5 min. Primary CYP1B1 monoclonal antibody was diluted in DAKO antibody diluent (1:25) and incubated on the sample tissue for 30 min at room temperature. Sections were washed with TBS-Tween and incubated with DAKO ChemMate™ biotinylated link antibody (ready to use) for 30 min. After washing, DAKO ChemMate™ streptavidin labelled peroxidase was applied for 30 min. Sections were washed in TBS/Tween then 3,3-
diaminobenzidine (DAB) (20µl DAB/ml diluent) was applied for 5 min and sections were washed in running tap water for 4 min.

Sections were counterstained in Gills haematoxylin and irrigated with running tap water for 5 min, dehydrated then mounted in mountant (DPX), (Surgipath, Peterborough, UK).

2.3.4 Xenograft staining protocol for CYPIB1

The tumours were processed and embedded in paraffin wax and 3 µm sections were cut using a microtome. Section preparation including heat mediated antigen retrieval as described in 2.3.2 was followed. A ‘rodent block’ was applied (Lab vision, Newmarket, Suffolk, UK) to avoid binding of the secondary (anti-mouse) antibody to endogenous mouse immunoglobulins on the mouse tissue. Sections were blocked for 15 min with H2O2 (Lab Vision, Newmarket, Suffolk, UK) then washed in TBS (3 × 2 min). Ultra V block (Lab Vision, Newmarket, Suffolk, UK) was incubated for 5 min and then removed. Rodent block was applied and incubated for 1 h, then washed in TBS. After these initial steps, the samples were stained using the DAKO™ autostainer and the protocol described in 2.3.3.

2.3.5 Normal tissue MaxArray™

This protocol was also followed for the MaxArray™ normal human tissue array (Zymed Laboratory Inc., San Francisco USA). This tissue array was used to compare normal tissues with the CHART sections. Details of this array can be seen in Figure 2.4.
**Figure 2.4** Zymed MaxArray™. Paraffin core normal tissue array. Panel A shows the 30 cores embedded in paraffin and B the tissues present with their identification, C.

### 2.3.6 Visual analysis of staining

It was of utmost importance to have an internal control for the validation of the staining intensities. A bladder tumour positive control was used throughout the staining runs for CYP1B1 and the intensity of immunoreactivity of the bladder carcinoma was evaluated at the end of each run. Experiments where the staining was weaker/stronger than the previous run, the staining run was repeated.
Tissue sections were observed with light microscopy and graded semi-quantitatively as strong, medium or weak staining intensity, in relation to the bladder carcinoma. All xenograft samples were analysed in the same way as the clinical tissues (i.e. semi-quantitatively, by giving a score of 1 for weak, 2 for medium and 3 for strong). The clinical sections were also viewed and pre-malignant tissue graded by an independent observer (Dr. P. I. Richman, Clinical Pathologist, Mount Vernon Hospital). CYP1B1 immunoreactivity in the pre-cancerous tissue was also analysed and graded in the same way as the carcinoma tissue. Microscopic analysis of each stage of pre-malignancy was important when analysing the tissue. Hyperplasia was defined as an increase in the epithelial layer with an increase in one or more of its components. Where there was inflammation there may also be some cell crowding and cytological atypia. Rete processes (finger-like projections into the supporting tissue/stroma) may also be observed in hyperplastic tissue. Moderate dysplasia was graded by analysing the cytological atypia, mitotic activity and polarity. This occurs where the cells are atypical at the lower two-thirds of the mucosal layer and the nuclei become hyperchromatic (the nucleus/cytoplasmic ratio is high). Cells can often become keratinised in severe dysplasia and the nucleus can become pyknotic (contraction of nuclear contents). This is described in more detail in chapter 3.

2.3.7 Spectral imaging: background

Spectral imaging is a method of image analysis which allows quantification of staining intensity from immunohistochemically stained tissue samples. This technology was developed at the Gray Cancer Institute and is a novel, cost-effective way of capturing spectral imaging data using a variable dielectric optical filter (Barber et al., 2003; Barber et al., 2005). Other methods available are highlighted in the literature (Farkas et al., 1998; Rothmann et al., 1998). Spectral imaging microscopy has been previously used successfully for the analysis of CYP1B1 staining intensity in clinical samples (Carnell et al., 2004).

The set up of the spectral imaging apparatus is shown in Figure 2.5. A standard monochrome camera (Type 4912, Cohu Inc., USA), coupled to the spectral imaging device and an upright microscope (Optiphot, Nikon™, UK) was used to capture representative shots of the tissue to be analysed. This method of image acquisition will capture information about the clinical sample (for example the
optical density, (O.D)) at a number of wavelengths. The purpose of this study is to separate the haematoxylin stain from the brown DAB stain. Spectral information on the sample is collected covering the red, green and blue regions of the spectrum (covering the visible spectrum 400 to 700 nm). Dyes used in the staining process have characteristic absorption spectra. Changes in O.D. at each wavelength can be determined for each pixel within a captured image. Optical densities, measured by the spectral imager, were comparable with O.D measured by a spectrophotometer. This is highlighted in Figure 2.6 which shows the O.D measured by these two methods (reproduced by kind permission of Paul Barber). When coupled to a linear unmixer this technique will separate the different stains, where multiple stains have been used (allowing quantitative analysis of the each stain). This provides reliable automated data not only on visible immunoreactivity but on staining that may not be visible by eye.

Figure 2.5 Spectral imaging microscopy set up, showing the spectral imager coupled to the standard monochrome camera.
Figure 2.6 O.D generated with the spectral imager and a diode array spectrophotometer at three different wavelengths, from (Barber et al., 2003).

2.3.8 Spectral imaging: image capture and analysis

Figure 2.7 shows the procedure involved in capturing an image with the spectral imager. Firstly, three areas of the tissue section were selected (800 µm x 650 µm). In some cases limited tissue made it necessary to analyse fewer areas. Images were captured from 400 nm to 700 nm in 24 nm steps, and a false colour image was generated (panel B). The captured images were then unmixed to reveal the individual stains (panel C and D) using reference spectra for the stains used during the immunohistochemistry protocol (DAB and haematoxylin, panel E). Absorbance (optical density, O.D.) spectra were normalised to these reference spectra. A histogram of pixel frequency versus normalised absorbance, (panel F) was generated. The threshold for data analysis (0.15) of stained areas was chosen by eye to disregard the areas of the image containing obvious areas of stroma and was also set to include all areas of tumour that were to be analysed. This value was used for all tumour and tumour related (pre-malignant) tissue to be analysed. This threshold value was not set for analysing the normal tissue MaxArray™, as all of this tissue was to be analysed. If a threshold was applied this would affect the data generated from the weak to negative stained normal tissues. This is illustrated in Figure 2.8.
Figure 2.7 Spectral imaging microscopy linear unmixing process. Section of tissue was selected for analysis (A). A false colour image was generated (B) by the spectral imaging software using the spectral data from each pixel, across all acquired wavelengths. The two stains were spectrally ‘un-mixed’ from each other, giving DAB (C) and haematoxylin (D). Using a previous control stained slide, reference spectra for these two stains are determined (E) and the normalised absorbance of immunoreactivity was determined (F).
The mean normalised absorbance (MNA) values are given for thresholded data and for data where this was not applied. The mean normalised absorbance was dramatically different (0.177 compared to 0.045) when comparing threshold data to non threshold data respectively.

![Threshold data from the spectral imager.](image)

**Figure 2.8** Threshold data from the spectral imager. Spectral imaging at the same magnification to show the difference in mean normalised absorbance (MNA) between ± a threshold in tissue that has low immunoreactivity. Panel A shows a cerebellum core for a normal tissue MaxArray™, panel B shows a human tumour xenograft. Both samples have been stained with the CYP1B1 monoclonal antibody. Scale bar 100 µm

When no threshold was applied, the whole section was analysed. The mean normalised absorbance intensity values (for each image captured, for each sample) were averaged and the standard errors derived. Data was plotted and analysed, comparing visual and spectral data.

### 2.4 Nitric oxide prodrug metabolism

HPLC chemicals described in 2.4.1 were purchased from Fisher Chemicals, (Loughborough, Leicestershire, UK) unless otherwise stated.
2.4.1 Prodrug synthesis and aim of assay

Prodrugs for nitric oxide were synthesised at the GCI by Dr. Saraj Ulhaq. Prodrugs were designed to bind at the active site based on CYP1B1 using sequence homology modelling. Upon metabolism, these benzo- or pyrido-fused indole oxime prodrugs will release nitric oxide, which is quantified using the autooxidation product nitrite (Stratford et al., 1997). Gentest supersomes™ were used for all metabolism studies with the prodrugs. Prodrugs were analysed on their ability to release nitric oxide, and the rate of loss of the parent prodrug was also evaluated using high performance liquid chromatography (HPLC). Samples were analysed using an autosampler (Waters™, 717), and pump (Waters™, 616), with columns and buffers described in detail later (see section 2.4.3).

2.4.2 NADPH regenerating system

The following procedure was used for both nitric oxide detection and prodrug loss. The original batch of Gentest supersomes™, once thawed were aliquotted and stored at -80 °C. Before each assay, the aliquot of Gentest supersomes™ was thawed and tested for activity using the EROD assay using a published protocol (Burke et al., 1994). Assay incubation volumes totalled 200 µl with 10 µl of CYP enzyme (Gentest supersomes™) used (typically 10 pmol P450 protein). Incubations were carried out in plastic HPLC v-shaped polypropylene snap cap 200 µl vials (SMI, Gloucestershire, UK). Glucose-6-phosphate (4.95 mM), glucose-6-phosphate dehydrogenase (0.4 units), NADPH (0.49 mM) and magnesium chloride (2.97 mM) were made up in phosphate buffer pH 7.4 (100 mM) and Gentest supersomes™ were added and incubated at 37°C. After 5 min incubation, prodrug (10 mM in DMSO, 50 µM final concentration) was added to initiate the reaction. Samples were taken directly from the assay incubate (which was in the autosampler). This resulted in aliquots taken from the assay mixture at the end of the previous injection/separation. Parent prodrug/nitrite was measured over time (approximately 20-30 min depending on the reaction). Each assay was performed in triplicate.
2.4.3 HPLC analysis

2.4.3.1 Nitric oxide analysis

Nitrite ions can be measured by absorbance at 210 nm but other components in the incubation mixture will adsorb at this wavelength, therefore, HPLC with electrochemical detection is the preferred method to give a cleaner system (Stratford et al., 1997; Stratford 1999).

The potential at which nitrite can be oxidised using the glassy carbon electrode was determined by injecting a fixed concentration of nitrite and altering the mV on the electrochemical detector and recording the peak area. Figure 2.9 shows the optimisation of this procedure for the glassy carbon electrode. Freshly prepared sodium nitrite (5 μM) was used as a standard for nitrite assays. Buffer was freshly prepared for each experiment for measurement of nitrite production. The samples were incubated in the autosampler and aliquots taken every 4 min for analysis. Based upon the standards, the concentration of nitrite in each aliquot was determined, and the rate of nitrite production was calculated using regression analysis. For each prodrug a negative control was performed, whereby all of the assay components were incubated minus the enzyme (Gentest supersomes™). The data calculated from these controls was taken away from the supersomal assay data. HPLC analysis of assay incubates for nitrite were performed isocratically using a 150 mm x 4.6 mm anion exchange column (Exsil™ SAX) at a flow rate of 1.8 ml/min. The column was housed at 30°C and the assay vial incubated at 37°C in a Waters™ 717 autosampler with a Waters™ 616 pump. Data was acquired using Waters™ Millennium software. Nitrite ions were detected using an ESA™ coulochem 5100 A electrochemical detector equipped with a porous graphite cell set at +0.65 V. A Waters™ 2695 autosampler/pump was also used with a Dionex™ ED40 electrochemical detector with a glassy carbon electrode set at +1.05 V for nitrite detection.
Figure 2.9 Detection of nitrite using the glassy carbon electrode. Change in peak area by the alteration of voltage on the detector.

The eluents for both autosamplers were A: 5 % acetonitrile, 40 mM KH₂PO₄, 7 mM H₃PO₄ and B: 75 % acetonitrile using isocratic elution (98 % : 2 %, A:B). Eluents were degassed with Helium (Waters™ 616) or by vacuum degassing (Waters™ 2695). Column/column housing and assay incubation conditions were the same for the Waters™ 2695 autosampler. Samples were taken every 4 min for analysis.

2.4.3.2 Prodrug analysis

Assay incubates for prodrug loss evaluation contained the same reagents as those for the nitrite production analysis. Aliquots were taken from the assay incubation mixture every 5 min. The concentration of drug at each time point was calculated by the known original concentration at the start of the assay (50 µM). Linear regression analysis calculated the loss of prodrug over time and related this to protein concentration of the Gentest supersomes™. As with the nitrite detection a negative control was included, with the incubation of all of the assay components minus the enzyme. The rates were corrected for the controls.

Prodrug loss was analysed from assay incubates housed in the same conditions as in 2.4.3.1. Eluents were degassed and the column temperature was...
under the same conditions as described in 2.4.3.1. Flow rate was 1.8 ml/min and prodrug analogues were separated using isocratic conditions using a 100 mm × 3.2 mm anion exchange column (Hichrom™ RPB). The eluents were A: 5 mM KH₂PO₄, 5 mM H₃PO₄ 5 mM heptane sulphonic acid and B: 75 % acetonitrile. Prodrug loss was measured by absorbance at 264 nm using a Waters™ 2996 diode array detector. Table 2.1 below summarises the conditions and percentages of eluents used to detect the 4 different prodrug analogues over time for loss in the assay incubate. Samples were taken every 5 min for analysis.

Table 2.1 Conditions for elution of prodrug analogues from the prodrug assay incubates.

<table>
<thead>
<tr>
<th>Prodrug analogue</th>
<th>Elution time (min)</th>
<th>Eluent conditions (% A: % B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCI 500</td>
<td>2.37</td>
<td>20:80</td>
</tr>
<tr>
<td>GCI 503</td>
<td>1.99</td>
<td>40:60</td>
</tr>
<tr>
<td>GCI 504</td>
<td>1.69</td>
<td>40:60</td>
</tr>
<tr>
<td>GCI 510</td>
<td>2.66</td>
<td>10:90</td>
</tr>
</tbody>
</table>

2.5 Statistical analysis

Data was analysed using JMP statistical software package, version 5.1, © SAS Institute, Inc. When analysing the data from the head and neck study, ordinal data was analysed using Chi-squared tests (for a likelihood ratio). Continuous data differences/associations were tested using analysis of variance (ANOVA) and correlations were tested using Spearman Rank’s correlation co-efficient. Correlations between spectral imaging and visual analysis were analysed using ANOVA. Prodrug data was analysed by comparing the means using the student’s t-test. Values of p < 0.05 were considered as significant in all cases.
Chapter 3

Target validation of cytochrome P450 1B1 in head and neck squamous cell carcinoma and associated pre-malignant tissue
3.1 Introduction

Head and neck squamous cell carcinoma (HNSCC) is the most common cancer of the upper aero-digestive mucosa (Quon et al., 2001) and comprises all cancers arising from squamous cells in the region (Figure 3.1).

Figure 3.1 (A) Schematic cartoon of areas within the head and neck region affected by HNSCC (www.cancerbackup.com). (B) Cartoon depicting the anatomical regions within the head and neck (www.oncologychannel.com).
HNSCC is known to progress through a series of distinct pre-malignant stages, namely hyperplasia and dysplasia (illustrated in haematoxylin and eosin (H and E) stained sections Figure 3.2 panels A and B) (Crowe et al., 2002). These pre-neoplastic lesions will often precede malignancy (Crowe et al., 2002; Hittelman 2003; Lonardo & Sakr 2003), and macroscopic lesions (e.g. erythroplakia and leukoplakia), especially in the oral cavity, are indicative of asymptomatic oral cancer (Mashberg 1978; Prasad & Huvos 2001). The final stage in malignant progression is the formation of an invasive carcinoma or carcinoma in situ (Figure 3.2, Panel C). Pearls of keratin indicate that the tissue is overtly malignant and well differentiated. As this disease follows distinct pathological stages, it makes it an ideal model to study the expression of CYP1B1 during malignant progression (Prasad & Huvos 2001).

Figure 3.2  H & E stained patient samples showing the pathological progression of HNSCC. Panel A shows hyperplasia. Panel B shows a dysplastic patient sample and panel C shows a well differentiated HNSCC. The tumour islands are visible (T) and pearls of keratin (Ke) have formed. Scale bar 100 μm.
This distinct aetiology of disease progression can be attributed to the ‘field
cancerisation’ effect (Slaughter et al., 1953) which can be attributed to carcinogens
in tobacco smoke (Blot et al., 1988). Smoking and alcohol consumption have been
shown to be responsible for cancers at this site and worldwide, it is estimated that
smoking is responsible for 41 % of laryngeal and oral/pharyngeal cancers in men
and 15 % of women (Stewart & Kleihues 2003). In 75 % of cases of HNSCC in the
United States, tobacco and alcohol consumption is thought to play a considerable
role (Blot et al., 1988).

3.1.1 Anatomy of the head and neck region

When analysing tissues of head and neck origin, it is common practice to
divide the whole area into three distinct regions, the oral cavity, the hypopharynx
and the oropharynx. The oral cavity is defined as the area extending from the
vermilion border of the lips to a plane between the junction of the hard and soft
palate superiorly and the circumvallate papillae of the tongue inferiorly. This region
includes the buccal mucosa, upper and lower alveolar ridges, floor of the mouth,
retromolar trigone, hard palate, and anterior two thirds of the tongue. The
oropharynx is the tonsillar fossa, soft palate, and base of tongue. The hypopharynx is
divided into the pyriform sinus, posterior pharyngeal wall, and postcricoid region.
The majority of the HNSCC sections used in this study were taken from the oral
cavity, namely the floor of mouth and tongue, however, other sites were also
analysed. Samples were taken from the larynx and columella (for analysis in this
study) which have to be considered as separate entities. Further information on
individual sites can be seen in appendix 1.

The normal pathology of the head and neck tissue is shown in Figure 3.3.
The squamous mucosa is the inner layer of any hollow organ which consists of the
epithelial layer, below which sits the lamina propria (Figure 3.3, panel A). This layer
gives the epithelial layer movement. Figure 3.3, panel B shows the distinct layers
with the supportive underlying muscle or muscularis mucosae (King 2004).
3.1.2 Protocols for grading pre-malignant tissue

Pathological grading of pre-malignant tissue is carried out under a range of different protocols including that of the World Health Organisation (WHO) (WHO 1997), Crissman (Crissman et al., 1993) and Ljubljana (Hellquist et al., 1999). The classification system advocated by WHO exploits the extent of atypical cells to grade dysplasia. Mild dysplasia is defined where atypia is confined to the lower third of the epithelium and the remainder is normal. In moderate dysplasia, the lower two thirds of the mucosa are occupied with atypical cells and the nucleoli are more pronounced. In severe dysplasia the atypical cells occupy more than two thirds of the mucosal layer. This system is commonly used for grading cervical sections and can also be employed for screening head and neck biopsies. Keratinisation may also be evident at this stage. Formation of pearls in the tissue is defined as high grade keratinising dysplasia.

In the system highlighted by Crissman (Crissman et al., 1993), keratinisation and cytological features determine the grade of dysplasia. The tissue can also be analysed non-morphologically, with the inclusion of cytological/biological markers. This method gives very detailed information on the biology of the tissue but this...
classification method could be time consuming and costly; therefore, this is not preferred and not often used clinically.

Atypical hyperplasia and other gross microscopic changes are accounted for in the Ljubljana classification system (Hellquist et al., 1999). Here, the thicknesses of the component basal and parabasal layers are analysed. In abnormal hyperplasia these layers are increased, occupying most (over half) of the mucosa. In atypical hyperplasia the nucleus is abnormal, and dyskeratosis occurs even though the shape and structure of the mucosa is preserved.

Discrepancies have been documented between these systems (Michaels 1997) and this is why it is of the utmost importance for consistency in pathology departments. The WHO system has been routinely used at Mount Vernon Hospital and was used by the clinical pathologist in this study (Dr. Paul. I. Richman, Department of Pathology, Mount Vernon Hospital). Pre-malignant tissue was separated into the different stages namely, hyperplasia and dysplasia. However, mild, moderate and strong dysplasia patient samples were grouped together.

3.2 Aims and objectives

There have been a number of studies looking at cytochrome P450 1B1 (CYP1B1) protein expression in a range of human malignancies (Murray et al., 1997; McFadyen et al., 1999; McFadyen et al., 2001b; Gibson et al., 2003; Carnell et al., 2004). However, no previous studies have investigated CYP1B1 protein expression during the malignant progression of cancer. The focal point of this study is HNSCC, as this particular cancer provides an excellent model to give an insight into expression of CYP1B1 during early stage tumourigenesis (Voravud et al., 1993). Carcinoma samples used in this study also contained pre-malignant lesions, which will be analysed together with the carcinoma tissue for CYP1B1 protein expression. The aims of this chapter are to:

1. Determine the distribution and frequency of CYP1B1 in HNSCC.
2. Compare CYP1B1 expression in pre-malignant versus malignant tissue.
3. Quantify staining intensities using spectral imaging microscopy.
4. Investigate possible correlations between CYP1B1 and clinico-pathological parameters.
This information will help to further validate CYP1B1 as a target for cancer therapeutics.

3.3 Results

3.3.1 Cytochrome P450 1B1 over-expression in HNSCC

Table 3.1 shows the number and frequency of expression of CYP1B1 in HNSCC and associated pre-malignant tissues (the latter is discussed in more detail in section 3.3.2.1). CYP1B1 protein was over-expressed in HNSCC, and this was the first study to identify protein of this CYP isoform in this cancer. Samples from HNSCC were stained for CYP1B1 protein using a monoclonal antibody and visually analysed.

Table 3.1 CYP1B1 expression in HNSCC and associated pre-malignant tissues

<table>
<thead>
<tr>
<th>tissue type</th>
<th>No. positive / No. stained</th>
<th>staining intensity</th>
<th>visually scored, number(frequency)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>strong</td>
<td>moderate</td>
</tr>
<tr>
<td>hyperplasia</td>
<td>14/14</td>
<td>2 (14 %)</td>
<td>8 (57 %)</td>
</tr>
<tr>
<td>dysplasia</td>
<td>22/22</td>
<td>2 (9 %)</td>
<td>9 (41 %)</td>
</tr>
<tr>
<td>HNSCC</td>
<td>66/70</td>
<td>12 (17 %)</td>
<td>30 (43 %)</td>
</tr>
</tbody>
</table>

Appendix 1 details the analysis of each section, with all pathological features listed, (degree of heterogeneity and staining in other, non-cancer tissue was noted). The majority of the HNSCC sections displayed moderate CYP1B1 staining intensity (43 %), 6 % of the total HNSCC sections were negative, with 17 % displaying strong CYP1B1 staining intensity. Weak CYP1B1 staining intensity was displayed by 34 % of the patients. Tumours representing each group of CYP1B1 staining intensity, are shown in Figure 3.4 (panels A-D). The brown diaminobenzidine stain indicates CYP1B1 localisation.
Figure 3.4 Representative HNSCC tumour sections illustrating the immunohistochemical detection of CYP1B1 protein. CYP1B1 is specifically localised to the cytoplasm of tumour cells (insert, panel A, ▲) when using a specific CYP1B1 monoclonal antibody. (A) strong CYP1B1 staining at low magnification (× 100), (B) medium staining of CYP1B1 in tumour cells, (C) weak and (D) negative CYP1B1 staining. In all cases CYP1B1 is mainly absent from the surrounding stromal tissue. Scale bar 100 μm except in insert in panel A (20 μm).

The tumour cells in panels A-C are positive and the supporting stroma is negative for CYP1B1. In most cases the entire tumour cell population was positive for CYP1B1, although the protein was heterogeneously expressed.

3.3.1.1 CYP1B1 expression at different anatomical sites

There was no pattern in CYP1B1 staining at different sites; equally, the intensity of CYP1B1 stain showed no particular relation to site (Figure 3.5). CYP1B1 staining was observed in all areas throughout the head and neck region with CYP1B1 protein localised in the cytoplasm of tumour cells (Figure 3.6, panels
Table 3.2 lists where the primary tumours were taken from for this study. Some tumours were more differentiated than others with keratin pearls (Figure 3.6, panels A, C and D). Supporting stroma was devoid of CYP1B1 protein. Statistically, there was no correlation between anatomical site and CYP1B1 visual score ($p = 0.26$). It was noted that in excess of two-thirds of hypopharynx (66%) and larynx (75%) specimens had weak CYP1B1 staining intensity and no tumours from these sites exhibited strong staining. The columella and oral cavity had the most even distribution of CYP1B1 staining intensity compared with the 3 other anatomical sites.

Table 3.2 Number of specimen samples taken from each anatomical site of the primary cancer

<table>
<thead>
<tr>
<th>anatomical site of tumour origin</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>oral cavity</td>
<td>31</td>
</tr>
<tr>
<td>oropharynx</td>
<td>16</td>
</tr>
<tr>
<td>hypopharynx</td>
<td>3</td>
</tr>
<tr>
<td>larynx</td>
<td>4</td>
</tr>
<tr>
<td>columella</td>
<td>9</td>
</tr>
<tr>
<td>other</td>
<td>7</td>
</tr>
</tbody>
</table>

Figure 3.5 Visual scores at each anatomical site in HNSCC samples. The visual staining is shown for each site. Some sites were devoid of strong staining (hypopharynx and larynx). Statistical analysis showed that there was no significant difference between anatomical site and CYP1B1 staining intensity in HNSCC.
Figure 3.6 Expression of CYP1B1 protein in different tissues in the head and neck region. (A) columella, (B) ear, (C) mandible, (D) mouth, (E) neck node and (F) tongue. All of these tumours displayed strong staining when the whole section was analysed. Scale bar 100 μm.
3.3.1.2 Intra-patient heterogeneity of CYP1B1 protein expression

This study showed that CYP1B1 protein was heterogeneously expressed within patient samples (Figure 3.7). Areas of strong CYP1B1 stain could be found adjacent to areas of weak stain, irrespective of site and overall staining intensity. Although many samples exhibited homogeneous staining, a proportion (47 %) of tissues showed distinct heterogeneity in staining. This was defined as tissue in one field of view showing a different visual score to an area in another field of view on the same tissue. The rest of the carcinoma tissues (53 %) did show some staining heterogeneity but this was not as marked as in other samples. Figure 3.7, panel A an example of a weakly immunostained section (W) is shown adjacent to a negative area (N). Figure 3.7, panel B, medium CYP1B1 staining intensity (M) is adjacent to weak CYP1B1 staining intensity (W), and in panel C strong (S) areas can be seen next to medium and weak immunoreactive areas. Overall visual scores were scored as the staining intensity that covered the majority of the section.

Figure 3.7 Intra-patient heterogeneity in HNSCC. A HNSCC section taken from the mouth is weak/negative (panel A), the tongue (panel B) which stained medium, panel C is from the mandible and stained strong for CYP1B1. Key: weak (W), medium (M), strong (S) and negative (N). Scale bar 100 μm.
3.3.1.3 CYP1B1 protein expression in recurrent disease

In this study CYP1B1 was identified in recurrent disease of head and neck origin (Figure 3.8). Here we have shown that CYP1B1 stain was localised to the cytoplasm of recurrent cancer tissues whilst the supporting stroma was negative. Although the sample size of this group (11) was limited, this result warrants further investigation in HNSCC related tissues. Details of patient samples can be seen in appendix 1.

3.3.1.4 Spectral imaging for CYP1B1 in carcinoma tissues

Patient samples were separated into individual anatomical sites and the normalised mean absorbance derived from three sample fields per section (800 µm x 650 µm) as detailed in Figure 3.9. The lowest mean normalised absorbance was 0.17 ± 0.02 found in a tumour from the oropharynx and the greatest value of 0.77 ± 0.40 was measured in a tumour from the oral cavity. A mean normalised absorbance value of 0.26 was determined for the carcinoma tissue (Figure 3.9).

Figure 3.8 CYP1B1 staining in recurrent disease. All CYP1B1 protein is localised to the cytoplasm of the metastatic cancer cells and is absent from supporting stromal tissue. Staining is heterogeneous. Panel A is from a strong CYP1B1 stained section, panel B from a medium stained and C from a weak stained section. Scale bar 100µm
Figure 3.9 Individual patient spectral imaging data for the HNSCC samples. Each sample is shown with the mean for 3 fields that were analysed with spectral imaging microscopy. Data is grouped into the different sites within the head and neck region. ‘Other’ refers to metastatic tissues (of HNSCC origin), ear and parotid tissue. Mean figures are shown for each anatomical site. The mean value for all HNSCC is shown (---).

There were no significant statistical differences between the mean values in each of the individual sites. The highest mean was shown in the columella (0.30). The oral cavity displayed the largest variation in mean normalised absorbance between anatomical sites (range in mean normalised absorbance = 0.59). Figure 3.10 shows that spectral imaging data correlated well with visual scores and this was statistically significant (p = < 0.0001).
3.3.2 Expression of CYPIB1 protein in pre-malignant tissues

It was demonstrated that CYPIB1 protein was expressed during the early stages of cancer progression. CYPIB1 protein was present in pre-malignant tissues (n=36, 100 % positive) of HNSCC, namely, hyperplasia and dysplasia. There were no pre-malignant tissues that were negative for CYPIB1 as assessed using the CYPIB1 selective monoclonal antibody. Further details can be found in appendix 2.

Table 3.1, in section 3.3.1, shows the CYPIB1 staining intensity at each stage of pre-malignancy. This tissue was obtained from the 95 original samples initially stained for CYPIB1. Some of these tissues also contained HNSCC tissue, whilst others exclusively contained pre-malignant squamous mucosa (determined by H & E staining). Samples containing tissue of different pathological types (i.e. hyperplasia with dysplasia) within the squamous mucosa, were not analysed for CYPIB1 in this study.

In summary the percentage of weakly stained sections increases during malignant progression (from 29 % in hyperplastic tissue to 50 % in dysplastic tissue). Conversely, the percentage of sections that displayed medium CYPIB1 staining intensity decreased (from 57 % in hyperplasia to 41 % in dysplasia). These
data suggest that there was a trend for CYP1B1 staining to decrease throughout malignant progression without distinct patterns of staining.

### 3.3.2.1 CYP1B1 protein expression in hyperplastic tissue

Figure 3.11 shows CYP1B1 protein expression in hyperplastic tissue. Figure 3.11, panels A-C show the range of CYP1B1 staining intensity in the rete pegs (characteristic of hyperplasia). As with the carcinoma tissue, the protein was localised to the cytoplasm of the pre-malignant cells and the nucleus was devoid of protein (Figure 3.11, panel D). There was some heterogeneity of CYP1B1 protein expression; however, CYP1B1 expression did not localise to any specific layer in the squamous epithelium (see Figure 3.3). The majority of the hyperplastic tissue displayed medium CYP1B1 staining intensity (53.3 %) whilst 13.3 % exhibited strong staining and 33 % were weak.

### 3.3.2.2 CYP1B1 protein expression in dysplastic tissue

CYP1B1 was also over-expressed in dysplastic tissues of all grades. Figure 3.12 shows dysplastic tissue with cytoplasmic cellular localisation, the same as in hyperplastic tissue. There was no distinct pattern of localisation within the epithelial compartment. Figure 3.12, panels A-C show intrapatient heterogeneity of staining. Panel D shows a high magnification image of this tissue and this distinctly shows CYP1B1 protein in the cytoplasm of the cells with the stroma showing no CYP1B1 staining. There was no occurrence of nuclear staining in any of these tissues, clearly visible in panel D. This high magnification picture also shows a pearl of keratin (K) that is present in the dysplastic squamous mucosa.
Figure 3.11 CYP1B1 localisation in hyperplastic head and neck squamous mucosa. Panels A-D demonstrates the heterogeneity of CYP1B1 in hyperplasia. A: Weak staining (× 100), B: medium staining in hyperplasia (× 100). CYP1B1 is clearly localised in the rete processes (rp, ↝) with the surrounding stromal tissue unstained. C: Strong staining in hyperplastic mucosa (× 100). D. High power magnification (× 400) of indicated field in C Cytoplasmic localisation (↝) of the CYP1B1 protein in hyperplasia. Panels A – C scale bar 100 μm, panel D scale bar (40μm).
Figure 3.12 CYP1B1 localisation in dysplastic head and neck squamous mucosa. Panels A-D show CYP1B1 heterogeneity in dysplastic mucosa where representative sections show, weak (A), medium (B) and strong (C) CYP1B1 staining. Panel D shows a high power magnification (× 400) of dysplasia from indicated area in C with cytoplasmic CYP1B1 protein localisation. Scale bar 100 μm, except panel D (40μm).

The grade of dysplasia did not affect CYP1B1 staining, as the protein was expressed at all stages to the same degree. All of the sections that contained pre-malignant dysplastic tissue were positive for CYP1B1 protein with the majority (50 %) expressing mild staining.

3.3.2.3 Spectral imaging for CYP1B1 in pre-malignant tissue

Spectral imaging was used to quantify staining in pre-malignant tissues. Using this technique, CYP1B1 expression was quantified in the tissues stained for CYP1B1. The mean normalised absorbance ± standard deviations are shown in Table 3.3.
Table 3.3 Mean normalised absorbances determined by spectral imaging microscopy for CYP1B1 staining intensity during the malignant progression of HNSCC.

<table>
<thead>
<tr>
<th>tissue type</th>
<th>spectral imaging microscopy mean normalised absorbance ± SD</th>
<th>Significance (p value) student’s t test</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNSCC</td>
<td>0.26 ± 0.10</td>
<td>Hyperplasia and carcinoma (0.002)</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>0.35 ± 0.12</td>
<td>Hyperplasia and dysplasia (0.09)</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>0.29 ± 0.10</td>
<td>Dysplasia and carcinoma (0.18)</td>
</tr>
</tbody>
</table>

Three sections per patient were analysed with the spectral imager. When averaged and analysed together, it is clear that there is a trend for CYP1B1 protein expression to decrease during malignant progression, displaying the same trend as the visual scoring system. Therefore, inclusion of different pathological stages when capturing the sample field was not favourable. Some sections had to be excluded because the pathological stage was heterogeneous within the mucosal layer.

Spectral imaging microscopy gives a quantitative value for diaminobenzidine stain, facilitating the measurement of CYP1B1 staining intensity; areas of weak CYP1B1 expression which may not have been detected by eye, are quantified by spectral imaging. Both methods are comparable when analysing staining intensity and spectral imaging microscopy (see appendix 3), further validating this method. These data are also shown in Table 3.3. Figure 3.13 shows that there was variability in mean normalised absorbance in pre-malignant tissues of HNSCC. Characteristics of tissue and staining patterns were also observed and recorded. There was a statistical difference between hyperplasia and HNSCC tumours with regards to CYP1B1 protein expression (respective spectral imaging absorbencies, 0.35 ± 0.12 versus 0.26 ± 0.10, p < 0.001). Expression in the tumour was similar to that in the dysplastic tissue with a mean normalised absorbance of 0.26 ± 0.10 compared to 0.29 ± 0.08. CYP1B1 expression was still high however, even with the decreasing trend.
Figure 3.13 Individual patient spectral imaging data for each pathological stage of malignant progression of HNSCC. Each patient sample was analysed in 3 different fields and the mean was taken. Each bar in the above chart is the mean of 3 fields. Fields that were analysed only contained the named stage tissue.

The spectral imaging data showed that all sections, in the pre-malignant stages, display mean normalised absorbance above 0.2. This bar graph shows there was considerable heterogeneity in normalised mean absorbance of CYP1B1 at the different stages of malignant progression. The lowest values for hyperplasia and dysplasia were 0.26 ± 0.07 and 0.20 ± 0.04, respectively.

3.3.3 CYP1B1 in normal tissues

CYP1B1 was detected in some of the tissues associated with the head and neck region. Visual analysis indicated that these tissues did not express the CYP1B1 protein to the same extent and intensity as the HNSCC and pre-malignant tissues. Figure 3.14 shows the normal tissue array cores (MaxArray™) of tissue from the head and neck region including samples of glandular origin, stained for CYP1B1.
Figure 3.14 Sample core tissue from the normal tissue array (MaxArray™) stained for CYP1B1. Panel A normal oesophagus, B normal tonsil, C normal salivary gland, D, normal thyroid gland. All images are taken at × 100 magnification and scale bar 100 μm.

Figure 3.14 panels A and B show normal oesophagus and normal tonsil, both exhibiting some staining for CYP1B1. Panel A clearly shows the squamous mucosal layer positive for CYP1B1 protein. It is clear from these samples that the glands, in particular, stain positive for CYP1B1 and the supporting tissue was negative. Figure 3.14 panel C shows CYP1B1 expressed in the glands. The tissue core from the thyroid gland shown in Figure 3.14 panel D shows that non-specific binding may be occurring in this section as there are areas of strong DAB stain. This staining pattern is addressed in the discussion. Generally, the surrounding tissue was negative for CYP1B1 protein. Request for sample data/origin (i.e. patient details), from the manufacturers of this array was unsuccessful. (This may have given information regarding smoking status, age and gender).
3.3.3.1 Spectral imaging for CYP1B1 in normal tissues

The normal tissue array was analysed for CYP1B1 staining intensity using spectral imaging microscopy. Table 3.4 shows spectral imaging data from these tissues on the Zymed normal tissue MaxArray™ (section 2.3.5). These data show that tissues of head and neck origin exhibited a mean normalised absorbance below 0.2. However, the values from spectral imaging microscopy indicate that when applying the same threshold as that applied for the carcinoma, the values are lower than that of the lowest value for the carcinoma with a mean normalised absorbance of 0.17 ± 0.02. As illustrated in Figure 3.14, there are proteins (mucin) within the glandular structures that will bind the antibodies. This will result in high mean normalised absorbance values. Therefore complementary visual evaluation of the tissue is essential.

<table>
<thead>
<tr>
<th>tissue type</th>
<th>spectral imaging microscopy</th>
<th>mean normalised absorbance ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>oesophagus</td>
<td></td>
<td>0.20 ± 0.04</td>
</tr>
<tr>
<td>tonsil</td>
<td></td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>salivary gland</td>
<td></td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>thyroid gland</td>
<td></td>
<td>0.20 ± 0.05</td>
</tr>
</tbody>
</table>

3.3.3.2 CYP1B1 expression in non carcinoma cells in patients with HNSCC

Supporting stroma in the HNSCC patient tissue stained positive for CYP1B1 in some cases. Figure 3.15 shows infiltrating immunological cells (Langerhans cells) stained positive for CYP1B1, giving staining which was very specific. These cells are specifically stained and positive staining has not been attributed to binding to non-specific proteins. In relation to the rest of the stroma these cells occupy a low percentage of this tissue.
3.3.4 Comparison of CYP1B1 with other clinico-pathological parameters: an overview

Different clinical parameters were statistically analysed with CYP1B1 visual scoring. Prior to analysis of these data, an overview of the parameters tested is detailed. Clinico-pathological parameters (including tumour grade, T stage, N stage, and biological markers (such as p53 and bcl-2)) can give an insight into disease progression and outcome.

Tumour grade is an important clinico-pathological feature for determining the treatment and prognosis of many different cancers including HNSCC. Grading depends on a variety of factors including cellular and nuclear pleomorphism, degree of keratinisation, frequency of epithelial pearls, mitotic rate and atypical mitosis. For the sections in this study, originating from the pilot CHART\(^1\) trial, the tumours were graded 1 to 4, (where 1 is most similar to normal mucosa). Grade 4 indicates that the cells are anaplastic (loss of structural differentiation) and usually more aggressive.

T stage is mainly determined by the tumour size but also incorporates invasion into other structures and mobility of structures such as the vocal cord. A T1 tumour is 2 cm or less in greatest dimension, T2 is more than 2 cm but not more than 4 cm, T3 is more than 4 cm and T4 is a tumour with extension to bone, muscle, skin, antrum, or neck.

\(^1\) CHART – continuous hyperfractionated accelerated radiotherapy
Node staging evaluates and classifies the involvement of lymph nodes in cancer progression. N0 signifies no evidence of regional lymph node involvement; N1 is evidence of involvement of movable homolateral regional lymph nodes, N2 is evidence of involvement of movable contralateral or bilateral regional lymph nodes and N3 is evidence of involvement of fixed regional lymph nodes. Finally, time to local recurrence is defined as the time from the date the patient was first seen until the tumour relapsed. Survival is defined as the time between the date first seen until the time that the patient died.

3.3.4.1 Correlation with other clinico-pathological parameters

These data were tested for statistical significance with spectral imaging data and visual data; the p values of which are tabulated in Table 3.5. These data show that there was no statistical significance when analysing the clinico-pathological with spectral imaging mean normalised absorbances. Conversely, the visual data of CYP1B1 expression showed that there was a statistical correlation with p53 staining intensity, duration of S phase and a trend for CYP1B1 to be expressed in tumours of the primary disease. There is a clear difference in significance between spectral imaging and visual scores. This is addressed in the discussion.

Table 3.5 Correlation of CYP1B1 with various clinico-pathological parameters. Statistical significance is shown (* = p< 0.05)

<table>
<thead>
<tr>
<th>Clinico-pathological feature</th>
<th>P value (spectral imaging)</th>
<th>P value (visual)</th>
</tr>
</thead>
<tbody>
<tr>
<td>site</td>
<td>0.68</td>
<td>0.26</td>
</tr>
<tr>
<td>grade</td>
<td>0.10</td>
<td>0.39</td>
</tr>
<tr>
<td>T stage</td>
<td>0.08</td>
<td>0.31</td>
</tr>
<tr>
<td>N stage</td>
<td>0.61</td>
<td>0.21</td>
</tr>
<tr>
<td>pri/rec</td>
<td>0.79</td>
<td>0.02 *</td>
</tr>
<tr>
<td>p53</td>
<td>0.47</td>
<td>0.03 *</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>0.41</td>
<td>0.62</td>
</tr>
<tr>
<td>Labelling index</td>
<td>0.07</td>
<td>0.10</td>
</tr>
<tr>
<td>Ts</td>
<td>0.10</td>
<td>0.02 *</td>
</tr>
<tr>
<td>Tpot</td>
<td>0.29</td>
<td>0.52</td>
</tr>
</tbody>
</table>

3.3.4.2 Tumour grade

Table 3.6 shows the distribution of tumour grades in this study whilst Figure 3.16 shows the relationship between grade and CYP1B1 expression levels.
There was similar and even distribution of expression across the tumour grades with no apparent trend for CYP1B1 to be expressed preferentially at one stage compared to another. On analysis with CYP1B1 stain, there was a trend for CYP1B1 expression to be stronger in the lower grade, better differentiated tumours but this does not reach significance (p = 0.33). This would imply that CYP1B1 is independent of the factors that govern tumour grade.

**Table 3.6** Grade, N stage and T stage of tumours (n = 70), analysed immunohistochemically for CYP1B1 protein expression.

<table>
<thead>
<tr>
<th>Grade</th>
<th>N stage</th>
<th>T stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>number</td>
<td>number</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>41</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>No data</td>
<td>1</td>
<td>No data</td>
</tr>
</tbody>
</table>

**Figure 3.16** CYP1B1 staining with grade of HNSCC patients. Weak and negative staining has been grouped together. There is a tendency for CYP1B1 to be stronger in lower grade tumours but this does not reach significance when analysed using a chi-squared test.

### 3.3.4.3 Tumour stage (T stage)

The distribution of T-stages is shown in Table 3.6 and in Figure 3.17.
The T stage of patients was correlated with the CYP1B1 staining intensity. There was no significant association between CYP1B1 expression and T stage (p = 0.31) although there was an absence of strongly expressing tumours in the T3 category.

3.3.4.4 Nodal stage (N stage)

Table 3.6 illustrates the distribution of N stages in this cohort of patients and Figure 3.18 illustrates the proportions of CYP1B1 staining intensity at each N stage of the tumour.
There were no significant associations between N stage and CYP1B1 expression. As the node stage is a positive or negative parameter the data was separated as such to analyse CYP1B1 low (0 and 1 visual scores) stained and high (2 and 3 visual scores) stained patients with node positive and negative patients. This enabled the data to have more statistical strength as the group sizes are larger. It also revealed some interesting correlations. Without grouping CYP1B1 (into high and low CYP1B1 expressing tumours) and node stage (positive and negative) the correlation p value was 0.21, indicating no correlation. When the node positives and negatives were analysed with the 3 groups of CYP1B1 stain the p value was 0.17. After grouping the CYP1B1 stains into high and low expressors the statistical data revealed an interesting, near significant correlation (p = 0.06). Node stage is the only parameter where this type of grouping can be performed as the node involvement is a positive or negative status whereas all of the other clinical parameters are not.

3.3.4.5 Correlation of CYP1B1 protein expression with other parameters

Figure 3.19 shows that there was no correlation between CYP1B1 and p53 expression. Strongly stained sections were mainly expressed in medium stained p53
tumours. However statistical analysis showed that these staining patterns did not have any significant correlation \( p = 0.55 \).

![Diagram showing the relationship between CYPIB1 stain and intensity of p53 in HNSCC. CYPIB1 staining intensity is indicated in the key and for p53 staining intensity ranges from negative (0) to strong (3).]

Figure 3.19 Relationship between CYPIB1 stain and intensity of p53 in HNSCC. CYPIB1 staining intensity is indicated in the key and for p53 staining intensity ranges from negative (0) to strong (3).

Figure 3.20 shows the relationships between CYPIB1 expression and proliferation associated parameters. These included labelling index (LI), duration of s phase (Ts) and potential doubling time (Tpot). There was a trend for CYPIB1 to be associated with decreasing labelling index and a significant reduction of the duration of S phase. In addition there was no relationship with DNA ploidy status, as diploid and aneuploid tumours had the same distribution of CYPIB1 expression.
Figure 3.20 Relationship between CYP1B1 expression and proliferation. Panel A shows the labelling index (LI), panel B shows the duration of S phase (Ts), panel C shows the potential doubling time (Tpot).
3.3.4.6 CYP1B1 and survival parameters (time to local recurrence and survival)

CYP1B1 was analysed and compared with this parameter. These data are presented in Kaplan Meier plots in Figure 3.21. There was no statistical significance between time to local recurrence and CYP1B1 expression (p = 0.74). The maximum time to local recurrence was 66 months. Patients with strongly stained CYP1B1 tumours had the shortest time to local recurrence (51 months).

![CYP1B1 staining intensity](#)

**Figure 3.21** The relationship between CYP1B1 (visual score) and local tumour control. The negative tumours were grouped with the weak stained patients to give the data stronger statistical power. Statistical analysis was performed with log rank survival analysis.

There was no correlation between CYP1B1 and overall survival. The curve can be seen in the Kaplan Meier plot in Figure 3.22. When analysed statistically CYP1B1 did not have any significance on overall survival of the patients (p = 0.89).
In summary, these data show that there was no correlation with site, grade, stage, n stage or bcl-2 status. CYP1B1 is more likely to be expressed in the primary rather than the recurrent disease. All of the ordinal data sets used in this study were analysed using the JMP software package statistical analysis package (© SAS Institute Inc.), and more details can be seen Chapter 2 (section 2.5). The ordinal data was analysed using a chi squared test which will compute a likelihood ratio. Continuous data was analysed using analysis of variance and a correlation was tested using Spearman Rank’s correlation co-efficient. (Data in consultation with Prof. George D. Wilson, Karmanos Cancer Institute, Detroit, USA).

3.4 Discussion

CYP1B1 protein expression has been reported in the literature in a range of different solid tumour types (Murray et al., 1997; McFadyen et al., 1999; McFadyen et al., 2001b; Gibson et al., 2003; Maeccker et al., 2003; Carnell et al., 2004). However, the present work is the first study to identify CYP1B1 in HNSCC. The protein was expressed throughout the tumour mass and showed no indication of expression in specific areas of the tumour (i.e. adjacent/distal from vasculature or adjacent/distal from stromal tissue). CYP1B1 was also absent from the supporting
stromal tissue. Expression of CYP1B1 occurs in a range of anatomical sites including squamous cells of the columella, ear, mouth and tongue. Moreover, this enzyme was also present in pre-malignant tissues, namely hyperplasia and dysplasia. In HNSCC, the majority of the tissue stained moderate for CYP1B1, the protein being restricted to the cell cytoplasm in all samples analysed. This observation is consistent with other studies that have shown the cytoplasmic localisation of CYP1B1 in tumour cells, but not in the supporting stromal tissue (Murray et al., 1997; McFadyen et al., 1999; McFadyen et al., 2001b). Other studies noted nuclear localisation of the CYP1B1 protein (Muskhelishvili et al., 2001), which is at odds with the notion that most phase I metabolising enzymes including the P450s reside in the endoplasmic reticulum (Timbrell 1992). The present work has also shown that CYP1B1 was present in tissues from recurrent disease. Other studies have reported CYP1B1 protein expression in metastatic ovarian cancer tissues with corresponding expression at the primary site (McFadyen et al., 2001b). This work provides preliminary evidence for CYP1B1 in the recurrent disease.

This study observed heterogeneity in CYP1B1 staining, which contrasts with previous studies that specifically state that CYP1B1 staining was not heterogeneous in all samples analysed (Murray et al., 1997; McFadyen et al., 1999). The present work shows intra-patient heterogeneity in CYP1B1 stain, which conflicts with previous studies where CYP1B1 staining was homogeneous across the tissue section (Murray et al., 1997). This may be due to the choice of immunohistochemical protocol employed. Murray and colleagues used a signal amplification method not used in this protocol. This present work observed a range of staining, from extremely strong to weak or negative, under the same staining conditions and would argue that heterogeneity of CYP1B1 stain in HNSCC is genuine. The degree of tumour differentiation did not have an effect on staining for CYP1B1, as some highly keratinised and well differentiated tumours had similar expression to the less differentiated tumours. There appears to be a spontaneous or as yet, undefined pattern of expression, as CYP1B1 staining did not follow any pattern.

The tumour material used in this work had previously been analysed for other biological markers, in the CHART pilot trial. There were no emerging trends when comparing different pathological markers and biomarkers including p53 status, T and N stage. The finding that CYP1B1 protein expression was independent
of p53 protein expression is valuable as some reports have shown poor prognosis in p53 negative patients.

Bcl-2 expression also showed no correlation with CYP1B1. The presence of bcl-2 in tumours is widely disputed and some studies indicate that it is a marker of favourable outcome for HNSCC (Wilson et al., 2001) whilst others dispute this (Gallo et al., 1996). Node status (positive/negative N stage) was analysed with weak/strong staining for CYP1B1 expression and yielded a p value of 0.06, suggesting that there may be some correlation with node involvement. Node status was performed on the primary disease and CYP1B1 expression was performed on the tumour available for this study, which may have been metastatic in origin, a factor which must be considered when interpreting these data. Overall, these data have not shown any correlations with other clinico-pathological markers. From a target validation stance, these results are desirable as cancer patients can be treated with CYP1B1 therapeutics irrespective of their tumour size, grade and site. The lack of prognostic correlation implies that CYP1B1 will not define outcome and leads to the tendency for CYP1B1, to be a more diagnostic tool for cancer. Clearly there is a difference in the statistical significance shown with spectral imaging and visual analysis. This may be due to the fact that spectral imaging data is continuous and the visual data is grouped (i.e. strong medium, weak).

The present work is the first to show CYP1B1 expression in pre-malignant tissues (hyperplastic and dysplastic squamous epithelium). Expression was independent of the depth of the epithelial layer and showed no preference for localisation within this layer. Previous reports have stated that protein expression can be dependent on the pathological stage of the disease, for example p53 expression changes in the oral mucosal layer at different pre-malignant timepoints, which was quantified using image analysis (Shin et al., 1994). In some sections CYP1B1 was expressed in the basal epithelium and in other cases it was evenly expressed throughout the epithelial layer. This indicates that CYP1B1 protein expression was independent of cell type as atypical cells can occupy different percentages of the mucosal layer depending on the severity and grading of dysplasia. pre-malignant targeting is also achievable, as CYP1B1 was expressed in hyperplastic and dysplastic tissues and was present, locally, to the growing tumour mass. CYP1B1 was also expressed in normal tissues of head and neck origin.
However, the antibodies used in the immunohistochemical protocol will bind to some glands (Polack & Van Noorden 1997) resulting in positive staining for CYP1B1. These ‘sticky’ antibody sites within these structures (confirmed with negative controls) are due to non-specific binding to the protein, mucin, which was present in some glands.

The two methods of analysis used the present study to grade the amount of antibody binding to the CYP1B1 antigen, visual scoring analysis and spectral imaging microscopy, were compared head to head (see appendix 3). The scores from visual analysis correlated well with the spectral imaging scores. However, spectral imaging microscopy provides an unbiased method of quantification for analysing staining in immunohistochemically stained sections. It can also detect ‘brown’ stain in very weak sections that was not obvious to the eye. Comparisons between different clinical tumours can also be made using spectral imaging (providing the samples were processed and stained using the same protocols).

The expression of CYP1B1, as measured by spectral imaging, appears to decrease throughout the malignant progression (when comparing hyperplasia, dysplasia and carcinoma). However, it is important to stress that although this was the case, the protein was still differentially expressed in pre-malignant/tumour tissue as opposed to the stroma, which is imperative for cancer therapy (McFadyen et al., 2004a). Understanding the role of CYPs in pre-malignant tissue is important for two reasons. Firstly, CYP1B1 protein expression could be a risk factor in carcinogenesis (via xenobiotic metabolism and subsequent toxicity of carcinogens) and secondly, activation and metabolism of therapeutic drugs could improve treatment. Published work in the literature illustrated that CYP1B1 mRNA was present in Barrett’s metaplasia, although no immunohistochemistry was performed to confirm localisation within the mucosal layer (Hughes et al., 1999). The authors proposed that the presence of this isoform amongst a plethora of other CYP isoforms may be a risk factor for carcinogenesis. In summary, this present study has shown that spectral imaging analysis can be used to quantify the amount of stain (i.e. CYP1B1 staining intensity) in formalin fixed paraffin embedded tissue, giving rise to the prospect of individualised therapy based on CYP1B1 expression.

The aryl hydrocarbon agonist pathway (see Chapter 1, section 1.2.2), is involved in the constitutive and inducible expression of CYP1 family isoforms. It is
therefore important to consider that the over-expression of CYP1B1 in tissues such as the squamous mucosa, may be due in part to the inducibility of the enzyme by compounds in cigarette smoke. However, as the tissue progresses towards forming an invasive cancer, the impact of these chemicals will be less, as the tissue is distal from the exposure site, and expression could be influenced by tumour micro-environmental factors.

A recent study by Bandiera and colleagues (Bandiera et al., 2004) investigated the role of the proteasome in the degradation of CYP1B1. It was suggested that CYP1B1 protein over-expression within solid tumours could be due to non-functional proteasomal pathways in cancer tissue. This pathway is known to be active and functional in normal tissues and could explain why the protein is never detected in normal tissues, whilst the mRNA is detected. This has been shown in many studies looking at normal tissue CYP1B1 mRNA (Baron et al., 1998; Rieder et al., 1998; Cheung et al., 1999; Piipari et al., 2000; Finnström et al., 2001; Spivack et al., 2001; Chang et al., 2003b; Bertrand-Thiebault et al., 2004). Various studies in the literature have shown CYP1B1 mRNA expression in cells involved in immunological responses, including monocytes and macrophages (Baron et al., 1998), mononuclear cells (Dassi et al., 1998; Tuominen et al., 2003), and lymphocytes (Spencer et al., 1999). The mRNA expression seen in many tissues (section 1.3.2), including those that are cancerous, may not be important as ultimately it is the protein and the metabolic activity of that protein that will determine the outcome of therapy.

Allelic variants in CYP isoforms is an area of growing importance in cancer biochemistry and cancer risk (Li et al., 2000; Tanaka et al., 2002; Chang et al., 2003a; Bandiera et al., 2004) as this could govern the rate of turnover of carcinogens, especially with respect to HNSCC. Changes in amino acid composition can result in increased activities of these enzymes. In a previous study, HNSCC susceptibility was analysed by looking at an array of polymorphisms (Ko et al., 2001). The authors showed the CYP1B1*3 codon 432 polymorphism was a susceptibility factor in smoking related HNSCC. Polymorphisms in this gene have been examined and have been shown to play a role in renal cell carcinoma (Sasaki et al., 2004), endometrial cancer (Sasaki et al., 2003) and prostate cancer (Chang et al., 2003a). However, the present work does not investigate CYP1B1 polymorphisms
and would be a priority for future work to ascertain the functionality of the CYP1B1 protein. Mutated forms of genes and allelic variants which are considered as risk factors of disease, (Thier et al., 2002) could be detected using PCR based analysis.

The regulation of CYP1B1 is not fully understood and there have been studies to show that oxidative stress (Barouki & Morel 2001) and hypoxia (Fradette et al., 2002; Fradette & Du Souich 2004) can play a role in the regulation of P450s. However, in the case of this study hypoxia appears not to play a role in protein expression, as CYP1B1 was expressed adjacent to well oxygenated tissues (i.e. bordering blood vessels). Further investigation is needed using vascular markers (CD31) in combination with double staining protocols for CYP1B1 and pimonidazole (which is an intrinsic hypoxia marker (Raleigh et al., 2001)).

CYP450 therapy strategies have included exploiting isoforms that are present and abundant in other organs in the body, such as the case of CYP3A4 with the prodrugs cyclophosphamide and iphosphamide (Roy et al., 1999). However, CYP3A4 is expressed in the liver and this strategy may result in toxicity in other tissues as well as the desired tumour tissue. Gene therapy using CYPs have been attempted (Patterson et al., 1999; Waxman et al., 1999; Baldwin et al., 2003; McFadyen et al., 2004a) however, such strategies lack the specificity that is necessary for tumour cell toxicity. Avoidance of activation in non tumour tissue is desired, but certain CYP isoforms used in this approach are expressed in other tissues, implying that non-tumoural metabolism is very likely to occur.

The metabolic capabilities of CYP1B1, including the metabolism of oestradiol, suggests that this enzyme is important in hormone dependent tissues (Spink et al., 1998); CYP1B1 protein expression may be a contributing factor in cancer in tissues of this nature. As head and neck squamous mucosa is in contact with many carcinogens present in cigarette smoke, this has been implicated as a risk factor in disease progression (Slaughter et al., 1953; Stewart & Kleihues 2003).

This study has explored CYP1B1 in a clinical tumour (HNSCC) and has shown that this protein is expressed at low levels in selected anatomically related normal tissues. Future work would seek to ascertain functional activity and allelic variants and establish the presence of CYP reductase in clinical samples. In the initial validation stages, CYP1B1 is a potential target for cancer therapy, as it could provide intra-tumoural activation of CYP1B1 activated prodrugs.
Chapter 4

Constitutive and inducible cytochrome P450 1B1 protein expression in a primary human tumour xenograft model
4.1 Introduction

CYP1B1 has been identified as a tumour related P450 with the possibility of exploitation in targeted cancer therapy (Murray et al., 1997; Patterson & Murray 2002). Chapter 3 showed that CYP1B1 protein is over-expressed in head and neck squamous cell carcinoma and targeting this enzyme with cancer therapeutics is achievable. Additionally, the presence of CYP1B1 protein in hyperplasia and dysplasia allows for targeting of pre-malignant tissues. There is no literature present for CYP1B1 expression in a human tumour xenograft, therefore, the necessity to find an *in vivo* tumour model expressing this CYP isoform is imperative for testing CYP1B1-activated prodrugs for cancer therapy.

Prior to the identification of CYP1B1 in 1994 (Sutter et al., 1994), it was demonstrated that both CYP1A family members, CYP1A1 and CYP1A2, were present in breast and colon xenografts, when treated with CYP inducers, including, 3-methylcholanthrene and β-naphthaflavone (Smith et al., 1993). Since then no studies have investigated CYP1 protein expression in human tumour xenografts with the view of using them for evaluating prodrug efficacy *in vivo*. Other studies have investigated the expression of CYP1B1 protein in explanted cells from humans, but this has mainly centred around toxicological investigations into the effect of the environmental pollutant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and the identification of this protein as a potential biomarker of exposure (Spencer et al., 1999; Baccarelli et al., 2004; van Duursen et al., 2005).

The classical TCDD induction pathway by the Aryl hydrocarbon (Ah) receptor (Mandal 2005), with regards to CYP1A1 mRNA and protein expression, has been extensively reported for cells (Moore et al., 1993; Nebert et al., 1993; Xu et al., 2000), lymphoid tissues (Germolec et al., 1996), brain and liver (Huang et al., 2003). However, effects of TCDD on CYP1B1 gene and protein expression, *in vitro* and *in vivo*, have not been studied extensively (Walker et al., 1999; Hukkanen et al., 2000). CYP1A1 was induced in the livers of treated animals with different doses of TCDD *in vitro* (Walker et al., 1999). Treatment of tumour bearing mice with TCDD and subsequent analysis of CYP1 protein expression in tumours has not been extensively studied, although other known CYP1-inducers such as β-naphthoflavone, phenobarbital, dexamethasone, and 1,4 bis 2-(3,5dichloro-
pyridloxybenxexe) have been reported in the literature (Smith et al., 1993). Additionally, Phortress™ is known to induce CYP1A1 activity in human mammary carcinoma xenografts (Fichtner et al., 2004). Dose response relationships which investigate CYP1A1 and CYP1B1 mRNA have also been addressed in liver tissues from mice treated with TCDD (Abel et al., 1996). The ED50 values for CYP1A1 and CYP1B1 mRNA induction was 1.3 and 0.08 μg/kg TCDD, respectively, in C57BL/6J mice, but the dose was higher in DBA/2J mice (3.4 and 1.5 μg/kg).

It is important to identify a human tumour xenograft which expresses the target enzyme as the presence of the enzyme which is responsible for the activation of a prodrug, is essential for in vivo prodrug evaluation. Two breast cancer cell lines were considered as candidates for human tumour xenografts; one of which has been reported to show constitutive CYP1B1 protein expression (MDA-MB-468, personal communication with Prof. G. A. Potter) and the other, MCF-7, has been grown successfully in vivo in other studies (Kelland et al., 2001; Varvaresou et al., 2004; Chhipa et al., 2005). Based on the clinical results in chapter 3, where CYP1B1 protein was expressed in head and neck cancer, a primary head and neck cell line (UT-SCC-14) was selected for evaluation as a xenograft model.

Hypoxia and CYP responsive genes both rely upon the aryl hydrocarbon nuclear translocator protein (ARNT), also termed HIF-1β (Nie et al., 2001) (described in detail in chapter 1). Preliminary studies were planned to investigate if hypoxia per se could have an effect on CYP1B1 protein expression, where the aim was to establish a possible role for hypoxia on the regulation of constitutive and inducible expression of CYP1B1 in vitro.

4.1.1 Expression of CYP1B1 in breast cancer cells in response to TCDD

The basis of these initial cell studies was to examine CYP1B1 protein levels ± TCDD to give insight into the phenotypic expression in the cells that were anticipated to be used for human tumour xenograft models. The literature surrounding CYP1B1 protein levels in tumour cells grown as xenografts is nonexistent and a better understanding of CYP1B1 protein levels in vivo could serve to
provide a human tumour xenograft model for testing CYP1B1 activated therapeutics.

There have been studies reported in the literature which have looked at the effects of TCDD treatment on CYP1B1 mRNA levels in breast cancer cells, *in vitro* (Dohr *et al.*, 1995; Spink *et al.*, 1998). These reported studies have served as a basis to investigate other factors and compounds that may contribute to the constitutive and inducible protein and/or mRNA expression levels of CYP1B1 in breast, liver and lung cancer cells (Hukkanen *et al.*, 2000; Chun *et al.*, 2001a; Guo *et al.*, 2001). CYP1A1 and CYP1B1 mRNA levels in the breast cancer cell lines, MDA-MB-157, MDA-MB-231 and MDA-MB-436 cells have been reported (Dohr *et al.*, 1995; Spink *et al.*, 1998; Angus *et al.*, 1999), where induction of CYP1B1 mRNA was observed in response to TCDD (1-100 nM). CYP1B1 enzyme activity (by measuring oestradiol hydroxylation at the 4 position) was also seen to increase in response to treatment with 10 nM TCDD (Spink *et al.*, 1998). However, protein levels (by immunoblotting) in the MDA-MB-468 breast cancer cell line have not been investigated to date. The MCF-7 breast cancer cell line has been well characterised in terms of CYP1B1 mRNA expression and its activity with oestradiol (a CYP1B1 substrate which yields 4-hydroxyoestradiol upon metabolism by CYP1B1) (Spink *et al.*, 1994). One particular study identified that MCF-7 xenografts were oestradiol dependent (Gierthy *et al.*, 1993), which would be considered if these cells were to be grown as human tumour xenografts. Induction studies on MCF-7 cells have revealed that TCDD can induce the CYP1B1 protein (identified in microsomal samples of treated cells) with matched increased oestradiol activity profiles (Spink *et al.*, 1994). However the time scales of induction have not been investigated which is necessary for phenotypic analysis in response to TCDD.

### 4.2 Aims and objectives

Various carcinoma cell lines were proposed as CYP1B1 human tumour xenografts, however further validation of these cell lines as tumour models was vital. Therefore, this chapter aims to:
1. Investigate CYP1 (primarily CYP1A1 and CYP1B1) protein expression (±
   TCDD) in selected breast tumour cell lines and a head and neck cell line in vivo
   and their corresponding human tumour xenografts.
2. Quantify CYP1B1 protein expression using spectral imaging microscopy in
   these selected human tumour xenografts.
3. Identify a suitable CYP1B1 expressing human tumour xenograft for the
   evaluation of prodrug efficacy in vivo.

4.3 Results

4.3.1 Expression of CYP1A1 and CYP1B1 isoforms in breast carcinoma cells

   As described in section 4.1.1, many studies have investigated expression of
   the CYP1 isoforms in cells of breast cancer origin. Immunoblotting and
   immunohistochemistry were employed in this study to look at the effects of a known
   CYP1 enzyme inducer in vitro and in vivo on CYP1B1 protein expression.

4.3.1.1 CYP1A1 and CYP1B1 protein expression: effect of TCDD on MDA-
   MB-468 cells

   The effect of TCDD on the oestrogen receptor negative breast carcinoma cell
   line, MDA-MB-468, was investigated using western blot analysis with a polyclonal
   antibody for CYP1A1 and polyclonal antibody for CYP1B1. TCDD (10 nM) was
   incubated with the cells for different time intervals after which, cells were lysed and
   protein levels were measured and western blotting performed (see chapter 2). Protein levels of the two CYP1 isoforms (CYP1A1 and CYP1B1) analysed in this
   experiment were detected by western blotting (Figure 4.1). The MDA-MB-468 cells
   showed constitutive expression of both CYP1A1 and CYP1B1, which was inducible
   following treatment with TCDD. Both isoforms gave immunodetectable bands at 52
   kDa for CYP1B1 and 58 kDa for CYP1A1. However, the polyclonal antibody has
   been stated to recognise a 60 kDa band in western blotting (product information
   from Gentest™). Previous reports have confirmed that a 52 kDa band is detected
   with the monoclonal antibody (McFadyen et al., 1999). Induction of CYP1A1 and
CYP1B1 protein was measured between 4-72 h post treatment with TCDD (10 nM). Induction of both isoforms occurred 4 h post TCDD (10 nM) treatment. However, CYP1A1 (Figure 4.1, panel A) was induced to higher levels than CYP1B1 (Figure 4.1, panel B), over time, and increased up to 72 h, a pattern which was not mirrored by CYP1B1. Expression of CYP1B1 (panel B) peaked at 18 h and protein levels remained elevated up to 72 h.

![Figure 4.1](image)

Figure 4.1 Western blot analysis for CYP1A1 (panel A) and CYP1B1 (panel B) protein in MDA-MB-468 cells using polyclonal antibodies. Cells were lysed at various time points after the addition of 10 nM TCDD. Supersomal™ CYP1A1 and CYP1B1 (0.5 µg) were used as controls. The blot was run, stripped and re-probed for the different proteins. Actin (panel C) is shown to illustrate even protein loading.

4.3.1.2 Growth of MDA-MB-468 cells as a human tumour xenograft

As the MDA-MB-468 cells showed both constitutive and inducible CYP1B1 expression in vitro, growth of these cells in mice as human tumour xenografts was assessed for testing CYP1B1 activated prodrugs. However, it was clear from preliminary studies that these cells grew very slowly in mice with a long latency period of up to 15 days (Figure 4.2) and once tumours were established in vivo, > 80
% necrosis was observed in the tumour mass (Figure 4.2, panel B). Figure 4.2, panel A, illustrates the growth characteristics of the MDA-MB-468 cells grown as human tumour xenografts (data supplied by Free Radicals in Cancer Research Group and F. Hylands, Tumour Microcirculation Group).

**Figure 4.2** Growth curve for the MDA-MB-468 xenograft tested at the GC1. Cells were implanted subcutaneously ($2 \times 10^6$ cells in 0.05 ml) The MDA-MB-468 tumour took over 30 days to reach a suitable treatment volume (panel A). Panel B shows H & E stained tumour and constitutive expression of CYP1B1 in viable tumour tissue (panel C) of the same tumour. Widespread necrosis (n) can be seen in this tumour adjacent to areas of viable tumour cells (v). TCDD experiments were not carried out as the cells constitutively expressed CYP1B1 (panel C). The inset in panel C shows a high magnification ($\times 400$) picture of CYP1B1 staining the viable tumour cells in the tumour represented in panel C. Scale bar 100 μm.
These data show that the MDA-MB-468 takes two weeks before the tumour becomes palpable, ultimately taking 45 days to reach a volume of 200 mm$^3$. Immunohistochemistry shows that CYP1B1 protein levels in the MDA-MB-468 tumours were constitutively high without the intervention of TCDD treatment for induction of the desired protein (Figure 4.2, panel C). Necrotic areas do not stain for CYP1B1 whilst areas of viable CYP1B1 expressing tumour cells (brown stain) can be seen around the edge. Localisation of the CYP1B1 protein is cytoplasmic (Figure 4.2, insert, panel C).

In summary these cells and human tumour xenografts were not analysed further due to poor in vivo growth characteristics, despite exhibiting constitutive CYP1B1 expression in vivo.

4.3.1.3 CYP1 protein expression: effect of TCDD on MCF-7 breast cancer cells

The MCF-7 breast carcinoma cell line was evaluated when it was established that the MDA-MB-468 cells displayed poor growth characteristics in vivo, despite displaying constitutive CYP1B1 protein expression. Western blotting was performed on MCF-7 cells treated with TCDD (10 nM) and analysed at various times after treatment. Blots were probed with CYP1A1 and CYP1B1 polyclonal antibodies (see chapter 2). A polyclonal antibody was used for analysis of CYP1B1 in vitro and was used in conjunction with the monoclonal antibody for in vivo CYP1B1 analysis. Figure 4.3 illustrates CYP1A1 levels over time with treatment of TCDD (10 nM).
CYP1A1 protein displayed very low constitutive levels (time 0 h), in the MCF-7 tumour cell line. However within 4 h CYP1A1 protein had been induced which increased over time, up to 72 h. Levels of CYP1A1 induction was lower than that displayed in the MDA-MB-468 cells on a µg protein basis.

As described in section 4.1, hypoxia may play a role in expression of CYP1A1/CYP1B1, therefore experiments were carried out in hypoxia (0.2 %) ± TCDD, to investigate if this had any effect on CYP1B1 protein expression. These samples have been shown next to the air ± hypoxia samples. Figure 4.4 shows CYP1B1 protein levels in MCF-7 cells over time (up to 72 h). CYP1B1 protein was expressed constitutively and elevated levels of protein can be seen after 4 h treatment with 10 nM TCDD. CYP1B1 protein peaked at 20 h after treatment with TCDD (10 nM). There are no obvious differences between corresponding samples for air untreated versus hypoxia untreated and air treated versus hypoxia treated cells over time. Expression of CYP1B1 protein also decreases after 20 h TCDD treatment in air or hypoxia. The protein loading levels were confirmed with actin staining. These data imply that hypoxia does not regulate either the constitutive or inducible expression of CYP1B1 protein, at least in vitro.
Figure 4.4 Western blot analysis for CYP1B1 protein in MCF-7 cells. Cells were cultured ± hypoxia (0.2 % O₂), ± TCDD (10 nM), hypoxia plus TCDD. Times are shown (h) with the culture conditions (air (A) or hypoxia (H)). CYP1B1 supersomes™ (0.5 μg) were used as controls (C).
4.3.1.4 CYP1 protein expression: effect of TCDD on MCF-7 breast tumour xenografts

It was established that CYP1B1 was expressed constitutively in the MCF-7 tumour cell line, therefore these tumour cells were implanted into mice to be grown as xenografts. Figure 4.5 shows that control tumours expressed CYP1B1 when detected with the polyclonal antibody (Figure 4.5, panel A) and monoclonal antibody (Figure 4.5, panel B). Clearly there are sizes of protein detected using these antibodies (60 and 52 kDa) and Figure 4.5, panel A shows that the only immunodetectable band is at 60 kDa, which does not correspond with the in vitro data (Figure 4.4). This is addressed in the discussion. There was no apparent inducibility of the 60 kDa protein (using the polyclonal antibody, Figure 4.5, panel A), however when using the monoclonal antibody CYP1B1 levels were elevated with TCDD (52 kDa), 48 h after TCDD treatment and diminished post 48 h. The actin shows that the sample did not run well in the control lane and therefore interpretation of constitutive CYP1B1 is difficult due to the condensed band. Protein levels of CYP1A1 are also elevated in response to TCDD treatment (Figure 4.5, panel C) and this peaks at 24 h after TCDD treatment and decreases at 48 h with expression not detected after 96 h.

4.3.2 Expression of CYP1 isoforms in primary head and neck squamous cell carcinoma cells

The identification of CYP1B1 in head and neck cancer described in Chapter 3 has lead this study to investigate CYP1B1 protein expression in a primary head and neck tumour cell line (UT-SCC-14), with the view of growing these cells in vivo as xenografts for testing CYP1B1 activated prodrugs.
**Figure 4.5** MCF-7 cell xenografts treated with 50 µg/kg TCDD and stained for CYP1B1, CYP1A1 and actin at various times after TCDD treatment. CYP1B1 (52 kDa) and 1A1 (58 kDa) supersomes™ controls are shown (0.5 µg). Panel A shows CYP1B1 staining of xenografts with the polyclonal antibody, panel B shows CYP1B1 using a monoclonal antibody, panel C shows the CYP1A1 polyclonal antibody and even loading is demonstrated with actin stain on panel D.

### 4.3.2.1 CYP1 protein expression: effect of TCDD on UT-SCC-14 cells

Preliminary studies, *in vitro*, investigated expression of the CYP1 isoforms ± TCDD in the UT-SCC-14 cell line (Figure 4.6). Western blotting analysis showed that CYP1A1 was constitutively expressed, although at a lower level than breast cancer cells (MCF-7 and MDA-MB-468, Figure 4.3 and Figure 4.1, respectively) and very slightly induced by TCDD. CYP1B1 was not constitutively expressed, nor was it induced by TCDD (10 nM, 21 h incubation). Even loading of samples was demonstrated with actin.

<table>
<thead>
<tr>
<th>CYP controls</th>
<th>Time (h) post TCDD (50 µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1B1 1A1</td>
<td>C 24 48 96</td>
</tr>
</tbody>
</table>

- CYP1B1 (52 kDa) polyclonal
- CYP1B1 (52 kDa) monoclonal
- CYP1A1 (58 kDa)

[Image of Western blot showing protein expression over time]
Figure 4.6 Western blot analysis to illustrate CYP1A1, CYP1B1 and actin in UT-SCC-14 cells. Sample lanes show cells cultured in air (A) and hypoxia (H), ± TCDD (10 nM, 21 h). Panel A shows CYP1A1 protein using a polyclonal antibody, panel B shows CYP1B1 protein using the polyclonal antibody and panel C uses the monoclonal actin antibody.

4.3.2.2 Growth of UT-SCC-14 cells as a xenograft

Growth of the UT-SCC-14 cells were examined in vivo and within two weeks from implantation, the cells had grown to form xenografts that were 200 mm³ in size (Figure 4.7) and displayed no necrosis (data supplied by V. M. McErlane and F. Hylands). This is in contrast with the MDA-MB-468 tumour, which showed massive necrosis (Figure 4.2, panel B) and took 3 times longer to reach the same volume. Therefore the fast growing characteristics of this tumour were favourable for using it as an in vivo model for drug evaluation. CYP1B1 staining also showed that this protein can be induced and is localised to the cell cytoplasm (Figure 4.7, insert panel C).
Figure 4.7 Growth curves for the UT-SCC-14 xenograft tested at the GCI (panel A). H & E stained tumour in panel B and inducible expression of CYP1B1 is shown in panel C of the same tumour (48 h post 50 μg/kg). Insert shows cellular localisation of CYP1B1 at × 400 magnification. Scale bar 100 μm

4.3.3 Western blotting for CYP1 in HNSCC xenografts

From initial in vitro studies, the UT-SCC-14 cell line expressed CYP1A1 protein upon treatment with TCDD, whilst CYP1B1 protein was not induced (in conditions of hypoxia (0.2 % O₂) or air), as shown in Figure 4.6. When these cells were implanted into mice and allowed to grow as xenografts, it was observed that the UT-SCC-14 tumour is fast growing (Figure 4.7) compared with the MDA-MB-468 tumour (Figure 4.2), reaching a volume of 200 mm³ (12 days compared with 45
days). It was imperative that CYP1B1 protein was investigated in this cell line in vivo to establish if protein expression changed (i.e. in vivo compared to in vitro). This was achieved not only by immunohistochemistry but also by western blotting analysis of tumour lysates using selective monoclonal and polyclonal antibodies towards CYP1A1 and CYP1B1.

4.3.3.1 Western blotting to detect CYP1B1 in UT-SCC-14 xenografts

Constitutive and inducible CYP1B1 protein expression was investigated in the UT-SCC-14 xenograft. These data can be seen in Figure 4.8. At each dose and within the control tumour material it was apparent that a band was detected at approximately 60 kDa. The protein corresponding to this size did appear to decrease after 4 hours with DMSO, but this did not happen at the 96 h time point, and levels were comparable with the control, 4 h tumour. However the protein at 52 kDa, which corresponds to CYP1B1 of the control was not detected in the control tumours, and was not affected by the vehicle alone (Figure 4.8, panel A). After 5 μg/kg TCDD (Figure 4.8, panel B), there were a couple of bands detected in all samples over time. The 60 kDa band that was detectable in the control samples was also detected in this treatment group, and appeared to increase up to 96 h in a time dependent manner. This 60 kDa CYP1B1 protein was constitutively expressed at time zero and showed little induction after 4 h treatment of 5 μg/kg TCDD, after 24 h CYP1B1 was present but not induced much, compared with controls. Levels of the 52 kDa protein were induced after treatment with 5 μg/kg TCDD up to 72 h. CYP1B1 protein expression measured at 52 kDa decreased slightly over time after 72 h and was notably lower at 96 h.
**Figure 4.8** Western blot analysis for CYP1B1 protein, using a monoclonal antibody in the UT-SCC-14 xenograft. Tumours were excised at different times after different doses of TCDD. Panel A shows CYP1B1 protein from tumours of control animals from 5 μg/kg treated mice (B), from 15 μg/kg treated mice (C), and 50 μg/kg treated mice (D). Even loading of protein was confirmed with ponceau stain, (data not shown)
CYP1B1 was detected at 15 µg/kg of TCDD (Figure 4.8, panel C). The 60 kDa band was detected in all samples at this dose over time and appeared to remain at the same level over time. The 52 kDa band did not appear to be induced 4 h after TCDD but was present after 24 h TCDD treatment. There were no notable changes in protein levels at 48 and 72 h compared to the level of protein at 24 h.

Treatment of mice with 50 µg/kg TCDD resulted in CYP1B1 protein expression at 60 kDa in the xenografts, which remained at the same level over time (Figure 4.8, panel D). This protein did appear to be expressed to the same level as the control tumour, over time at this dose of TCDD. However levels of the 52 kDa protein were induced after 4 h which was not evident with other doses. A time course induction profile was evident although CYP1B1 expression (detected at 52 kDa) remained at similar levels at 48 h to 96 h. These data show that CYP1B1 (52 kDa) is not constitutively expressed in the UT-SCC-14 tumour but is inducible with TCDD, to different levels, with different time course profiles, using different doses of the inducer.

The two different CYP1B1 antibodies (monoclonal and polyclonal) used in this study detected different protein residues on the CYP1B1 protein. Therefore it was important to characterise the protein profiles in the TCDD treated xenografts using both antibodies. The 60 kDa CYP1B1 band was the main protein detected in the xenografts using the polyclonal antibody (Figure 4.9). Western blotting using the polyclonal antibody for CYP1A1 can be seen in Figure 4.10. Levels of CYP1B1 protein at 60 kDa did not appear to change over time or with different doses of TCDD. A faint band above 60 kDa was detected which was present both in the treated mice and control tumours. This band was not the same in all of the samples and did not appear to follow any pattern in expression over time or at different doses. These data indicate, using this antibody that a 60 kDa immunoreactive protein is not inducible with TCDD at 5, 15 or 15 µg/kg and is constitutively expressed in the UT-SCC-14 tumour. However, a 52 kDa band is induced over time at higher doses of TCDD when using the monoclonal antibody (Figure 4.8).
Figure 4.9 Western blot analysis for CYP1B1 protein, using a polyclonal antibody in the UT-SCC-14 xenograft. Tumours were excised at different times after different doses of TCDD. Even loading of protein was confirmed with ponceau stain, (data not shown).

4.3.3.2 Western blotting to detect CYP1A1 in UT-SCC-14 xenografts

CYP1A1 protein expression was examined in the UT-SCC-14 tumours using stripped and re-probed blots from the CYP1B1 blots (Figure 4.10). The polyclonal antibody used in this study showed induction of CYP1A1 protein at doses of 15 and 50 μg/kg only. CYP1A1, in control tissue, did not exhibit constitutive protein expression and was not affected by DMSO (vehicle). CYP1A1 was also not expressed at any time point during treatment of 5 μg/kg TCDD. When animals were treated with 15 μg/kg TCDD CYP1A1 protein was seen only after 48 h and 72 h but not at 96 h. After 50 μg/kg TCDD CYP1A1 protein was induced after 24 h, 48 h and
appeared to peak after 72 h. CYP1A1 protein levels were diminished after 96 h. These data suggest that CYP1A1 protein is expressed at higher doses of TCDD \textit{in vivo} in comparison to CYP1B1.

![Figure 4.10](image)

**Table 4.1.1** Western blot analysis for CYP1A1 protein, using a polyclonal antibody in the UT-SCC-14 xenograft. Tumours were excised at different times after different doses of TCDD. Supersomal™ controls are shown, these samples were from the same blots but other CYP isoform controls were excluded from the blot.

4.3.3.3 CYP1 protein expression: effect of TCDD on UT-SCC-14 xenografts

The UT-SCC-14 xenograft grew more efficiently than the MDA-MB-468 tumours as there were no areas of necrosis and viable tumour cells occupied the majority of the tumour. When analysing clinical tumours, the supporting architecture is evident. However in UT-SCC-14 xenografts grown in mice, the architectural organisation is not clear, the tumour mass is the main component of the xenograft and supporting stroma occupies a small percentage of the tumour mass.

The UT-SCC-14 cells, once established as xenografts were analysed for expression of CYP1 proteins. CYP1B1 expression levels were analysed in mice treated with different doses (0, 5, 15 and 50 \(\mu g/kg\)) of TCDD, using immunohistochemistry with a selective monoclonal antibody. Visual analysis of staining intensity of CYP1B1 was performed as detailed in chapter 2 (Table 4.1).
For this experiment 2 tumours per treatment group were analysed. Staining of xenografts was also analysed using spectral imaging microscopy. Time course induction of CYP1B1 protein with TCDD has not been investigated in tumour xenografts, (dose response with CYP1A1 mRNA has been investigated previously (Abel et al., 1996)). Therefore CYP1B1 protein was analysed at different doses and different times after administration of TCDD.

Constitutive expression in the UT-SCC-14 tumour can be seen in Figure 4.11 panels A-D. Tumours from mice treated with TCDD are shown in Figure 4.12 - Figure 4.14. The CYP1B1 protein was not expressed in the stroma of the xenograft as indicated in panels A and D of Figure 4.11. This is as expected as the stromal tissue is of mouse origin and is not related to the tumour tissue. Localisation of the protein can be seen in the cell cytoplasm (Figure 4.11 insert panel D). Tumours represented in Figure 4.11 (panels B and D) have been treated with vehicle (DMSO) alone, and tumours in panels A and C were controls. Treatment ± DMSO, as detected by immunohistochemistry, does not appear to alter CYP1B1 protein levels.

<table>
<thead>
<tr>
<th>Dose of TCDD (μg/kg)</th>
<th>Time after i.p. TCDD administration (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>50</td>
<td>1</td>
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</table>

Table 4.1 Visual analysis of staining intensity in the UT-SCC-14 human tumour xenografts. Scores denote: weak - 1, medium - 2 and strong - 3, CYP1B1 staining intensities, treated with different doses of TCDD at different times after administration of the Ah agonist (i.p.).
Figure 4.11 Human tumour xenografts (UT-SCC-14) from control animals stained for CYP1B1. Representative sections shown are from different time points ± vehicle (DMSO). Panel A was taken after 4 h, panel B after 4 h with DMSO, panel C after 96 h, panel D after 96 h with DMSO. Stromal tissue (s) is indicated in panel A. Insert panel D shows ×400 magnification with cytoplasmic localisation (>). Scale bar 100 μm.

Figure 4.12 shows the induction of CYP1B1 in the UT-SCC-14 xenograft by TCDD (5 μg/kg) compared to control tumours, however CYP1B1 protein expression returns to control levels by 96 h. CYP1B1 staining intensity at 48, 72 and 96 h were not visually different from the control tumours (Table 4.1). CYP1B1 protein was localised to the tumour cell cytoplasm in all time points at 5 μg/kg (Figure 4.12 insert panel A). Stromal tissue was negative for CYP1B1 and is a constant observation throughout all stromal tissue in tumours at this dose of TCDD.
Figure 4.12 Human tumour xenografts (UT-SCC-14), treated with 5 μg/kg TCDD (i.p) and stained for CYP1B1. Representative sections are from different time points. Panel A was taken after 4 h, B after 24 h, C after 48 h, D after 72 h, E after 96 h of TCDD treatment. Stromal tissue (s) is shown insert, panel A. Cytoplasmic localisation (>>) is shown in insert panel A (× 400 magnification). Scale bar 100 μm.

A TCDD dose of 15 μg/kg (Figure 4.13) shows that the intensity of CYP1B1 staining after 4 h of TCDD treatment is less than that at 5 μg/kg, which was confirmed by visual analysis (Table 4.1). Nevertheless CYP1B1 staining at this dose of TCDD clearly shows that the protein is over-expressed in a time dependent fashion (i.e. the staining gets stronger), with a slight decrease at 72 h, even though expression at 96 h is strong. CYP1B1 protein is localised to the cytoplasm of the
cells (Figure 4.13, insert, panel E). Stromal tissue is devoid of CYP1B1 protein and this is shown in Figure 4.13, insert panel A.

**Figure 4.13** Human tumour xenografts (UT-SCC-14), treated with 15 μg/kg TCDD (i.p.) and stained for CYP1B1. Representative sections shown are from different time points within the stated dose. Panel A was taken after 4 h, B after 24 h, C after 48 h, D after 72 h, E after 96 h of TCDD treatment. Stromal tissue (s) is shown in panels A, C and E. Cytoplasmic localisation (at × 400 magnification) is shown in insert panel E (►). Scale bar 100 μm. Stroma (S) is shown.

The maximum dose of TCDD administered to the UT-SCC-14 xenograft bearing mice was 50 μg/kg. The xenografts were more strongly stained for CYP1B1 in comparison to 5 and 15 μg/kg over time (Figure 4.14). After 4 and 24 h (Figure 4.14, panel A and B) there is no visual increase in CYP1B1 protein compared with controls as determined by brown 3,3-diaminobenzidine (DAB) stain (Table 4.1).
However, at 48 h the amount of CYP1B1 protein increases to the level (medium staining) shown after 96 h of 15 μg/kg TCDD.

**Figure 4.14** Human tumour xenografts (UT-SCC-14), treated with 50 μg/kg TCDD (i.p.) and stained for CYP1B1. Representative sections are from different time points. Panel A was taken after 4 h, B after 24 h, C after 48 h, D after 72 h, E after 96 h of TCDD treatment. Scale bar 100 μm. stroma (s) is shown in panel A. high magnification, × 400, is shown with cytoplasmic localisation (▲)

CYP1B1 staining after 50 μg/kg is greater at each time point than compared with 5 μg/kg. Visual analysis of staining intensity from 48 h is maintained 72 h and 96 h at a strong level after 50 μg/kg TCDD (Table 4.1). The protein is localised to the cytoplasm of the tumour cells (as shown in Figure 4.14, inset panel E). Stromal tissue was negative for CYP1B1 and this is shown in panel A and E. Upon visual
analysis it is clear that there is a difference between the 3 doses and also differences within each dose at different times after administration of TCDD.

4.3.4 Spectral imaging to quantify CYP1B1 protein in the UT-SCC-14 xenografts

Chapter 3 demonstrated that spectral imaging is a useful tool for the quantification of staining intensity of CYP1B1 in immunohistochemical sections. This method has been employed for the measurement of CYP1B1 staining intensity in human tumour xenografts. As with the clinical tumours, three images were captured per tumour (this gave the data more statistical strength). Spectral imaging for CYP1B1 staining was carried out and these data are presented in Figure 4.15, with the statistical differences of treated tumours at different times compared to control tissue shown in Table 4.2. The controlled mean normalised absorbance is shown by the line at 0.18. The patterns of CYP1B1 expression shown in Figure 4.15 mirror that seen from visual analysis in Figure 4.11 - Figure 4.14.

4.3.4.1 Constitutive and inducible protein expression in human tumour xenografts post TCDD.

There is a decrease in CYP1B1 expression in the 5μg/kg group over time after administration of TCDD. This corresponds well with the immunohistochemical data in Figure 4.12. The spectral imaging scores can be seen to decrease at 96 h to absorbances comparable with the control tumours. The highest mean normalised absorbance within this dose was after 4 h of administration (0.21 ± 0.02), and then at 24 h (0.20 ± 0.03) with the lowest after 72 h (0.18 ± 0.01).

A TCDD dose of 15 μg/kg resulted in an increase of CYP1B1 expression over time with a decrease at 72 h (again a pattern which was mirrored in the immunohistochemical data in Figure 4.13). Expression of CYP1B1 protein at 96 h was the greatest of all of the tumours examined (0.33 ± 0.07), which was nearly a 2 fold induction, compared to controls). Mean normalised absorbance after 4 h of TCDD administration was lower than that of the other 2 dose groups (0.18 ± 0.02) but did increase over time, with a slight decrease in absorbance at 72 h (0.19 ± 0.01).
The 50 μg/kg dose illustrated that there was an obvious induction of CYP1B1 protein over time (4 – 96 h) which was sustained up to 96 h after TCDD administration (1.4 fold induction compared to controls at 96 h). Initially after 4 h of TCDD the mean normalised absorbance was greater than that of control tumours (0.19 ± 0.01), and rose to 0.21 ± 0.01 after 24 h. Mean normalised absorbance does plateau at 48, 72 and 96 h after initial TCDD administration (with absorbances of 0.25 ± 0.01, 0.25 ± 0.02 and 0.26 ± 0.02, respectively). More importantly, this trend in CYP1B1 expression quantified by spectral imaging does mirror the visual immunoreactivity towards CYP1B1 (Figure 4.14). Only samples from this dose displayed a time dependent increase in CYP1B1 protein staining.

In summary, these data show that CYP1B1 protein expression can be manipulated in vivo and the treatment of mice with TCDD can induce CYP1B1 protein in the xenografts. CYP1B1 is induced to different levels with different doses over time (4 – 96 h). Quantification of CYP1B1 staining intensity from immunohistochemistry can also be quantified using spectral imaging microscopy. This method of quantification provides data that is comparable to visual analysis of staining intensity as shown in Table 4.1.

### 4.3.4.2 Statistical analysis of spectral imaging data within TCDD treatment groups

Statistical comparisons shown in Figure 4.15 are made between the treated tumours and control tumours. However, statistical comparisons between CYP1B1 staining intensity (as determined by spectral imaging microscopy) at different time points, within treatment groups, are detailed in Table 4.2. These data show that, within the 5 μg/kg dose group, there is a statistical significance between CYP1B1 staining intensity in the tumour 4 h post TCDD with the tumours taken at 48, 72 and 96 h after administration of TCDD (p < 0.05). This is also true for CYP1B1 staining intensity in the tumour taken 24 h after TCDD compared with tumours taken 48 h and 72 h post TCDD administration (p < 0.05).

Statistical significance in the 15 μg/kg group revealed fewer statistically significant correlations. It was clear that there was no statistical difference between CYP1B1 staining in tumours taken 4, 24, 48 and 72 h post TCDD administration,
when compared with each other. However, the tumour taken 96 h post TCDD did show a statistical significance in CYP1B1 staining intensity compared with all other tumours, taken at different times after TCDD, within this treatment group (p < 0.001).

**Figure 4.15** Spectral imaging mean normalised absorbances for CYP1B1 with human tumour (UT-SCC-14) xenografts grown in mice. Administered doses and times after tumour excision are shown in the key. Results shown are the means (± SD) of 6 experiments with 2 groups of animals (3 animals per group). TCDD was administered in DMSO. Controls were minus DMSO unless shown otherwise in key. Mean control data is shown (...) Significance between controls are also shown (** * p < 0.005, ** p < 0.05)

Statistical analysis of tumours in the 50 μg/kg treatment group revealed that there were more statistical differences, compared with the other doses. However, there were no statistical significances in CYP1B1 staining intensity between tumours taken at 48, 72 and 96 h post TCDD, when compared with each other. Difference in CYP1B1 staining intensity 4 h post TCDD was not statistically significant to that in the tumour taken after 24 h; but tumours taken 48, 72 and 96 h post TCDD did show a difference in CYP1B1 stain when compared with the 4 h tumour (p < 0.05). There was a statistical significance between CYP1B1 staining
intensity in the tumour taken 24 h post TCDD when compared with staining in the
tumours taken at 48, 72 and 96 h post TCDD (p < 0.05).

These data are important as it helps ascertain at what dose of TCDD and at
what time after TCDD administration is the level of CYP1B1 staining significantly
different to controls.

Table 4.2 Statistical significance for spectral imaging data on the UT-SCC-14
human tumour xenografts. Mean normalised absorbances for CYP1B1 staining
intensity at stated times after TCDD administration were compared for each dose.
Means were compared using the student's t test. P values of < 0.05 (**) and < 0.005
(***) are indicated.

<table>
<thead>
<tr>
<th>Groups compared (times)</th>
<th>P value</th>
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</thead>
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<tr>
<td></td>
<td>5 µg/kg</td>
</tr>
<tr>
<td>4 / 24</td>
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</tr>
<tr>
<td>4 / 48</td>
<td>0.017***</td>
</tr>
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</tr>
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</tr>
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4.4 Discussion

In order to exploit CYP1B1 in targeted enzyme/prodrug therapy for cancer it
is important to identify a suitable human tumour xenograft model to evaluate
prodrug efficacy in vivo. Previously, the inability to identify suitable experimental
models that solely express CYP1B1 has proven a hindrance to prodrug evaluation in
vivo. The aim of this chapter was to investigate CYP1 protein expression in a range
of selected tumour cells and their corresponding human tumour xenograft models.
This investigation employed both western blotting and immunohistochemistry with
spectral imaging microscopy to quantify staining intensity, reflecting target enzyme protein expression.

This study has shown that CYP1B1 is expressed constitutively and is also inducible in response to TCDD in cells of breast cancer origin (MCF-7 and MDA-MB-468) in vitro over time. CYP1A1 is also expressed constitutively and is induced in response to TCDD in vitro. The same pattern is exhibited in vivo with the MCF-7 xenograft levels of CYP1A1 and CYP1B1 are induced over time (up to 48 h). The MDA-MB-468 cells were established in vivo but did not show good growth characteristics (> 80 % necrosis), with the added complication of exhibiting intratumoural necrosis. Therefore, an alternative human tumour xenograft model had to be sought. Growth of MCF-7 tumour cells in vivo requires exogenous oestrogen which is supplied as pellets (implanted subcutaneously). This has proved a hindrance to the study as complications arose due to toxicity of the oestrogen in female nude mice.

Chapter 3 showed CYP1B1 protein was expressed in clinical head and neck squamous cell carcinoma from clinical samples. Moreover, a pilot study showed constitutive expression in vivo in a HNSCC primary human xenograft. Therefore examination of a primary tumour cell line of head and neck origin (UT-SCC-14) was the next choice of cell line. These cells grown as human tumour xenografts showed good growth characteristics in vivo, however western blotting analysis did show that CYP1B1 protein was not constitutive or inducible in vitro. Conversely, CYP1A1 protein measured using western blotting did show constitutive expression in vitro, which was not inducible by treating the cells with 10 nM TCDD. The protein level of the CYP1 enzymes had to be characterised in vivo as tumour microenvironmental factors could alter protein expression. In vivo analysis of the UT-SCC-14 xenograft involved immunohistochemistry and showed CYP1B1 protein was highly inducible upon treatment of the mice with TCDD. This is the first experiment which showed induction of CYP1B1 in a primary human tumour cell xenograft model. However induction after 5 μg/kg was not as pronounced as that showed after 15 μg/kg. Induction was greater after 48 h of TCDD treatment in both the 15 and 50 μg/kg groups (1.1 fold and 1.38 fold, respectively) compared with controls. These data show that the expression of CYP1B1 can be manipulated in
vivo and provides a model for testing CYP1B1 activated prodrugs. In all cases CYP1B1 was localised to the cytoplasm of the tumour cells. This agrees with other studies which have recognised cytoplasmic localisation of the CYP1B1 protein (Murray et al., 1997; McFadyen et al., 1999; McFadyen et al., 2001b).

There have been few investigations into CYP1B1 in tumour models, whereas most of the studies in the literature have focussed upon CYP1B1 mRNA induction in the liver in response to TCDD treatment (Abel et al., 1996; Walker et al., 1999; Badawi et al., 2000). These studies revealed that CYP1B1 induction is not as marked as CYP1A1 induction and where protein levels were analysed in the livers of treated mice, CYP1B1 was only expressed at higher doses of TCDD (37 ng/kg/day). Interestingly, constitutive CYP1B1 protein expression was below detection level (or not expressed) in control animals (Walker et al., 1999). Levels of mRNA in treated animals were 40 fold higher than CYP1A1. Doses in this study were lower at 125 ng/kg/day, for the mRNA studies and 35.7 ng/kg/day for protein (30 week treatment in total) (Walker et al., 1999). Other studies have shown that CYP1A1 mRNA is more inducible in mouse livers after TCDD treatment compared to CYP1B1 in a dose dependent manner (Abel et al., 1996). Therefore a more thorough investigation is needed to ascertain if TCDD induces CYP1B1 protein in the liver, as there could be a risk of activation in the liver of prodrugs. A squamous cell carcinoma (SCC) xenograft model that constitutively expresses low levels of CYP1B1, but not CYP1A1 has been identified in this present study. We have also shown in this HNSCC human tumour xenograft that CYP1B1 protein can be induced in vivo on treatment with TCDD, with the induction of CYP1A1 protein only at higher doses of TCDD.

Spectral imaging microscopy was employed to quantify the CYP1B1 staining intensity in the xenograft samples. This method of analysis proves a low cost alternative to visual scoring analysis (Barber et al., 2003) and the results generated are comparable to visual scores. However, it gives a more accurate value for staining intensity, compared with visual analysis. In this study it is clear that control tumour tissue displayed lower levels of CYP1B1 stain which is reflected in the low CYP1B1 mean normalised absorbance score. The visual pattern of ‘brown’ CYP1B1 stain appeared to increase over time with increasing doses of TCDD. This is reflected with increased spectral imaging mean normalised absorbances.
In the 5 μg/kg group the CYP1B1 staining shows that protein expression is initially high at 4 to 48 h and then decreases up to 96 h. This pattern is reflected in the mean normalised absorbances for this group where staining in all cases is higher than that of control tissue. At 15 μg/kg the visual analysis of CYP1B1 stain reveals an increase in expression which is maintained up to 96 h. The tumour shown at 72 h does appear to have lower CYP1B1 stain than 48 h, which is reflected with the mean normalised absorbance from spectral imaging analysis. The data presented for the 50 μg/kg group has shown that expression rises above that of controls at 4 h after TCDD administration and the amount of CYP1B1 stain rises dramatically after the 24 h time point. From visual analysis CYP1B1 appears to remain at a similar level from 48 to 96 h after TCDD, which is confirmed with spectral imaging analysis.

There is however one point which has come to the fore in terms of interpretation of spectral imaging data. Figures from data analysis using this form of microscopy indicate that there is not much difference in mean normalised absorbance between visually weak and visually strong stained sections. This is illustrated with the xenograft data between control tumours, which are weak/negative (0.18) and tumours at 96 h after 50 μg/kg TCDD, which are strong (0.26). Clearly, a difference of 0.08 is not great considering the variation in visual staining intensity. This has to be taken into account in data analysis. In summary, these data have shown that CYP1B1 visual scores and spectral imaging correlate and this method of quantification represents a powerful, unbiased tool for data analysis and is generally more accurate.

The different antibodies used in this study have revealed some interesting trends in CYP1B1 protein expression in vitro and in vivo. Two different weights of CYP1B1 immunoreactive protein have been identified at 52 kDa and 60 kDa. Interestingly when examining the polyclonal antibody, in vitro (MCF-7 and MDA-MB-468 cells), a 52 kDa band is more prominent with the 60 kDa visible, but faint. The majority of the western blots from cells cultured in vitro, were performed using the polyclonal antibody. When using the CYP1B1 polyclonal antibody in vivo, the 52 kDa band is not detected in both the MCF-7 breast cancer xenograft and the UTSCC-14 head and neck tumour xenograft with the 60 kDa band alone being detected. This 60 kDa band does not appear to change in response to TCDD treatment in both
xenografts tested. As immunohistochemistry was performed using the monoclonal antibody (which was used for the HNSCC clinical samples) it was important to complement these data with western blots using the same antibody. Protein was detected at 60 kDa but the 52 kDa protein was also detected in the xenograft samples, which was not detected using the polyclonal antibody. The protein at 60 kDa was not induced; conversely the 52 kDa protein was induced over time and with different doses of TCDD. Therefore it would be reasonable to suggest that inducible CYP1B1 was detected with immunohistochemistry and also by western blotting. These data have also shown that CYP1A1 protein was inducible in the UT-SCC-14 xenograft but at higher doses of TCDD.

There are some discrepancies in the literature regarding the size of CYP1B1 protein detected by western blotting; a 52 kDa band has been identified previously in vitro (McFadyen et al., 1999; Spivack et al., 2001; Lin et al., 2003) as well as a 60 kDa band (McKay et al., 1995; Malaplate-Armand et al., 2003). This may be due to the use of different antibodies which will detect different sizes of CYP1B1 protein for example the Gentest™ antibody used in this study is stated to detect a 60.8 kDa band, whereas the CYP1B1 monoclonal antibody recognises a 52 kDa sized CYP1B1 protein. There may also be a possibility of post-translation modification occurring which alters both the size and charge of the protein (Mann & Jensen 2003), but further work would clarify if this is occurring (discussed in chapter 6). Previous studies have stated that post-translational modifications of the CYP1B1 protein could occur in cancer cells (Bandiera et al., 2004). This could explain the change in weight of the CYP1B1 protein in these studies from in vitro to in vivo (western blotting). It may also explain why CYP1B1 is expressed constitutively in cancer, as modifications are known to stabilise proteins, making them less susceptible to degredation (Mann & Jensen 2003).

When identifying levels of CYP1B1 protein, there were a number of methods employed which included immunohistochemistry, spectral imaging microscopy and western blotting. This study has shown that visual analysis coupled to spectral imaging microscopy proves to be a useful tool for staining quantification. Western blotting was useful when identifying the size of immunoreactive protein but does not give information on cellular localisation of the protein and automation of staining is more difficult than immunohistochemistry with the DAKO autostainer.
Clearly the induction profiles with TCDD vary for each method. Immunohistochemistry coupled with spectral imaging proves to be a useful method of data analysis, as cellular localisation of protein can be identified which is not achievable with western blotting.

In conclusion a CYP1B1 expressing xenograft has been identified using a primary cell line of HNSCC origin. Other cell lines (MCF-7 and MDA-MB-468) which constitutively express CYP1B1 were identified in this study, but the growth characteristics and/or practicalities of growing the cells as xenografts were not favourable. This was due to a number of factors including spontaneous tumour necrosis as well as slow growth of xenografts. CYP1B1 expression can be manipulated \textit{in vitro} and \textit{in vivo}, using the planar aromatic hydrocarbon, TCDD. This compound can induce CYP1B1 protein at lower TCDD concentrations (5 and 15 \text{\textmu}g/kg) as well as at high doses (50 \text{\textmu}g/kg) in the UT-SCC-14 human tumour xenograft. The CYP1A1 isoform was not induced at lower concentrations but a time dependent increase in protein was exhibited \textit{in vivo} at 50 \text{\textmu}g/kg. Spectral imaging microscopy continues to prove a useful tool for identification of proteins identified immunohistochemically and can be coupled to other, less reliable, less automated methods of protein identification.
Chapter 5

Identification of cytochrome P450 CYP1-activated prodrugs for nitric oxide
5.1 Introduction

5.1.1 Prodrugs for cancer therapy

A prodrug (Albert 1958) is a chemical which is non-toxic and biologically inert, until it is transformed by the target enzyme to the actively toxic drug (Connors 1986). Prodrugs rely upon the delivery of a high concentration of anticancer drug to target tumour cells, whilst reducing non-specific toxicity in normal tissues. Classical enzyme/prodrug therapy for cancer relies on the over-expression of the target enzyme in tumour tissue, which is also usually expressed to a lesser degree in normal tissues. Therefore, this approach may be limited by normal tissue toxicity associated with prodrug treatment. CYP-activated prodrugs used clinically include cyclophosphamide (which can be activated in the liver (Chang et al., 1993)), ifosfamide (Jounaidi et al., 1998), paracetamol (Thatcher et al., 2000) and 4-ipomeanol (Rainov et al., 1998).

There are a number of strategies for enhancing target enzyme or cytochrome P450 expression in tumours including gene directed enzyme prodrug therapy (GDEPT) (Greco & Dachs 2001), antibody directed enzyme prodrug therapy (ADEPT) (Denny 2001; Xu & McLeod 2001) and virus directed enzyme prodrug therapy (VDEPT) (Xu & McLeod 2001; Tychopoulos et al., 2005). The identification of CYP1B1 in tumour tissue (Murray et al., 1997; Carnell et al., 2004; Oyama et al., 2005) has implicated this CYP1 isoform as a target enzyme for prodrug therapy of cancer (Rooney et al., 2004). CYP1B1 has a superior expression profile to other cytochrome P450s, as this CYP isoform is constitutively expressed in cancer, and not in normal tissues (Murray et al., 1997). This will circumvent problems associated with complex targeting strategies that are encountered with GDEPT, ADEPT and VDEPT.

The multifaceted roles of nitric oxide (NO\(^{\bullet}\)), described in chapter 1, form the basis for a diverse range of cancer therapies, from modification of blood flow (Worthington et al., 2000), cytotoxicity due to cellular oxidative/nitrosative stress (Tozer & Everett 1997b; Soler et al., 2000) and hypoxic cell radiosensitisation (Gray et al., 1958; Griffin et al., 1996). NO\(^{\bullet}\) can be produced in cancer cells using iNOS.
suicide’ gene therapy (Xie et al., 1995; Soler et al., 2000; Kuroki 2004; Wang et al., 2004).

CYP1B1 homology modelling (based on primary sequence homology with CYP2C5) has been used to guide prodrug design. This homology model aids in the design of prodrugs that will specifically interact with substrate recognition sites in the active site of CYP1B1 (Lewis et al., 2003). Novel benzo- or pyrido-fused indole oxime nitric oxide-producing prodrugs have been synthesised, with the potential of enhancing oxidative/nitrosative stress and/or radiosensitisation in tumour cells which activate the compound. In vitro evaluation of these compounds is necessary to investigate structure-activity relationships (SAR), thereby improving on existing drug design. The purpose of this study is to rationally design stable prodrugs for nitric oxide targeted at CYP1-family enzymes in the first instance.

5.1.2 Factors controlling substrate specificity for cytochrome P450 1B1

Molecular modelling and experimental determinations can assist in a better understanding of SARs of potential prodrugs. The genetic sequence of the CYP1B1 gene has been determined (Tang et al., 1996); however its crystal structure remains to be elucidated (Lewis et al., 2003). A three dimensional model of the CYP1B1 enzyme was constructed based on primary sequence homology with the mammalian CYP2C5 isoform, whose crystal structure is known (Williams et al., 2000), and critical residues, which are important for substrate binding in the CYP1B1 active site, were determined, using oestradiol as a model substrate. Orientation of oestradiol in the putative CYP1B1 active site illustrated that critical residues were responsible for substrate/enzyme interaction. (It was important that the substrate was correctly orientated above the haem moiety for 4-hydroxylation). Residues involved in hydrogen bonding included Ser 122 and Thr 510, whereas Phe 231 was responsible for \( \pi-\pi \) stacking (Lewis et al., 2003). Therefore, modelling of this type has shown that the CYP1 family enzymes exhibit specificity for planar aromatic/heterocyclic substrates which facilitate \( \pi-\pi \) stacking and hydrogen bond interactions with amino acid site residues (Lewis et al., 2003). Additionally, it has been proposed that the overall planarity of the substrate may be used to modify prodrug selectivity for individual CYP isoforms. It was also determined that the
more tightly bound CYP1B1 substrates tend to possess high log $P$ values\(^2\). The precise lipophilic nature of the substrate will affect which CYP isoform conducts its metabolism. Hence, it was concluded that the higher the log $P$ value for a compound, the more avidly it will bind to the active site, which is as expected, but compounds with very high log $P$ values will not bind as well, due to increased hydrophobicity. Some well established CYP1B1 substrates have log $P_{expt}$ values of 3.86 (17β-oestradiol), 3.50 (bufuralol) and 6.35 (Benzo(a)pyrene), with an overall average log $P$ for substrates of CYP1B1 of 3.73 (Lewis et al., 2004).

These studies form the basis for understanding that Log $P$, hydrogen bonding and $\pi-\pi$ stacking are fundamental in the development of CYP1B1-activated prodrugs. The planar nature of the aforementioned compounds is characteristic of a CYP1 substrate when compared with other CYP isoforms (which can recognise acidic/basic compounds, in the case of CYP2C8 and CYP2B6, respectively (Lewis et al., 2004)).

5.1.3 Rational design of prodrugs for nitric oxide

A series of 10 potential prodrugs for nitric oxide have been synthesised by Dr Saraj Ulhaq (Senior Scientist, Cancer Research UK Free Radical Research Group) with a series of substituents (at the $R^1$, $R^6$ and $R^8$ positions) around a core benzo- or pyrido-fused indole core bearing a $\alpha$-hydroxyamino (NOH moiety) functional group as shown in Figure 5.1. In this preliminary study, a range of substituents will be used to vary the electronic properties, the hydrophobicity and planarity of these putative prodrugs for nitric oxide, in order to understand the structural features which control the efficiency of nitric oxide release by, and selectivity for members of the CYP1-family of P450 enzymes.

$R^1$ and $R^8$ substitutions were made to prevent competition with denitrification of the $N$-hydroxyamino functional group to release nitric oxide, (although GCI 503, 506 and 507 were the only compounds which did not have a $R^8$ substitution). All of the GCI 500 series prodrugs contained a methyl or in one case a trifluoromethyl substituent at $R^8$ in order to prevent aromatic hydroxylation at this position. Indole-3-carbaldehyde oximes have been shown to generate a radical-cation which

\(^2\) Log $P$ is a measure of lipophilicity of the compound and is determined by (octanol:water partition coefficient)
deprotonates from the $N^1$ position to generate an indolyl radical in competition with deprotonation of the NOH moiety to form an iminoxyl radical ($\textgreater C=\text{NO}^\bullet$). Therefore, deprotonation from $N^1$ is prevented by the inclusion of a methyl substituent at the $R^1$ position, although other solubilising and aromatic groups have been introduced at this position.

![Chemical structure](image)

<table>
<thead>
<tr>
<th>Prodrug</th>
<th>Substituent</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCI 500</td>
<td>$N$</td>
</tr>
<tr>
<td>GCI 502</td>
<td>$N$</td>
</tr>
<tr>
<td>GCI 503</td>
<td>$N$</td>
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<tr>
<td>GCI 504</td>
<td>$N$</td>
</tr>
<tr>
<td>GCI 505</td>
<td>$N$</td>
</tr>
<tr>
<td>GCI 506</td>
<td>$N$</td>
</tr>
<tr>
<td>GCI 507</td>
<td>$\text{CH}$</td>
</tr>
<tr>
<td>GCI 509</td>
<td>$N$</td>
</tr>
<tr>
<td>GCI 510</td>
<td>$N$</td>
</tr>
<tr>
<td>GCI 511</td>
<td>$N$</td>
</tr>
</tbody>
</table>

**Figure 5.1** Structure of the benzo- or pyrido-fused indole oximes designed as prodrugs for nitric oxide
5.2 Aims and objectives

The study aims were:

1. Screen 10 prodrugs for nitric oxide (NO\textsuperscript{*}) by CYP1 family members plus CYP3A4.
2. Determine the stoichiometry of prodrug loss compared to NO\textsuperscript{*} release.
3. Identify structure-activity relationships, in order to understand structural features which confer selectivity to CYP1-family members.

5.3 Results and discussion

Nitric oxide production (measured indirectly as nitrite ions from auto-oxidation of nitric oxide) was measured from the 10 prodrug analogues. Auto-oxidation of nitric oxide occurs in oxygenated aqueous solution (Stratford 1999) and generates NO\textsubscript{2} as shown in reaction (1). The prodrug analogues were initially screened for specific activities for nitric oxide production at a fixed concentration of 50 μM with four different CYP supersomes\textsuperscript{TM} using HPLC analysis (see chapter 2, section 2.4.1). It is important to highlight that the concentration of prodrug used in these preliminary experiments was above the K\textsubscript{m} of most, but not all representative substrates for CYP1B1 (Lewis et al., 2003).

\[
4\text{NO}^\ast + \text{O}_2 + 2\text{H}_2\text{O} \rightarrow 4\text{NO}_2^- + 4\text{H}^+ \quad (1)
\]

These CYP isoforms include three members of the CYP1 gene family (CYP1A1, CYP1A2 and CYP1B1) and the CYP3 isoform, commonly found in the liver, CYP3A4 (Rooney et al., 2004). Nitric oxide production was analysed using HPLC with electrochemical detection. A prodrug was discarded from further study if the rate of nitric oxide production fell below a threshold of 0.5 pmol/min/pmol P450, unless evidence for selective activation by a CYP1-family member was obtained from this initial screen. Rate of nitric oxide production was calculated from time course experiments and where applicable, prodrug loss rates were determined. Figure 5.2 shows selected chromatograms for nitric oxide production and prodrug loss; where panels B and D illustrate the change in nitric oxide/prodrug over time. Figure 5.3 shows the kinetic traces from selected prodrug analogues, GCI 503 and GCI 510 with CYP1B1 and CYP1A1, respectively.
Figure 5.2 HPLC chromatograms of nitrite production and prodrug loss for GCI 510 (50 μM) with CYP1A1. Panel A shows the chromatogram with the initial peak of nitrite at time 0 min (−) and after 45 min (—), panel B shows an overlay of chromatograms illustrating increase over time of nitrite ions. Panel C shows the decrease in parent prodrug at time 0 and after 45 min. Panel D shows an overlay of the chromatograms illustrating a decrease in prodrug over time. Glucose-6-phosphate (4.95 mM), glucose-6-phosphate dehydrogenase (0.4 units), NADPH (0.49 mM) and magnesium chloride (2.97 mM) were made up in phosphate buffer pH 7.4 (100 mM) and Gentest supersomes™ were added and incubated at 37°C. After 5 min, prodrug (10 mM in DMSO, 50 μM final concentration) was added to initiate the reaction. Loss of prodrug and nitrite production were determined every 5 and 4 min, respectively.
The enzyme kinetics were analysed in the linear phase for all prodrugs with each CYP isoform. Stoichiometric relationships between nitric oxide production and prodrug loss were investigated for the selected prodrug analogues highlighted in section 5.3.1. The data can be seen in Figure 5.4, where prodrug loss and nitric oxide production is shown for GC1 500, 503, 504 and 510. Figure 5.5 gives a diagrammatic representation of the rate of nitric oxide release and Table 5.1 details the specific activities measured for the GC1 500 series prodrugs and includes prodrug loss and rate of nitric oxide production by the CYP1-family enzymes plus CYP3A4. Selected prodrugs were then evaluated to compare the stoichiometry of nitric oxide release to loss of prodrug as well as selectivity for the target enzymes.

**Figure 5.3** Kinetic traces showing loss of prodrug with corresponding nitrite production; GC1 503 with CYP1B1 (panel A) and GC1 510 with CYP1A1 (panel B). Results shown are the means ± SD of three independent experiments. Linear fits are shown. Rates were established from the linear phase of each experiment. Assay conditions are shown in the legend for Figure 5.2.
Figure 5.4 Stoichiometric analysis (prodrug loss and nitrite production) for the selected GCI prodrugs. Nitrite production (panel A) (pmol nitrite/min/pmol P450) and corresponding prodrug loss (panel B) (pmol prodrug/min/pmol P450). The threshold value (---) above which drugs were further analysed for prodrug loss is shown. Mean specific activity is shown ± SD of three independent experiments. Prodrug loss was performed at 264 nm and nitrite production was measured with electrochemical detection.

5.3.1 Nitric oxide production with the benzo- or pyrido-fused indole oxime nitric oxide prodrugs

This HPLC-based assay quantified nitric oxide release from each of the 10 prodrugs proposed in this study, upon incubation with four CYP isoforms. For
further information, rates of nitric oxide release and prodrug loss are detailed in Figure 5.5 and Table 5.1.

5.3.1.1 Role of the quinoline nitrogen in nitric oxide release.

The quinoline nitrogen, present in all prodrugs except GCI 507, may be crucial for hydrogen bonding and selectivity towards the CYP1B1 active site. Relative to GCI 503, nitric oxide release decreased when the nitrogen in the quinoline core is removed and this is marked with CYP1B1, where a 7-fold decrease in nitric oxide rate was observed from $1.60 \pm 0.23$ to $0.23 \pm 0.03$ pmol/min/pmol P450. The other CYP isoforms, CYP1A1, CYP1A2 and CYP3A4, showed a decrease in nitric oxide production (1.2, 1.5- and 4-fold decrease, respectively). Therefore, the rationale for including nitrogen in the core structure is justified, and suggests that this nitrogen may play a possible role in hydrogen bonding in the CYP1B1 active site. Substitutions around the core GCI 503 molecule can be investigated in order to achieve CYP1B1 specificity, whilst maintaining activation by other CYP isoforms low.

5.3.1.2 Effect of substitution at the R^6 position on the efficiency of nitric oxide release.

The first prodrugs to be investigated contained either electron withdrawing or electron donating substitents at the R^6 position para to the quinoline nitrogen. The electronic properties of these chemical moieties are reflected by Hammet $\sigma$ constants; whereby a positive value represents an electron withdrawing group, relative to hydrogen, conversely a negative value indicates electron release (Hansch & Leo 1995).
Figure 5.5 Nitrite production from GCI nitric oxide producing prodrugs activated by CYP1A1, CYP1A2, CYP1B1 and CYP3A4 supersomes™. Results shown are the means ± SD of three independent experiments. The threshold above which compounds are further analysed is illustrated (- - -). The assay conditions are shown in the legend for Figure 5.2. Specific activities are given in detail in the legend in Figure 5.2.
Table 5.1 Specific activities of nitric oxide production and prodrug loss for the benzo- or pyrido-fused indole oxime prodrugs. Rates of nitric oxide production are shown for four CYP isoforms and selected analogues were evaluated further for prodrug loss. Mean specific activity is shown ± SD of three independent experiments. (ND = not determined)

<table>
<thead>
<tr>
<th>Prodrug</th>
<th>CYP1A1</th>
<th>CYP1A2</th>
<th>CYP1B1</th>
<th>CYP3A4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{NO}_2^-$</td>
<td>Prodrug loss</td>
<td>$\text{NO}_2^-$</td>
<td>Prodrug loss</td>
</tr>
<tr>
<td>GCI 500</td>
<td>0.56 ± 0.06</td>
<td>3.72 ± 0.31</td>
<td>0.11 ± 0.08</td>
<td>0.19 ± 0.69</td>
</tr>
<tr>
<td>GCI 502</td>
<td>0.35 ± 0.17</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>GCI 503</td>
<td>0.54 ± 0.07</td>
<td>2.92 ± 0.18</td>
<td>0.52 ± 0.15</td>
<td>0.58 ± 0.37</td>
</tr>
<tr>
<td>GCI 504</td>
<td>1.66 ± 0.14</td>
<td>0.87 ± 0.5</td>
<td>0.35 ± 0.10</td>
<td>0</td>
</tr>
<tr>
<td>GCI 505</td>
<td>0.24 ± 0.03</td>
<td>ND</td>
<td>0.10 ± 0.06</td>
<td>ND</td>
</tr>
<tr>
<td>GCI 506</td>
<td>5.08 ± 0.25</td>
<td>ND</td>
<td>3.46 ± 0.78</td>
<td>ND</td>
</tr>
<tr>
<td>GCI 507</td>
<td>0.48 ± 0.15</td>
<td>ND</td>
<td>0.35 ± 0.06</td>
<td>ND</td>
</tr>
<tr>
<td>GCI 509</td>
<td>0.56 ± 0.04</td>
<td>ND</td>
<td>0.22 ± 0.01</td>
<td>ND</td>
</tr>
<tr>
<td>GCI 510</td>
<td>15.16 ± 0.72</td>
<td>15.64 ± 0.65</td>
<td>2.25 ± 0.09</td>
<td>0</td>
</tr>
<tr>
<td>GCI 511</td>
<td>6.58 ± 0.47</td>
<td>ND</td>
<td>1.36 ± 0.08</td>
<td>ND</td>
</tr>
</tbody>
</table>
These prodrugs include GCI511 (trifluormethyl, $\sigma_p 0.54$), GCI504 (chloro, $\sigma_p 0.23$), GCI503 (hydrogen, $\sigma_p 0.00$), GCI509 (methy, $\sigma_p -0.17$), and GCI502 (methoxy, $\sigma_p -0.27$). These substituents may affect hydrogen donor/acceptor interactions with the quinoline nitrogen and amino acid residues in the active site of the enzyme. Rate of nitric oxide production for GCI 503 was $0.16 \pm 0.23$ pmol/min/pmol P450 for CYP1B1 but was slower for the other CYP1-family members and CYP3A4. The $pK_a$ values for these prodrug analogues were determined (data supplied by Mr. Kantilal B. Patel, Cancer Research UK Free Radical Research Group); GCI 500, $< 1$, GCI 502, $5.20 \pm 0.07$, GCI 503, $3.09 \pm 0.07$ and GCI 509, $3.36 \pm 0.07$.

Addition of increasing electron donating groups at the $R^6$ position (methyl (GCI 509) $>$ methoxy (GCI 502)) gradually decreased nitric oxide release from CYP1B1. However, the effects on nitric oxide production upon the addition of electron withdrawing groups at this position is less clear. There is an increase in nitric oxide rate with trifluoromethyl with CYP1B1 (1.5-fold) and CYP1A1 (12-fold), but this is also matched with CYP3A4 to a greater extent (almost 20-fold). Therefore this substitution results in a loss of selectivity for CYP1 isoforms. (Addition of trifluoromethyl at $R^3$, also in GCI 511, is not anticipated to affect the size of the prodrug analogue as it is similar in size to a methyl group). Conversely, the rate of CYP1B1-mediated nitric oxide production decreased when compared with another electron withdrawing group, GCI 500, -Cl, this effect was greater with CYP1B1 than CYP1A1.

The observed changes in rate of nitric oxide production from the different prodrug analogues seems unlikely to be related to the $pK_a$ of the quinoline nitrogen which is deprotonated at physiological pH for GCI 500, 502, 503 and 509. Further investigations are necessary to determine if these substituents at $R^6$ affect the prototropic equilibrium between the radical cation $[>\text{C}=\text{NOH}]^{\bullet^+}$ and the neutral iminoxyl radical $[>\text{C}=\text{NO}]^{\bullet}$ (Everett et al., 2001), believed to be precursors to the release of NO$^\bullet$.

Rate of loss of parent prodrug was determined for selected analogues and is shown in Figure 5.4 and detailed in Table 5.1. The rate of loss of GCI 503 is ~ 3-fold higher than the rate of nitric oxide production for CYP1B1, which suggests that other mechanisms of metabolic activation may be occurring. This is more prominent for CYP1A1 where there is a 5-fold greater increase in prodrug loss compared with
nitric oxide production. The data suggest that it is likely that there is more than one binding site in the CYP1B1/CYP1A1 active site, which does not allow nitric oxide release from the NOH moiety.

A methoxypyridine group (GCI 505) was added at the R⁶ position to investigate steric, as well as electronic effects. All CYP isoforms had low rates of nitric oxide production, which were below the stated threshold of 0.5 pmol/min/pmol P450, indicating that a bulky substituent at the R⁶ position is not tolerated. Collectively the data presented for R⁶ substitutions clearly show no major gain in selectivity towards CYP1B1, hence it was decided not to pursue chemistry involving substitution at this position any further.

5.3.1.3 Effect of substitution at the R¹ position on the efficiency of nitric oxide release.

Introduction of a phenethyl group at the R¹ position (GCI 510) resulted in a dramatic improvement in not only nitric oxide production with CYP1A1 (15.16 ± 0.72 pmol/min/pmol P450), but also an equal corresponding rate of prodrug loss (15.64 ± 0.65 pmol/min/pmol P450). This 1:1 stoichiometry of nitric oxide production to prodrug loss was not matched with any of the other CYP-isoforms for GCI 510. Selectivity for CYP1A1 over CYP3A4 was established, however, there was a marked difference in nitric oxide production with CYP1A1 compared to CYP1B1 (20-fold lower). A substitution at R¹ is not conjugated to the quinoline core; therefore, it can be assumed that the phenethyl group may be having an electronic effect but more likely to be impacting on the π-π stacking ability of the molecule with aromatic amino acid residues in the active site of the enzyme.

Other prodrug analogues with R¹ substitutions were investigated to explore prodrug solubility. GCI 504, with a methylpyridine group at R¹ illustrated poor CYP1B1 enzyme selectivity, conversely CYP1A1-mediated nitric oxide release was increased 3-fold compared with GCI 503. This suggests that the log P value for this analogue may have more of an effect with CYP1A1 than CYP1B1. A dramatic increase in nitric oxide production was observed for all CYP isoforms except CYP1B1 upon substitution at R¹ with a solubilising diethylaminoethyl substituent. However, CYP1 selectivity was lost as CYP3A4 nitric oxide production was 3-fold
higher than CYP1A1. Clearly the data show that solubilising substituents may have detrimental effects on target enzyme selectivity.

5.3.2 Identification of a CYP1A1 specific prodrug for nitric oxide

The rationale for prodrug evaluation highlighted in 5.1.3, revealed that GCI 510 met all of the criteria mentioned and therefore was considered as the lead compound for CYP1A1 activation in this series of prodrugs, with a nitric oxide production rate of $15.16 \pm 0.72 \text{ pmol/min/pmol P450}$. There was a linear increase of nitric oxide over time as measured by HPLC (as shown in Figure 5.3). This compound also showed that there was a proportional loss of prodrug to nitric oxide production over time as measured by HPLC (Figure 5.4). Moreover, there was stoichiometric production of nitric oxide from the prodrug relative to the loss of the prodrug with GCI 510 and CYP1A1, which was not mirrored in all cases of CYP-mediated metabolism of GCI 510 (Figure 5.4). CYP1A2 and CYP1B1-mediated metabolism of GCI 510 showed prodrug loss could not be measured. In the case of CYP3A4 the rate of nitric oxide production was more than that of the loss of parent prodrug. Statistical analysis revealed that nitric oxide production was significantly higher for CYP1A1 compared with the other CYP isoforms (Figure 5.6).

5.3.2.1 Identification of GCI 510 prodrug metabolites

Upon CYP metabolism of the aryl oxime prodrugs ($>\text{C}=\text{NOH}$), the corresponding aldehyde ($>\text{C}=\text{O}$) is expected to be produced in analogy to the NOS-catalysed oxidation of $N$-hydroxy-L-arginine ($>\text{C}=\text{NOH}$) to citrulline ($>\text{C}=\text{O}$) and NO$^*$ (Stuehr et al., 1991). A peak was observed with the same retention time as authentic aldehyde (Figure 5.7, panel A) which also matched the UV spectrum of the authentic sample (Figure 5.7, panel B). The chromatogram in Figure 5.7 shows GCI 510 aldehyde was detected at 3.55 min. GCI 510 is shown at 2.7 min. Quantification of prodrug GCI 510 aldehyde was not possible as standards were not available for analysis at the time of experimentation.
Figure 5.6 Statistical analysis of CYP-mediated metabolism of GCI 510. Means are shown with the 25\textsuperscript{th} and 75\textsuperscript{th} percentiles. Results are from three independent experiments. P values (from student’s t-test analysis) were calculated and significant figures are indicated as \( < 0.005 \) (***), or \( < 0.001 \) (****).

Clearly, this needs to be addressed in further work to allow full stoichiometric analysis of aldehyde production relative to nitric oxide production. Equal proportions of GCI 510 aldehyde (as estimated by area under the curve) and nitric oxide do not appear to be generated over time, which could be due to further oxidation of the aldehyde. During the assay this product does not appear to increase linearly warranting further investigation. Although aldehyde production was not quantitative it appeared to reach a maximum (as shown by area under the curve) and then declined, suggesting that it may be a substrate for the enzyme itself. Further experiments are needed to clarify this.

The chromatogram in Figure 5.8, panel A illustrates the elution of prodrug and shows that a polar product is detected prior to the parent prodrug at 1.8 min. This potential metabolite appeared to increase over time (Figure 5.8, panel B), and was not present in the control samples. The spectra shown in Figure 5.8 panel C suggests that this product is related to the parent GCI 510 prodrug. It is not known if this product is directly associated with metabolism of the parent prodrug (for example a hydroxylated product) or if it is related to further oxidation of the GCI 510 aldehyde.
Figure 5.7 Detection of the GCI 510 aldehyde over time upon metabolism by CYP1A1. Prodrug loss was measured by HPLC with absorbance at 264 nm (panel A). Absorbance spectra for the GCI 510 aldehyde is shown (panel B) along with the reference library spectra for the GCI 510 aldehyde. The assay conditions are shown in the legend for Figure 5.2.
Figure 5.8 HPLC chromatogram illustrating prodrug loss measured by absorbance detection at 264 nm; identification of a possible further metabolite. Panel A shows the chromatogram with the initial peak of parent prodrug at time 0 min (••••) and after 50 min (—). A product of metabolism was also detected. Panel B shows an overlay of chromatograms illustrating the increase of polar product (highlighted) over time when CYP1A1 was incubated with GCI 510 (50 μM). Panel C suggests the spectra of this peak is derived from the parent GCI 510. The assay conditions are the same as in the legend for Figure 5.2.
This observation needs further investigation to identify the structure and origin of this metabolite. Hydroxylation of the parent prodrug may occur by CYPs and further experiments are necessary to identify the metabolite profile of this prodrug.

In summary the data show that different rates of nitric oxide were produced from the 10 different prodrugs. Stoichiometric analysis showed GCI 510 gave loss of prodrug which was comparable with the rate of production of nitric oxide. The corresponding aldehyde was also generated, but in addition a more polar product which could be derived from GCI 510 was observed, and increased over time.

5.4 Summary

This chapter has aimed to evaluate the capacity of benzo- or pyrido-fused indole oxime prodrugs to produce nitric oxide using a cytochrome P450 screen including CYP1-family enzymes plus CYP3A4. Nitric oxide is not only a blood flow modifier (Tozer & Everett 1997a) but it is also a potent radiosensitiser (Gray et al., 1958; Mitchell et al., 1993; Griffin et al., 1996) and cytotoxin (Halliwell & Gutteridge 1985). Hence, these preliminary studies seek to identify lead prodrugs that can be taken further to establish if cytotoxicity can be gained in vitro. Stoichiometric analysis was undertaken and SAR investigated. The data strengthen existing, limited, studies into the development of CYP1B1 activated prodrugs for targeted cancer therapy; giving an insight into the properties of the groups added onto the core molecule which will interact with the CYP1B1 active site, optimising nitric oxide release from these prodrugs. A CYP1B1 partially-selective prodrug was identified, however, the rate of nitric oxide production was low and the stoichiometry of rate of nitric oxide production and prodrug loss was not equal, suggesting that there may be other metabolites of the parent prodrug, possibly hydroxylated products, which are typical reactions for CYP mono-oxygenases (Spink et al., 1998). Conversely, a CYP1A1-selective prodrug analogue, GCI 510 was identified. There was 1:1 stoichiometry of nitric oxide and prodrug loss but further evaluation into metabolite profiles for the two selected prodrugs (GCI 510 and 503) is necessary in future experiments. Key interactions in the CYP1A1 active site could be investigated further with homology modelling. Similar studies with CYP1B1 may assist in identifying CYP1B1-selective prodrugs for nitric oxide.
Chapter 6
General discussion and future directions
For effective tumour cell kill, anticancer drugs need to be active at the tumour site, whilst minimising systemic toxicity. This can be achieved in many ways, for example using gene directed enzyme prodrug therapy (GDEPT), antibody directed enzyme prodrug therapy (ADEPT) or by the expression of a functional enzyme in cancer cells, which is absent or only expressed at low levels in normal tissues. For the basis of this study, we are exploiting the presence of the cytochrome P450 1B1 monooxygenase (CYP1B1) in human cancer cells, for CYP1B1 activated prodrug therapy.

The literature has shown the presence of CYP1B1 in a range of solid human malignancies (Murray et al., 1997; McFadyen et al., 1999; Iscan et al., 2001; Gibson et al., 2003; Carnell et al., 2004; Oyama et al., 2005). Chapter 3 demonstrates that CYP1B1 protein is expressed in human head and neck squamous cell carcinoma (HNSCC), as well as in associated pre-malignant tissues. This is an important observation for two reasons. Firstly, CYP1B1 protein has not been looked for in HNSCC to date, and secondly, this is the first study to identify CYP1B1 protein during the initial stages of cancer progression. A previous study in the literature did identify the enzyme in hyperplasia and prostatic intraepithelial neoplasia (PIN) of the prostate (Carnell et al., 2004) which indicates a possible link with CYP1B1 expression and premalignancy; however, hyperplastic prostatic tissue is not a definitive indication of premalignancy and malignant progression (Bostwick et al., 1992). Conversely, hyperplastic/dysplastic tissues in the head and neck region will often precede the formation of a carcinoma in situ, as shown by a well characterised malignant progression profile (Hittelman 2003). This part of the study has shown that CYP1B1 is expressed to a high frequency (94 %) in HNSCC and associated pre-malignant tissue (100 %) and proves to be an attractive target for cancer therapy.

HNSCC is widely attributed to smoking and alcohol use (Blot et al., 1988). Tobacco smoke contains many chemicals, including polyaromatic hydrocarbons (PAH) (Stewart & Kleihues 2003), (which can induce CYP enzymes (Villard et al., 1998; Zevin & Benowitz 1999; Port et al., 2004), which are known to be involved in carcinogenesis (Kaminsky & Spivack 1999). As the squamous mucosa is in contact with the airways in the head and neck region, induction of CYP1B1 by these chemicals seems plausible in pre-malignant tissue but unlikely in the carcinoma tissue. Spivack and colleagues identified that CYP1B1 mRNA levels are modulated by exposure to carcinogens in tobacco smoke in normal lung tissue (Spivack et al.,
Unfortunately, smoking status of the patients analysed in this study was not available - this would have been beneficial in determining if smoking had an influence on CYP1B1 protein over-expression in HNSCC. Studies in the literature have stated that mRNA expression in the head and neck region may be due to the induction of the protein from aryl hydrocarbon receptor agonists (PAHs) in cigarette smoke (Thier et al., 2002; Port et al., 2004). The role of CYP1B1 in the metabolism of oestradiol into the carcinogenic 4-hydroxyoestradiol (McFadyen et al., 1999; van Duursen et al., 2003; Tsuchiya et al., 2004) may account for its over-expression in hormone dependent cancers, however, the reason for the high frequency of expression of CYP1B1, in all cancers studied to date, still remains unclear.

Spectral imaging microscopy has proved a useful tool for analysing and quantifying staining intensities (Barber et al., 2003). It has been used to quantify CYP1B1 protein expression in prostate carcinoma and compare its expression with CYP1B1 in bladder carcinoma (Carnell et al., 2004). If CYP1B1 targeted therapies were to be employed clinically, this technique would possibly facilitate the eligibility of CYP1B1 in personalised anti-cancer therapy. This has been highlighted previously in the literature, where geno- and phenotyping tests are devised to elucidate expression of individual CYP isoforms, prior to dmg treatment (Hasler 1999). The choice of drug for treatment can then be made accordingly (Pelkonen & Raunio 1997; Kirchheiner & Brockmoller 2005).

Due to the retrospective nature of this study, no enzymology studies could be undertaken in the tumour tissues to evaluate functionality of the CYP1B1 protein detected. Previous studies have demonstrated functionally active CYP1B1 in renal cell carcinoma samples but not in the corresponding normal tissue (McFadyen et al., 2004b); which is expected due to the lack of protein expressed in normal tissues (Murray et al., 1997). Specific CYP1B1 functional enzyme assays include oestradiol hydroxylation (Hayes et al., 1996) as well as ethoxyresorufin deacetylation (Burke et al., 1994), using appropriate inhibitors (McFadyen et al., 2004b). Oestradiol hydroxylation by CYP1B1 yields 4 hydroxyoestradiol which will be further metabolised to form 4 methoxyoestradiol, (Spink et al., 1994; van Duursen et al., 2003), whereas CYP1A1 activity is indicated by the formation of 2-hydroxyoestradiol and 2-methoxyoestradiol (Spink et al., 1998). The metabolites are quantified using HPLC (Spink et al., 1994). Ethoxyresorufin is O-dealkylated by all CYP1 isoforms to yield resorufin which can be measured over time using
spectrofluorimetry. Specific CYP1 isoform activity is elucidated using inhibitors such as 2,4,3',5'-tetramethoxystilbene (TMS) for CYP1B1 (Chun et al., 2001b) and resveratrol/furafylline for CYP1A1 (Yueh et al., 2005).

Allelic variants of CYP1B1 have been proposed as influencing cancer susceptibility (Li et al., 2000; Goodman et al., 2001; Sasaki et al., 2003; Han et al., 2004) in many solid tumour types, as well as altering the catalytic activity of the CYP1B1 protein (Bandiera et al., 2004). Allelic variation of CYP1B1 has been established as a risk factor in HNSCC which was linked with smoking status, thus increasing the rate of activation of carcinogens in cigarette smoke (Ko et al., 2001). Rare allelic variants of CYP1B1, which are catalytically inactive, are associated with familial glaucoma (Stoilov et al., 1998). Bandiera and colleagues showed the CYP1B1 variant, CYP1B1.4 (RALS) showed a decrease in activity with matched decrease in immunodetectable protein (Bandiera et al., 2004), which was attributed to proteasomal degradation of the protein. Consequent inhibition of this degradation pathway (using a proteasomal inhibitor, MG132) increased stability of this active CYP1B1 variant (as measured using the ethoxyresorufin O-dealkylase (EROD) assay). Therefore it was proposed that disruption of the proteasome in cancer (as opposed to functional proteasome in normal tissues) results in expression of CYP1B1 in tumour tissues (Bandiera et al., 2004). Variants of the CYP1B1 protein were not examined in the current study, but work in the literature provides an insight into the possible aetiology of cancer and cancer susceptibility, which would be important in future work.

Investigation of CYP1B1 with other clinicopathological parameters yielded some correlations. CYP1B1 staining intensity (as measured visually) was more likely to be present in the primary disease compared with recurrent disease, and there was an association with p53 staining (i.e. strong CYP1B1 with strong p53). Mutated p53 has been proposed in playing a role in cancer development, including HNSCC (Boyle et al., 1993). Investigation into possible co-localisation of these two proteins would prove important, with a possible impact on therapy.

In summary, future work related to this component of the study would entail investigations into CYP1B1 protein expression in patients who smoke versus those that do not, correlating CYP1B1 protein expression with amount of exposure (i.e. cigarettes per day). As CYP1B1 protein has been found to be overexpressed in HNSCC, this justifies evaluation of functional activity of CYP1B1 in these tissues.
using EROD and oestradiol hydroxylation assays. Analysis of allelic variants of CYP1B1, using RNA analysis, with complementary protein determination (using existing protocols for immunohistochemistry and western blotting), with enzymology studies (EROD and oestradiol hydroxylation) would give a comprehensive analysis of disease progression and susceptibility. This would also establish if existing allelic variants changed the catalytic activity of the CYP1B1 protein detected in the same tissue. As discussed previously, CYP1B1 'personalised therapy' could also benefit from this analysis as catalytic activity will ultimately alter drug metabolism rates. Double staining protocols could be used to ascertain whether there is a correlation in the localisation of p53 and CYP1B1, using double staining protocols coupled to spectral imaging. Previous reports in the literature have proposed hypoxia and oxidative stress as playing a role in the regulation of CYP1 isoforms (Morel & Barouki 1998; Morel et al., 1999; Husbeck & Powis 2002); so further double staining of CYP1B1 with the endothelial cell marker CD31 (to establish is CYP1B1 is localised near to oxygenated blood vessels) and the redox regulated protein, thioredoxin, would seek to investigate if CYP1B1 has a relationship with redox signalling, in clinical tumours and/or xenografts. This chapter has shown that a CYP1B1 human tumour xenograft was identified, providing an in vivo model for testing CYP1B1-activated prodrugs.

The second phase of this study was to investigate CYP1B1 expression in a range of cells that could be used as xenografts, with the anticipation of using them as models for testing CYP1B1 activated therapeutics. In the literature a tumour model which expresses CYP1B1 has not been documented; however, the results presented here (chapter 4) indicate that a primary head and neck squamous cell carcinoma cell line (UT-SCC-14), can be grown as a xenograft in mice and expresses CYP1B1 constitutively (at low levels) and is inducible (CYP1A1 to a lesser extent) using a known aryl hydrocarbon agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).

Previous studies have investigated the effects of TCDD on CYP1B1 mRNA and protein levels in vitro, without translating the observations to in vivo studies. Chapter 4 describes CYP1B1 protein expression in three different cancer cell lines (two breast (MCF-7 and MDA-MB-468) and one primary head and neck squamous cell carcinoma (UT-SCC-14)) in vitro and in vivo. Additionally, a time course of CYP1B1/CYP1A1 induction was demonstrated in vivo and in vitro. This was
important in gaining an understanding of CYP1B1 induction profiles in the selected cell lines and their corresponding human tumour xenografts. The HNSCC cell line (UT-SCC-14) was chosen for the xenograft model as the other cell lines were deemed unsuitable. The MDA-MB-469 cell line produced xenografts that had a necrotic core and showed poor growth characteristics; which is not optimal for drug testing. The MCF-7 xenograft produced tumours but required oestrogen implants to grow (which resulted in toxicity in the strain of mouse used). Additionally, immunohistochemical analysis revealed these tumours were positive for CYP1A1 protein (personal communication, V.M McErlane and F. Daley), which is not desirable for analysing CYP1B1 activated drugs.

In the UT-SCC-14 xenograft, CYP1B1 protein was expressed at low levels constitutively and was induced in response to TCDD at doses of 5, 15 and 50 μg/kg. CYP1A1 was only induced at higher doses of TCDD. This observation is consistent with some studies (Eltom et al., 1998) but in disagreement with others (Schaufler et al., 2002), which compare CYP1B1 and CYP1A1. The presence of CYP1B1 was investigated using western blot techniques coupled with immunohistochemistry and spectral imaging. Western blotting techniques employed in chapter 4 showed that CYP1B1 protein in vivo measured 60 kDa (polyclonal antibody), this size of protein was expressed to a much lesser degree in vitro (using various carcinoma cells lines). Previous studies have identified a 52 kDa protein from cells in vitro (McFadyen et al., 1999; Spivack et al., 2001; Lin et al., 2003), conversely other reports have identified the 60 kDa band (McKay et al., 1995; Malaplate-Armand et al., 2003), as shown in the xenografts in this present work. The predicted size of CYP1B1 is 60.8 kDa as this protein has 543 amino acids. Chapter 4 showed that two different molecular weight CYP1B1 proteins (52 and 60 kDa) were detected by immunoblotting. This may be due to post-translational modifications of the protein (Mann & Jensen 2003), as the 60 kDa band was not expressed to the same degree in vitro using the same cell line and antibody. These modifications will change the properties of protein (size and charge) and have been shown to increase protein stability (Mann & Jensen 2003). It may also be due to the use of different antibodies in this study. It could be postulated that the presence of CYP1B1 in cancer tissues could be due to post-translational modification.
In summary, future work for this aspect of the Ph.D. could seek to investigate a full profile of CYP1B1’s response to TCDD by investigating mRNA levels (using RT-PCR) and comparing the findings to existing CYP1B1 protein analysis; giving an insight into the inducibility of CYP1B1. Possible post-translational modifications could be assessed using mass spectrometry and sequencing analysis. This would ascertain if modifications were occurring, giving rise to the 60 kDa band.

Additional future work would need to address the validity of using this animal model for drug testing, as TCDD may induce CYP1B1 protein in normal tissues, in particular, the liver as shown in the literature for rats (Walker et al., 1999). To complement the observed CYP1A1 induction by TCDD (shown by western blotting in chapter 4), immunohistochemistry could be performed on the corresponding xenografts. Existing protocols optimised in this Ph.D. thesis (chapter 3 and 4), could be employed to determine protein expression in normal tissues and organs (western blotting and immunohistochemistry), as well as applying newer techniques for mRNA quantification (RT-PCR). Spectral imaging microscopy could be utilised to enable comparisons of CYP1B1 staining intensity in normal tissues and tumour xenograft tissue.

This study also focuses around developing selective prodrugs which are non-toxic until metabolically activated by the target enzyme, CYP1B1. Previous reports have identified diazeniumdiolate compounds (termed ‘NONOates’) that can generate NO$^+$ spontaneously in a pH dependent manner; but these drugs are not selective hence NO$^+$ can be generated throughout the body (Fitzhugh & Keefer 2000). Other studies utilise nitric oxide synthases (NOS) for the metabolism of $N$-hydroxyguanidine to release NO$^+$ (Mansuy & Boucher 2004), however, these compounds are not selective for activation in cancer cells due to the expression of NOSs at sites other than the tumour (Tozer & Everett 1997a; Alderton et al., 2001). Aryl oxime compounds, of which there are many analogues (Rehse & Brehme 1998) are metabolized by CYPs (Caro et al., 2001) but the specificity is still lacking for activation in cancer cells due to the presence of many CYP isoforms in the liver. Intra-tumoural nitric oxide release is an approach that has been proposed by many groups. The chemical and biological properties of nitric oxide (diffusibility
(Lancaster 1997), cytotoxicity (Halliwell & Gutteridge 1985) and radiosensitivity (Howard-Flanders 1957; Gray et al., 1958) make this molecule an attractive candidate for cancer therapy. Systemic NO* was shown to produce hypotension (Llorens & Nava 2003) and references therein) hence, gene therapy approaches were adopted, to ensure targeted NO* delivery. Gene therapy has been proposed as a viable tool for the generation of NO* for cytotoxicity (Worthington et al., 2002; Kuroki 2004; Wang et al., 2004) as well as changing the properties of the tumour vasculature via generation of NO* (Worthington et al., 2000). Previous homology modelling of CYP1B1 has identified key residues and interactions of oestradiol in the putative CYP1B1 active site (Lewis et al., 2003). Based upon this knowledge benzo- or pyrido-fused indole oxime prodrugs were synthesised to understand structural features which confer selectivity of the prodrug for the target enzyme(s).

Using a CYP isoform screen rate of nitric oxide and prodrug loss (for selected drugs) was established from 10 prodrug analogues (by measuring the surrogate marker, nitrite) (chapter 5). From these studies, structure-activity relationships (SARs) were established to ascertain which substituents, around the core structure, affected nitric oxide release from the prodrug, (due to differences in interaction with the CYP1B1 active site). The approach employed here also shows that drugs that have been designed around the active site of CYP1B1 using the oxime core structure, could allow for specific, enzyme activated NO* release, as opposed to using NO* donors which lack specificity (Morley & Keefer 1993; Fitzhugh & Keefer 2000; Keefer 2003).

We have identified a candidate molecule (GCI 503) which is activated by CYP1B1 and shows lower selectivity for other CYP isoforms tested (CYP1A1, 1A2, and 3A4). A CYP1A1 activated prodrug (GCI 510) was also identified which showed a 10 fold higher rate of nitric oxide production (upon metabolism with CYP1A1) compared with CYP1B1s metabolism of GCI 503 (15.16 ± 0.71 compared to 1.59 ± 0.23 pmol/min/pmol P450, respectively). Structure activity relationships concluded that extending the planarity of the substrate is advantageous and improves π-π stacking for CYP1A1 but this is not mirrored with CYP1B1. These factors may be important for the interaction of a substrate with the active site of CYP1B1 (Lewis et al., 2003; Lewis et al., 2004), and were described in detail in chapter 5.
Further work, based on the key findings in chapter 5, are addressed in detail. Sampling direct from the HPLC autosampler meant that fewer samples could be taken during the initial linear phases of metabolism. This could be circumvented by carrying out the reaction in a water bath and taking samples more frequently as required, depending on the activity of the enzyme. This could also protect the HPLC column, as crude proteins are pelleted by centrifugation, prior to running the sample on the HPLC apparatus. This would also ensure that run times could be extended and possible metabolites identified; problems encountered with quantification of prodrug addressed in chapter 5 would also be resolved.

Whole cell metabolism studies could be conducted to address whether metabolites identified with supersomes™ are generated *in vitro* using CYP1B1 expressing cell lines. The problems encountered in chapter 4 with concomitant CYP1A1 induction with CYP1B1 in cell lines, would be overcome using a Chinese hamster ovary (CHO) cell line (currently used in the laboratory and supplied from Dr. Thomas Friedberg, University of Dundee), which have been stably transfected with CYP1B1 (CHO.1B1). Preliminary experiments have shown that CYP1B1 protein can be identified in this cell line using western blotting (appendix 4), however supplementary experiments are needed to establish if this protein is functional (EROD with specific enzyme inhibitors and oestradiol hydroxylation). If CYP1B1 is active in the CHO.1B1 cells, cytotoxicity studies could then be employed *in vitro* upon incubation with the prodrug, to establish if sufficient NO* is released to elicit cell death. The HPLC separation protocol used for detecting nitric oxide in the CYP isoform screen (chapter 5) could be also be employed to establish if nitric oxide is formed *in vitro*, from the release of NO* from metabolism of the prodrug. However, solid phase extraction techniques would need to be used to concentrate the cell culture medium in the first instance.

In conclusion this Ph.D. study has shown that cytochrome P450 1B1 monooxygenase has been identified in head and neck cancer and was shown to be expressed during the malignant progression of this disease. This finding with the numerous studies of CYP1B1 expression in the literature identifies the enzyme as a target for cancer therapeutics. Additionally, a potential mouse tumour model has been identified, that preferentially expresses CYP1B1 compared with CYP1A1.
Prodrug analogues GCI 503 and GCI 510 have been shown to be activated *in vitro* by CYP1B1 and CYP1A1, respectively. This xenograft model can be utilised for testing these specific CYP1B1 benzo- or pyrido-fused indole oxime prodrugs (designed using CYP1B1 homology modelling) which yield nitric oxide upon metabolic activation.
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# Appendix 1

## Carcinoma tissues

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## Clinico-pathological status and pathology

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### Appendix 2

#### Pre-malignant tissues

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Appendix 3 Comparision of the visual scoring compared with the spectral image analysis. Selected patient samples are shown with the spectral imaging scores and the corresponding visual scores. Tissue samples from representative staining intensities are shown.
Appendix 4

Western blot analysis to show CYP1B1 expression in transfected Chinese hamster ovary cells (CHO) cells in relation to CHO wild type cells. Controls for supersomes™ are shown. Cells were provided by Dr. Thomas Friedberg, University of Dundee.
### Appendix 5

**Work presented at national and international conferences**

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**Other meetings/presentations**

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* Young Investigator Prize
** British Council Travel Award
*** Poster Prize
Published abstracts
