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**CYTOCHROME P450 mRNA PROFILE IN
HUMAN BREAST CANCER CELL LINES**

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Abstract

Cytochrome P450 enzymes (P450s) are involved in cancer development and treatment due to their roles in the oxidative metabolism of various endogenous (e.g. oestrogen) and exogenous (e.g. tamoxifen) compounds. It is well-known that intermediate P450 metabolites derived from oestrogen metabolism are associated with breast carcinogenesis. The main aim of this project was to profile the cytochrome P450 and P450-regulatory nuclear receptor mRNAs in a series of breast cancer cell lines (BCCs) and compare this profile with normal breast cells. This study used the qualitative reverse transcriptase-polymerase chain reaction (RT-PCR) to detect mRNA expression of target genes. Results showed CYP1B1, CYP2D6, CYP2J2, CYP2R1, CYP2U1 and CYP4X1 mRNA to be present in all cell lines. CYP2A6, CYP2C8, CYP2C18, CYP2F1 and CYP4Z1 mRNA were expressed in oestrogen receptor (ER)-positive Caucasian and ER-negative Afro-Caribbean BCCs. Although no differences in P450 mRNA were observed between the different ethnic groups, these preliminary findings suggest potential similarities in the ER-positive Caucasian and ER-negative Afro-Caribbean BCCs which warrant further investigation

The CYP4Z1 PCR product was identified as two distinct bands. Specific primer sets were used to demonstrate potential intron retention in CYP4Z1. Using established *in vitro* models for the study of regulatory mechanisms of CYP4Z1, T47D and ZR-75-1 breast cancer cell lines were used to determine the appropriate nuclear receptors (i.e. progesterone receptor, glucocorticoid receptor or peroxisome proliferator-activated receptor alpha). These findings suggest that there may be an alternative receptor mechanism involved in CYP4Z1 mRNA induction in these cells. In conjunction, pre-treatment of these two cell lines with the RNA synthesis inhibitor actinomycin D followed by the agonists showed a significant reduction ($p < 0.05$) of CYP4Z1 mRNA levels and inhibited CYP4Z1 induction by either progesterone, dexamethasone or pirinixic acid, indicating that these agonists have effects on CYP4Z1 mRNA transcription or stability. In contrast, cycloheximide differentially affected the level of CYP4Z1 mRNA induction by these agonists. Taken together, these results suggest that CYP4Z1 mRNA induction in T47D and ZR-75-1 is mediated through differential cell type specific regulatory mechanisms and there is evidence for differential regulation of the splice variants.

Keywords: Cytochrome P450 mRNA, cytochrome P450-regulatory nuclear receptor mRNA, Oestrogen receptor (ER)-positive and negative breast cancer cell lines (BCCs), Caucasian and Afro-Caribbean BCCs, CYP4Z1 alternative splicing, regulation of CYP4Z1

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List of Publications

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List of Abbreviations

A	adenine
ACTD	actinomycin D
AhR	aryl hydrocarbon receptor
AhRR	aryl hydrocarbon receptor repressor
AMV	avian myeloblastosis virus
ARNT	aryl hydrocarbon receptor nuclear translocator
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
B2M	β_2 -microglobulin
bp	base pair
C	cytosine
CAR	constitutive androstane receptor
cDNA	complementary deoxyribonucleic acid
CHX	cycloheximide
CO ₂	carbon dioxide gas
CYP	cytochrome P450
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMEM/F12	a 1:1 (v/v) mixture of DMEM and Ham's F12
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	dinucleotide triphosphate
dT	deoxythymidylic acid
dTTP	deoxythymidine triphosphate
DTT	dithiothreitol
ECACC	European Collection of Cell Cultures
EDTA	ethylenediaminetetraacetic acid
ER	oestrogen receptor
FBS	foetal bovine serum
G	guanine

GCR	glucocorticoid receptor
HIF-1 α	hypoxia-inducible factor-1 alpha
L15	Liebovitz's medium
LMP	low melting point
MEM	Minimal Essential Medium
M-MLV	moloney-murine leukemia virus
mRNA	messenger ribonucleic acid
NCBI	National Centre for Biotechnology Information
NNK	4-(methylnitrosamino)-1(3-pyridyl)-1-butanone
O ₂	oxygen gas
OD	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PGR	progesterone receptor
PPAR α	peroxisome proliferator-activated receptor alpha
PXR	pregnane X receptor
RAR	retinoic acid receptor
RNA	ribonucleic acid
RPMI	Rosewell Park Memorial Institute
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
RXR	retinoid X receptor
T	thymine
T _a	annealing temperature
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
T _m	melting temperature
U	Unit
UV	ultraviolet
V	volt
VDR	vitamin D receptor

Chapter 1

General Introduction

1.1 Introduction

According to a recent report by Cancer Research UK, breast cancer is the second most common cause of cancer mortality in women (<http://www.cancerresearchuk.org>). In 2005, it was responsible for more than 12,000 deaths in women and accounted for 17% of all female cancer deaths. The most important prognostic factor related to survival and tumour recurrence following surgery is node-positive breast cancer patients. The prognosis of these patients compared to patients with primary breast cancer is poor, despite improvements in chemotherapy (Wapnir et al. 2006). In addition, ethnic differences in breast cancer survival between Caucasian and Afro-Caribbean women have been well documented. A poorer prognosis and more aggressive breast cancer are found in Afro-Caribbean compared to Caucasian women (Curtis et al. 2008). However, it is unclear why, and there is an urgent need to identify the main factors involved in these differences. Clinically, the critical aspects concerning the use of chemotherapy associated with the poor prognosis of patients are tumour resistance and drug toxicity. Some of this variability may be due to the clearance and metabolism of the anticancer drugs used in breast cancer treatment.

Although antioestrogen therapy and aromatase inhibitors have long been shown to be effective in reducing the risk of recurrence and death in patients with oestrogen receptor (ER) positive tumours, the use of these treatments has been limited in patients with ER negative tumours (Chia et al. 2005). ER-negative breast tumours are more aggressive and have a poorer prognosis when compared with ER-positive tumours and it is therefore, clear that targeted therapies for this patient population are needed (Rochefort et al. 2003).

At the present time, the progression and invasion of breast cancer is not fully understood. However, lifetime exposure to oestrogen correlates with increased risk of breast cancer in women, suggesting that oestrogen plays a crucial role in breast carcinogenesis (Henderson and Feigelson 2000). Two main hypotheses have been discussed concerning oestrogen and breast cancer. Firstly, binding of oestrogen to ER increases cell division and DNA synthesis, resulting in DNA mutations in mammary cells (Yager and Davidson 2006). Secondly, intermediate metabolites derived from oestrogen metabolism cause DNA damage by producing free radicals, leading to initiation and promotion of breast cancer (Deroo and Korach 2006).

For the latter hypothesis, a better understanding of the mechanisms involved in biotransformation of endogenous and/or xenobiotic compounds by breast cancer cells is very important as it contributes to the development of new target therapies as well as the knowledge of breast cancer progression and invasion. These compounds are usually detoxified into inactive forms by enzymes existing in local cells. Cytochrome P450 enzymes (P450s) are one of the key enzymes involved in the oxidative metabolism of a wide range of endogenous (e.g. oestrogen, vitamin A and vitamin D) and exogenous (e.g. tamoxifen, cyclophosphamide, paclitaxel and some carcinogens) compounds (Nebert and Nelson 2005). It is important to note that certain chemicals which are not carcinogens (procarcinogens) can be transformed into carcinogens by cytochrome P450s (Guengerich 2004).

Currently, 57 functional cytochrome P450 enzymes have been classified in humans. The majority of these enzymes are predominantly expressed in the liver, but some cytochrome P450s are preferentially expressed in extrahepatic tissues such as the breast (Nebert and Nelson 2005). Previous studies demonstrated a differential expression profile of cytochrome P450s between tumours and their corresponding normal tissues, and some

cytochrome P450s have been identified as having tumour-specific expression. For instance, overexpression of CYP1B1 has been detected in various tumours including breast (Murray et al. 1997, McFadyen et al. 1999, Murray et al. 2001) and a novel member of the cytochrome P450 family 4, CYP4Z1 mRNA is preferentially expressed in breast carcinoma and associated with poor prognosis in primary ovarian cancer (Downie et al. 2005). More recently, CYP2W1 expression has been shown to be highly expressed in colon tumours (Karlgrén et al. 2006). The existence of tumour specific cytochrome P450 expression suggests that these enzymes could be targets for cancer therapy, and a better understanding of their roles in tumour progression and invasion may be beneficial to cancer research as a whole.

Several chemotherapeutic drugs used in breast cancer therapy (e.g. anthracyclines, taxane, cyclophosphamide, tamoxifen and aromatase inhibitors) are extensively biotransformed in the liver, thereby reducing the release of toxic metabolites present in the tumour cells.

Previous studies investigated the expression of cytochrome P450s in breast cancer cells from patients-derived samples (Albin et al. 1993, Huang et al. 1996, Murray et al. 1997, Iscan et al. 2001, Modugno et al. 2003, Haas et al. 2006), *in vitro* [i.e. cell lines (Sonneveld et al. 1998, Spink et al. 1998, Soulez and Parker 2001, McFadyen et al. 2003, Rieger et al. 2004, Savas et al. 2005, Fischer et al. 2007) and from xenografts (Smith et al. 1993)].

However, only a few cytochrome P450s (i.e. CYP1A1, CYP1B1, CYP2B, CYP2C and CYP3A) have been identified in breast cancer cells and the current knowledge of cytochrome P450 expression related to tumour characteristics in breast cancer is extremely limited. Therefore, this study was designed to identify the cytochrome P450 mRNA profile in a series of breast cancer cell lines derived from different tumour characteristics (i.e. ER status, invasive property and ethnicity) and compare this profile with a normal breast cell line.

1.2 Breast cancer

1.2.1 Overview of breast cancer

In the UK, breast cancer is the commonest cancer and the second most common cause of cancer death (after lung cancer) in women. According to a recent report by Cancer Research UK, breast cancer accounted for 29% of all female cancers. Each year more than 40,000 women are diagnosed with breast cancer in the UK compared to 324 new cases of breast cancer annually in men. In 2005, there were more than 12,000 deaths from breast cancer in the UK. Risk of developing breast cancer in women significantly increases with age particularly in women older than 50 (<http://www.cancerresearchuk.org>). The lifetime risk of being diagnosed with breast cancer in women is 1 in 9 (O'Halloran et al. 2004).

Over the last two decades, the number of new cases of breast cancer diagnosed has increased from 75 (in 1975) to 116 (in 2003) per 100,000 in the UK. Five year survival has also improved from 52% (1971-1975) to 80% (2001-2003). Some of this improvement is as a consequence of the National Health Service Breast Screening Programme (NHSBSP) which targets the female population aged between 50 and 64 for routine mammography. The service was set up in 1988 (Reddy and Given-Wilson 2004).

In addition, the fourth worldwide meta-analysis (2000-2005) by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) reported that interethnic variation in the incidence and mortality rate of breast cancer in women is widening. Caucasian women have a higher incidence of breast cancer whereas African women are more likely to die from breast cancer (Chia et al. 2005). Recently, Morris and colleagues (Morris et al. 2007) demonstrated that African-American patients with breast cancer appear to have more high-grade, more ER-negative and more aggressive tumours than Caucasian women. These observations suggest that differences in survival by race/ethnicity may be due to breast carcinoma characteristics. Further molecular studies are required to investigate this

correlation with survival and perhaps identify new therapeutic targets for this patient population.

The aetiology of breast cancer remains complex with multiple risk factors including hormonal receptor status (positive/negative), duration of oestrogen exposure (early menarche/late menopause), age (pre-/post-menopausal status), lifestyle (high fat/low fibre diet), racial/ethnic groups and familial history of breast cancer (Dumitrescu and Cotarla 2005). Oestrogen mediates its effects on growth, differentiation and function of target tissues particularly reproductive organs by binding to ER found in the nucleus of those cells that are targets for oestrogen action. The ER exists in two subtypes, ER α and ER β which are highly homologous and have distinct target tissue. ER α protein has been shown to be associated with breast cancer by several clinical and experimental studies, while the role of ER β in relation to breast cancer remains controversial (Deroo and Korach 2006). In addition, *in vitro* studies indicated that ER β appears to inhibit ER α activity (McDonnell 2004) and its expression is associated with the response of antioestrogen tamoxifen in ER-negative breast cancer (Gruvberger-Saal et al. 2007). Henceforth, the term 'ER status' in this thesis will refer to only ER α protein expression.

The nuclear oestrogen-ER complex binds to oestrogen response elements (EREs), resulting in transcriptional activation of oestrogen-responsive genes and this leads to the production of protein and a physiological response (Deroo and Korach 2006). In breast cancer cells, the ER signalling pathway is involved in increasing cell growth, cell division and DNA synthesis, and these can trigger DNA mutations in cells. Mutant cells can lead to uncontrolled proliferation in response to oestrogen stimulation and increase the risk of developing cancer in breast tissue (Yager and Davidson 2006). Therefore, targeting of the ER using antioestrogen therapy becomes an important therapeutic option in the treatment of breast cancer patients and this is described in the next section (McDonnell 2004).

The link between the metabolism of oestrogen and breast carcinogenesis is well documented. It is known that the carcinogenic effect of oestrogen can be attributed to the initiation of oestrogen metabolism by cytochrome P450s. A metabolite of 2-hydroxy-oestradiol is primarily catalysed by CYP1A2 and CYP3A4 in the liver, and by CYP1A1 in extrahepatic tissues including the breast. CYP1B1 which is mainly expressed in the breast, ovary and uterus catalyses the 4-hydroxylation of oestradiol. The quinone metabolites of 4-hydroxyoestradiol have been shown to act through the mechanisms that initiate and/or promote breast cancer by producing free radicals such as superoxides, and more reactive semiquinone-quinone intermediates, leading to the formation of DNA adducts (Tsuchiya et al. 2005, Yager and Davidson 2006).

1.2.2 The treatment of breast cancer

In general, breast cancer begins as a localised microscopic lesion which grows and spreads into the regional lymph nodes and metastases to other organs of the body, particularly the liver and lung, and may eventually lead to death. Traditional strategies for breast cancer therapy aim to reduce the risk of distant metastasis prevent a recurrence of tumour and improve overall survival. Clinically, a two-stage treatment strategy is commonly used to control the risk of local tumour recurrence. The first stage is surgery to control the regional tumour and this is usually supplemented with radiotherapy. Secondly, adjuvant hormonal therapy or chemotherapy is used to reduce risk of recurrence and improve long-term survival among women with breast cancer (Goldhirsch et al. 2007).

Guidelines for the use of adjuvant therapy in the management of early stage and metastases in breast cancer have developed over time. Two categories were recommended for patients with node-positive breast cancer. Adjuvant hormonal therapy (i.e. antioestrogen and aromatase inhibitors) is used for most patients who have ER-positive tumours but not recommended for women with ER-negative tumours. Another category, adjuvant

chemotherapy is advised for women with oestrogen-independent tumours (Harlan et al. 2006, Goldhirsch et al. 2007).

In hormonal therapy, a better understanding of the role of the ER signalling pathway and the action of oestrogen in breast carcinogenesis is allowing the development of therapeutic inventions, particularly in hormonal therapy which has been used to treat advanced and localised breast cancer in patients with ER-positive tumours. Antioestrogen therapy (e.g. tamoxifen and raloxifene) is commonly used as first-line therapy in pre-menopausal women. They exhibit antagonist properties by competitively inhibiting the interaction between oestrogens and ER blocking oestrogen action at the ER and reducing ER levels (Moulder and Hortobagyi 2008). However, Vogel and co-workers reported that the use of tamoxifen has been associated with a high risk of thromboembolism and ovarian cancer due to the similarity of its clinical efficacy to ovarian ablation. Raloxifene is as effective as tamoxifen in the prevention of invasive breast cancer and has a lower risk of tamoxifen's serious adverse effects (Vogel et al. 2006).

Aromatase (CYP19A1) is the key enzyme that converts androgen to oestrogen. Many aromatase inhibitors have been developed for the treatment of breast cancer and details of these are provided in Section 1.8. They are an important approach for reducing growth-stimulatory effects of oestrogen in post-menopausal oestrogen-dependent early stage breast cancer patients (Brueggemeier et al. 2005, Perez 2007). Long-term treatment of aromatase inhibitors in breast tumours have, however, resulted in resistance to tamoxifen, which may be due to hypersensitivity of ER to oestrogen, mutations in ER or alteration of ER expression. Cross-resistance between tamoxifen and aromatase inhibitor has also been reported in some breast cancer patients (Shao and Brown 2004).

In adjuvant chemotherapy, anthracycline based treatment such as the regimens of docetaxel and doxorubicin plus cyclophosphamide (TAC) are widely used as first-line therapy for node-positive breast cancer with ER-negative tumours. These regimens have been shown to have better antitumour activity than the classical CMF regimen (cyclophosphamide plus methotrexate and 5-fluorouracil) as adjuvant chemotherapy with TAC significantly improves the overall survival of breast cancer patients (Ahluwalia et al. 2005).

Doxorubicin (Adriamycin[®]) is an anthracycline antibiotic that interacts with DNA by intercalation and is widely used in the treatment of various types of cancers such as breast and ovary (O'Shaughnessy 2005). Cyclophosphamide (Cytosan[®], Neosar[®]) is an alkylating agent from oxazaphosphorine prodrug which is used against a wide range of human tumours such as breast and ovary (Moulder and Hortobagyi 2008). Docetaxel (Taxotere[®]) is a widely used taxane and the most active microtubule-interfering agents for breast cancer. It is not cross-resistant with anthracycline and does not interfere with the action of doxorubicin (Ahluwalia et al. 2005).

In addition, advances in adjuvant chemotherapy in patients who have ER-negative and node-positive breast cancer have been reported where biweekly doxorubicin and cyclophosphamide combined with paclitaxel results in a lower rate of recurrence and improved survival compared to low dose of the CAF regimen (cyclophosphamide and doxorubicin plus 5-fluorouracil) (Berry et al. 2006). However, these anticancer drugs used in the management of breast cancer have been shown to be associated with acute and long-term cardiovascular complications (Jones et al. 2007).

Indeed, these anticancer drugs offer an advantage of presenting a lethal threat to the tumour cell by killing rapidly proliferating cells. At the same time, they also have a disadvantage to the body as they affect normal cells undergoing rapid proliferation, for

example buccal mucosa, bone marrow, gastrointestinal mucosa and hair cells, contributing to the toxic effects, thus limiting the dose of cytotoxic drugs (O'Shaughnessy 2005).

Resistance to chemotherapeutics in breast cancer patients is another common phenomenon and a serious clinical problem, leading to reductions in the overall survival period. Drug resistance can be generated via a number of mechanisms, none of which are fully understood. However, tumour-specific physiological changes such as very low oxygen concentrations (tumour hypoxia) may facilitate the development of drugs that target the local gene expression responsible for malignant cells (McKeown et al. 2007). Tumour hypoxia in solid tumours has been demonstrated to be involved in tumour progression as well as resistance to chemotherapy and radiotherapy (Denny 2004). In addition, the presence of hypoxia is associated with aggressive phenotype and poor prognosis. This has led to the development of tumour-selective therapy such as hypoxia-activated prodrugs, hypoxia-activated gene therapy and treatments targeting the hypoxia-inducible factor-1 alpha (HIF-1 α) (Nagasawa et al. 2006, Brown 2007).

Due to problems with high drug toxicity and incidence of chemotherapeutic resistance, selective therapies that target the clinical and/or molecular characteristics of tumour cells are urgently required. Anticancer drugs are usually biotransformed to active and/or inactive form by certain drug metabolising enzymes. It is well recognised that cytochrome P450 enzymes (P450s) play a crucial role in the metabolism of various anticancer drugs (Section 1.8). Many cytochrome P450s have been found in extrahepatic tissues and certain cytochrome P450 genes have been defined as having tumour-specific expression.

Therefore, expression of individual cytochrome P450's in breast cancer cells is of interest as it may help identify a new target for cancer therapy resulting in more effective and less toxic treatments for breast cancer patients.

1.3 The use of breast cancer cell lines as models

In vitro systems such as cell culture have been widely established from several diseases including tumours, and have been characterised as models to represent the pathogenesis process *in vivo* (Weigelt et al. 2005). Tumour-derived cell lines have been shown to closely resemble patient tumour characteristics and reflect breast cancer cells *in vivo* (Burdall et al. 2003, Lacroix and Leclercq 2004). There are currently a limited number of breast cancer cell lines and only a few of these models (i.e. MCF7, T47D and MDA-MB-231) have been extensively used to investigate cell signalling pathways and genes involved in cancer progression (Burdall et al. 2003). Distinct features of available breast cancer cell lines have been previously detailed and classified by the origin of the cells, ethnic origin of patient, type of epithelial cells, oestrogen receptor status and *in vitro* invasive property. However, questions have been raised as to whether they are truly representative of the tissue of origin and cancer biology *in vivo*. The same type of breast cancer cell lines distributed to different environments (laboratories), grown under various culture conditions and after numerous passages could generate both genetic and phenotypic alterations in subsequent sub-populations. Serious consideration must be given to these factors when designing experiments, interpreting results and comparing findings with other studies (Lacroix and Leclercq 2004).

Table 1.1 shows a database of seven breast cancer cell lines MCF7, T47D, ZR-75-1, ZR-75-30, MDA-MB-157, MDA-MB-231 and MDA-MB-468 which were used in the present study. Of the available cell lines, most originated from pleural effusions, containing human breast adenocarcinoma cells from metastatic tumours. The advantages of using cells of this type are that they dissociate into single cells and generate viable tumour cells with low or no contamination by fibroblasts and tumour stroma cells (Lacroix and Leclercq 2004).

Table 1.1: Database of human normal breast and breast cancer cell lines used in this study (Combined and adapted from Lacroix and Leclercq 2004 and the Breast Cancer Cell Line Database at The University of Texas MD Anderson Cancer Centre website <http://www.mdanderson.org/>).

Cell line name	Age (years)	Site of origin	Pathology	Epithelial-mesenchymal transition (EMT)	ER protein status	<i>In vitro</i> invasive properties
Caucasian						
MCF7	69	Pleural effusion	Adenocarcinoma	Luminal-epithelial like	+	Low
T47D	54	Pleural effusion	Ductal carcinoma	Luminal-epithelial like	+	Low
ZR-75-1	63	Ascitic effusion	Infiltration ductal carcinoma	Luminal-epithelial like	+	Low
MDA-MB-231	51	Pleural effusion	Adenocarcinoma	Mesenchymal-epithelial like	-	High
Afro-Caribbean						
ZR-75-30	47	Ascitic effusion	Infiltration ductal carcinoma	Luminal-epithelial like	+	Low
MDA-MB-157	44	Pleural effusion	Medulla carcinoma	Mesenchymal-epithelial like	-	High
MDA-MB-468	51	Pleural effusion	Adenocarcinoma	Weakly luminal-epithelial like	-	Low

Molecular classification of breast cancer cell lines using epithelial-mesenchymal transition (EMT) is very important as EMT is associated with increased aggressiveness, and the invasive and metastatic potential of cancer cells (Sarrío et al. 2008). EMT is essential for the cell developmental process; it is an important component of tumour transformation and it can be characterised by cell adhesion, cell mobility and expression of E-cadherin. Specific characteristics of the initiation of invasive/metastatic cells are lack of cell adhesion, enhanced cell mobility and decreased E-cadherin expression. The cell lines are classified by their invasive properties, which are tested using the chemo-invasion assay that detects the migratory activity associated with matrix degradation (Matrigel in a Boyden chamber).

The cell lines can be divided into three phenotypes (Lacroix and Leclercq 2004). The first group, luminal epithelial-like cells highly express the epithelial markers (e.g. ER and E-cadherin) and they have weak *in vitro* invasiveness. This group consists of MCF7, T47D, ZR-75-1 and ZR-75-30 cell lines. The second phenotype is weakly luminal epithelial-like cells which is very similar to the first group as they are poorly invasive cells (i.e. MDA-MB-468). The last group, mesenchymal epithelial-like cells express high amount of vimentin but do not express the ER/E-cadherin gene. There are highly invasive breast cancer cell lines, such as MDA-MB-231 and MDA-MB-157 (Sommers et al. 1994, Pishvaian et al. 1999).

Breast cancer classification is based on standard histopathology which describes both the morphology and pathogenesis of the tumour. Normal breast is a bilayered structure that is composed of two types of epithelial cells, inner luminal secretory cells and outer contractile myoepithelial cells, and these cells are surrounded with basement membrane (Adriance et al. 2005). In breast tumour, it has been demonstrated that disruption of myoepithelial layer and degradation of basement membrane are required for tumour

invasion. In addition, tumour myoepithelial cells are associated with the loss of ER expression, a higher expression of invasion-related genes and a more aggressive cancer (Man and Sang 2005). Gordon and colleagues investigated myoepithelial markers (i.e. CK14 and $\alpha 6\beta 4$ integrin) in breast cancer cell lines, and demonstrated that MDA-MB-231 and MDA-MB-468 cells are defined as myoepithelial phenotype whereas MCF7, T47D and ZR-75 cells are represented as luminal phenotype (Gordon et al. 2003).

1.4 Cytochrome P450 enzymes (P450s)

1.4.1 Introduction to cytochrome P450s

Cytochrome P450 enzymes (P450s) have only been discovered and characterised in the last 50 years. The name cytochrome P450 was derived from the presence of a carbon monoxide binding pigment in rat liver microsomes which was first detected by Klingenberg (Klingenberg 1958). Six years later, this enzyme was found to have an unusual spectrum which has a very strong optical absorption peak at 450 nm (Omura and Sato 1964). The inactive form of cytochrome P450 has an absorbance maximum of 420 nm, known as cytochrome P420 (Tian et al. 1995).

Cytochrome P450s constitute a large superfamily of haem-containing proteins and are widely distributed among many organisms (e.g. bacteria, fungi, plants and animals). To date, 57 functional genes and 58 pseudogenes have been discovered and identified in humans (Nelson et al. 2004). These enzymes play a key role in several important biosynthesis and catalysis pathways (e.g. cholesterol, steroid hormones, bile acids, vitamins and arachidonic acids) as well as in the metabolism of various xenobiotic substrates including carcinogens and anticancer drugs (Guengerich et al. 2005, Nebert and Nelson 2005). The major cytochrome P450 enzymes with physiological roles in the metabolism of endogenous and xenobiotic compounds are summarised in Figure 1.1. Details of cytochrome P450 expression and functions are described in Section 1.5.

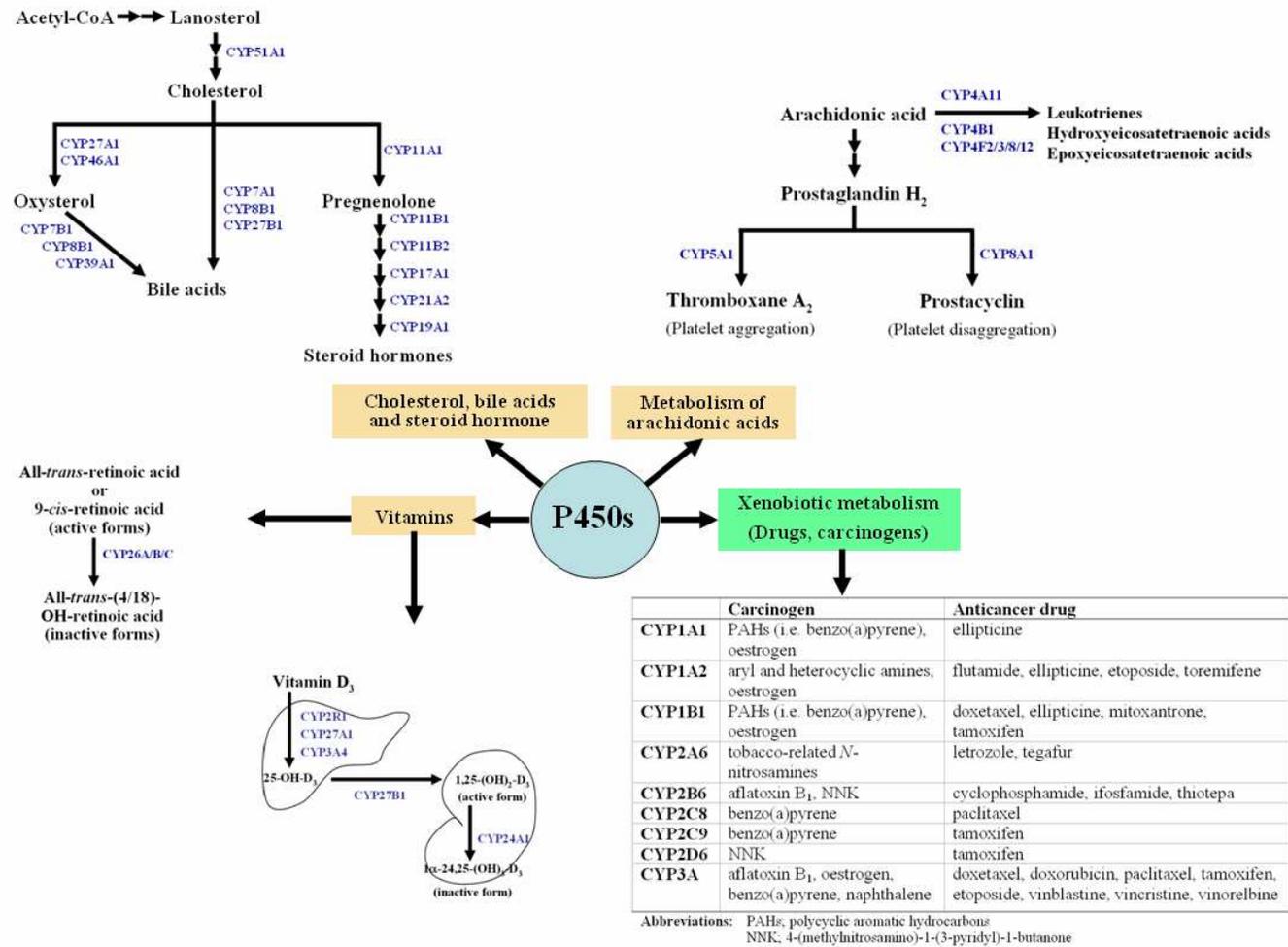


Figure 1.1: Cytochrome P450 enzymes implicated in the metabolism of endogenous and xenobiotic substrates.

1.4.2 Nomenclature

According to the standardised cytochrome P450 nomenclature, individual cytochrome P450s within a family are defined as having at least 40% amino acid sequence homology and the family can be further subdivided into subfamilies which generally have at least 55% amino acid sequence similarity. This is followed by an Arabic numeral designating the family, a capital letter for the subfamily and a sequential Arabic number for the individual gene (Nebert et al. 1991). CYP1A1, for example, has been categorised as belonging to the cytochrome P450 family 1 subfamily A and being the first individual gene identified in this subfamily (Figure 1.2).

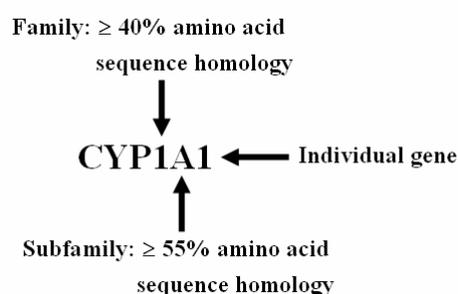


Figure 1.2: An example of human cytochrome P450 enzyme nomenclature.

1.4.3 Biochemical characteristic features

In eukaryotic cells, cytochrome P450 enzymes are primarily located in the endoplasmic reticulum or inner mitochondrial membrane. These enzymes act as the terminal oxidase in the mix-function oxidase system and are therefore also known as mono-oxygenases (Nelson et al. 1996). In a simplified form, cytochrome P450 function can be summarised as shown below (Montellano 1995).



The catalytic cycle of microsomal cytochrome P450 is shown in a schematic view in Figure 1.3. A functional cytochrome P450 system includes the active site of the catalytic reaction which is a haem moiety containing the iron ion (Fe^{3+}). The reaction begins when the substrate binds to the ferric form of cytochrome P450 in the active site (1). The

cytochrome P450-substrate complex is then reduced to the ferrous state (Fe^{2+}) by nicotinamide adenine dinucleotide phosphate-oxidase (NADPH)-cytochrome P450 reductase which serves as an electron donor protein (2). This production binds to molecular oxygen (O_2) (3) to generate asuperoxide complex which is unstable and easily auto-oxidised releasing O_2^- (4, 5). However, if the final step (the second reduction process) occurs this reaction can continue. This form converts to an activated oxygen atom which reacts with the substrate molecule (6), resulting in the hydroxylation of the substrate (7) (Coon 2003, Guengerich 2004).

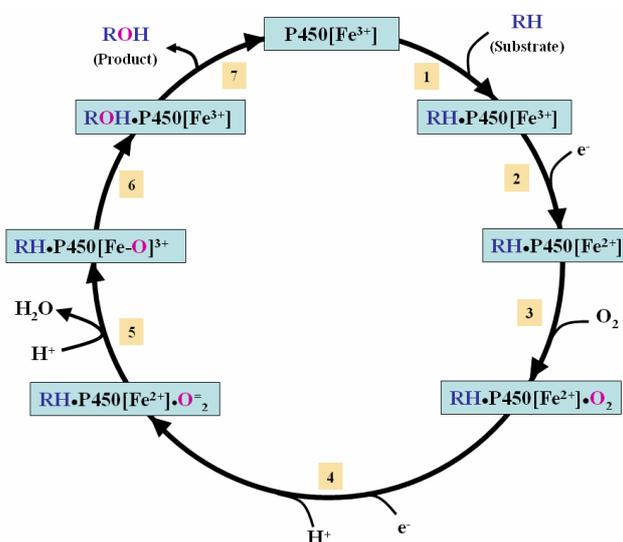


Figure 1.3: The catalytic reaction of cytochrome P450 enzymes (adapted from Coon 2003).

1.5 Cytochrome P450 expression and function

The 57 active cytochrome P450 genes are classified into 18 families and further subdivided into 43 subfamilies and they are located on 16 different chromosomes in the human genome which are summarised in Table 1.2. Enzymes belonging to families 1, 2, 3 and some enzymes of family 4 are responsible for the deactivation and/or activation of xenobiotic compounds, while the members of families 4 to 51 play a central role in the biosynthesis and catabolism of endogenous substrates. In the following sections, the importance and current knowledge of the cytochrome P450 genes are briefly described in terms of their expression and physiological roles in humans.

Table 1.2: Chromosomal location of the cytochrome P450 genes (Adapted from Nebert and Nelson 2005).

Chromosome No.	Cytochrome P450s
1	CYP2J2, CYP4A11/22, CYP4B1, CYP4X1, CYP4Z1
2	CYP1B1, CYP20A1, CYP26B1, CYP27A1
3	CYP8B1
4	CYP2U1, CYP4V2
6	CYP21A2, CYP39A1
7	CYP2W1, CYP3A4/5/7/43, CYP5A1, CYP51A1
8	CYP7A1, CYP7B1, CYP11B1/2
10	CYP2C8/9/18/19, CYP2E1, CYP26A1, CYP26C1
11	CYP2R1
12	CYP27B1
14	CYP46A1
15	CYP1A1/2, CYP11A1, CYP19A1
17	CYP17A1
19	CYP2A6/13, CYP2B6, CYP2F1, CYP2S1, CYP4F2/3/8/11/12/22
20	CYP8A1, CYP24A1
22	CYP2D6

1.5.1 CYP1 family

Three members CYP1A1, CYP1A2 and CYP1B1 are categorised in the cytochrome P450 family 1 (Nebert and Jones 1989, Nelson et al. 1996). The CYP1A1 (Jaiswal et al. 1986) and CYP1A2 (Ikeya et al. 1989) genes are located on chromosome 15q22-q24 and contain seven exons whereas the CYP1B1 gene is located on 2p21 and contains three exons (Tang et al. 1996).

CYP1A1 mRNA is mainly expressed in extrahepatic tissues including the breast (Huang et al. 1996, Hellmold et al. 1998, Iscan et al. 2001, Modugno et al. 2003). CYP1A1 catalyses the 2-hydroxylation of oestradiol and is induced by several procarcinogens, especially polycyclic aromatic hydrocarbons (PAHs) into reactive carcinogens which lead to the formation of DNA adducts (Tsuchiya et al. 2003, Shimada 2006). The induction of CYP1A1 by PAHs (e.g. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, benzo(a)pyrene and 3-methylcholanthrene) is mediated via the pathway of ligand-activated aryl hydrocarbon

receptor (AhR) complex (Kim and Guengerich 2005) which is described in Section 1.7.2.

Previous studies demonstrated that CYP1A1 mRNA appears to be expressed in both breast cancer tissues (Huang et al. 1996, Iscan et al. 2001, Modugno et al. 2003) and cell lines (Spink et al. 1998, McFadyen et al. 2003). These findings suggest that CYP1A1 may be involved in breast cancer initiation and progression because it has an important role in the metabolism of oestrogen and procarcinogens. The cancer chemotherapeutic prodrug ellipticine (an intercalative alkaloid) is activated by CYP1A1/1A2 to form DNA adducts and is currently in clinical trials (Aimova et al. 2007).

CYP1A2 is predominantly expressed in the liver but also at lower levels in extrahepatic tissues (Sterling and Cutroneo 2004). CYP1A2 mRNA has been detected in both normal human breast or breast tumours (Hellmold et al. 1998, Modugno et al. 2003). CYP1A2 can be induced by carcinogenic heterocyclic amines such as 2-amino-3,8 dimethylimidazo(4,5-f)quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo(4,5-b) pyridine (PhIP), which are produced in meat and fish during cooking (Guengerich and Shimada 1991, Kim and Guengerich 2005). It catalyses the 2-hydroxylation of oestrogen and this metabolite is believed to be associated with a reduced risk of breast cancer (Yager and Leihl 1996). Takata and colleagues investigated an association between genetic polymorphisms in enzymes involved in oestrogen metabolism and mammographic density in both breast cancer and healthy members of the Hawaiian population. Results showed an association between the CYP1A2 genotype and high levels of breast density, suggesting that CYP1A2 may be involved in the development of breast cancer (Takata et al. 2007). In addition, CYP1A2 is involved in metabolism of the anticancer drug flutamide (Chimax[®], Drogenil[®]), which is commonly used for the treatment of advanced prostate cancer (Goda et al. 2006).

CYP1B1 metabolises a wide range of procarcinogens (e.g. PAHs) and oestrogen (Shimada et al. 1996). Like CYP1A1, the induction of CYP1B1 by PAHs is mediated through the AhR/ARNT complex (Section 1.7.2). In addition, Hanna and co-workers demonstrated that inter-individual differences in breast cancer risk associated with oestrogen-mediated carcinogenicity may be related to CYP1B1 polymorphisms (Hanna et al. 2000). Recently, Yang and co-workers showed that high levels of CYP1B1 expression are associated with constitutive expression of AhR in human breast cancer but not with CYP1A1, suggesting interactions between CYP1B1 and AhR may be important in breast cancer initiation and progression (Yang et al. 2007).

CYP1B1 mRNA is expressed in several normal tissues but its protein expression has been detected primarily in tumours (i.e. breast, colon, lung, oesophagus, skin, lymph node, brain and testes) (Murray et al. 1997). In addition, Gibson and co-workers confirmed that overexpression of CYP1B1 protein was found in human colorectal tumours but lower levels were also found in normal colon cells (Gibson et al. 2003). Most importantly, CYP1B1 protein was not detected in human liver (Edwards et al. 1998). CYP1B1 overexpression in tumour cells is now recognised as a biomarker of the malignant phenotype (McKay et al. 1995, Murray et al. 1997, McFadyen et al. 2001, Roos and Bolt 2005, Barnett et al. 2007, Yang et al. 2007). Therefore, CYP1B1 is considered to be a potential novel cancer therapeutic target in a therapeutic setting, and details of how this enzyme is being targeted are provided in Section 1.8.

1.5.2 CYP2 family

CYP2 is the largest cytochrome P450 family in humans containing 13 subfamilies and 16 individual genes (Table 1.3). There are three members in the CYP2A subfamily (i.e. CYP2A6, CYP2A7 and CYP2A13) but one of these CYP2A7, has been proven to be a pseudogene (Ding et al. 1995). These genes are located on chromosome 19q13.2 and

contain nine exons (Fernandez-Salguero et al. 1995). CYP2A6 is mainly expressed in the liver but lower levels are also expressed in extrahepatic tissues including the breast (Hellmold et al. 1998). CYP2A6 plays a role in the oxidation of nicotine, coumarin (Yun et al. 1991) and anticancer drugs (i.e. tegafur and letrozole) (Rodriguez-Antona and Ingelman-Sundberg 2006). In addition, this enzyme is involved in the metabolic activation of procarcinogens, such as a tobacco-specific carcinogen 4-(methylnitrosamino)-1(3-pyridyl)-1-butanone (NNK) and the food mutagen aflatoxin B₁ (Gonzalez and Gelboin 1994). CYP2A6 mRNA and protein have been shown to be induced in human liver cells by dexamethasone and the induction by this compound is suppressed by the antagonist of glucocorticoid receptor (RU468), suggesting that the glucocorticoid receptor may play a role in the regulation of CYP2A6 (Onica et al. 2008).

Table 1.3: List of human CYP2 genes.

Subfamily of CYP2	Number of genes	Members
A	3	CYP2A6/7/13
B	1	CYP2B6
C	4	CYP2C8/9/18/19
D	1	CYP2D6
E	1	CYP2E1
F	1	CYP2F1
J	1	CYP2J2
R	1	CYP2R1
S	1	CYP2S1
U	1	CYP2U1
W	1	CYP2W1

In breast cancer, Bieche and co-workers investigated xenobiotic metabolising enzymes in breast tumours compared to normal breast. Results showed that higher levels of CYP2A6 mRNA were found in ER-positive tumours than ER-negative tumours or normal breast, suggesting that CYP2A6 could be used as a biomarker to predict an antioestrogen responsiveness (Bièche et al. 2004). It is important to note that CYP2A6 plays a role in the metabolism of a third-generation aromatase inhibitor letrozole which is widely used in

postmenopausal patients with oestrogen-dependent tumours (Ingelman-Sundberg et al. 2007). In addition, a recent study examined the regulation of CYP2A6 in the ER-positive breast cancer MCF7 cells and found that CYP2A6 is induced by oestrogen through ER. These results suggest that an interaction between CYP2A6 and oestrogen/ER may play a role in breast cancer initiation and progression (Higashi et al. 2007).

CYP2A7 is also expressed in the liver and shares 94% amino acid sequence homology with CYP2A6. However, CYP2A7 is a pseudogene as it has lost the haem-binding site (Wang et al. 2006). CYP2A13 is predominantly expressed in the respiratory tract (i.e. lung and trachea) (Su et al. 2000). This gene shares 93% amino acid sequence identity to CYP2A6 (Su et al. 2000) and their substrate-specificity is overlapping (He et al. 2004). However, the procarcinogen NNK is activated much more efficiently by CYP2A13 than CYP2A6, suggesting that CYP2A13 could be the primary enzyme in the human respiratory tract responsible for local metabolic activation of NNK and susceptibility to lung cancer (Su et al. 2000). A study by Zhang and colleagues (Zhang et al. 2007) demonstrated that a high level of CYP2A13 expression is associated with an increased risk of lung cancer particularly in smokers.

The CYP2B6 gene is located on chromosome 19q13.2 and contains nine exons (Miles et al. 1988). CYP2B6 constitutes 1-2% of the total cytochrome P450 expression in the liver (Mimura et al. 1993) and can be found in extrahepatic tissues such as lung, kidney (Nelson et al. 1996), heart (Thum and Borlak 2000) and breast (Hellmold et al. 1998, Iscan et al. 2001). CYP2B6 is highly inducible by phenobarbital, and its induction is regulated by the nuclear receptors constitutively active receptor (CAR) (Honkakoski and Negishi 2000) as described in Section 1.7.3. Extensive interindividual variability of CYP2B6 expression has been observed and polymorphisms cause functional alterations (Lamba et al. 2003), which have been shown to influence the pharmacokinetics of several drugs. CYP2B6 activates

anticancer prodrugs cyclophosphamide and ifosfamide into an active toxic form (phosphoramidate mustard and acrolein), so polymorphisms of this gene may impact on the activation of cyclophosphamide and the outcome of treatment (Zanger et al. 2007).

The human CYP2C subfamily contains four highly homologous genes CYP2C8, CYP2C9, CYP2C18 and CYP2C19 which are predominantly expressed in the liver (Shimada et al. 1994). These genes are located on chromosome 10q24.1-24.3 and contain nine exons (Gray et al. 1995). CYP2C8 plays a role in the metabolism of endogenous compounds such as arachidonic acids and retinoic acids (Zhao and Imig 2003). CYP2C8 is a major enzyme responsible for the inactivation of the anticancer drug paclitaxel which is an effective agent against a wide range of human cancers including breast cancer (Totah and Rettie 2005, Ingelman-Sundberg et al. 2007). Several research groups have investigated CYP2C mRNA in normal human breast and breast tumours but their results seem to be inconsistent. For example, Modugno and colleagues demonstrated that no CYP2C mRNA was detected in normal breast or breast cancer (Modugno et al. 2003) while two separate research groups found CYP2C mRNA in normal breast as well as breast tumour samples (Huang et al. 1996, Iscan et al. 2001). Such inconsistencies may be due to the use of matched samples, low number of samples or inter-individual variations of this enzyme.

CYP2C9, the major CYP2C form in human liver (~20%) metabolises several therapeutic drugs including the hypoglycemic agent tolbutamide, anticonvulsant phenytoin, anticoagulant warfarin, a number of nonsteroidal anti-inflammatory drugs flurbiprofen, diclofenac (Totah and Rettie 2005) and endogenous compounds arachidonic acids (Goldstein 2001). It has been shown to participate in the metabolism of anticancer prodrugs tamoxifen (Boruban et al. 2006) and ifosfamide (Schmidt et al. 2004). The genetic polymorphisms of the CYP2C9 gene have been demonstrated to have functional

consequences for clinical drug response and adverse drug effects (Ingelman-Sundberg et al. 2007).

CYP2C18 mRNA is expressed in human liver at very low levels (Furuya et al. 1991) but is also found in extrahepatic tissues (i.e. trachea, small intestine, ovary and testes) (Bièche et al. 2007). CYP2C18 shares 89%, 93% and 93% amino acid sequence identity to CYP2C8, CYP2C9 and CYP2C19 respectively (Goldstein and de Morais 1994). Zhu and Yu reported that a variant allele of the CYP2C18 gene, which is associated with decreased enzyme activity, affects anticancer ifosfamide efficacy in human hepatoma HepG2 cells (Zhu-Ge and Yu 2004).

CYP2C19 is a clinically important enzyme that metabolises a wide range of therapeutic drugs including the anticancer drugs tamoxifen and cyclophosphamide (Rodriguez-Antona and Ingelman-Sundberg 2006). Polymorphisms in the CYP2C19 gene cause poor metabolism of these drugs, resulting in drug toxicity or a severe adverse drug response to specific drugs. Approximately 3-5% of Caucasians and 12-23% of Asians have been shown to have a poor metaboliser phenotype (Mathijssen and van Schaik 2006).

Recently, Wu and colleagues investigated CYP2C19 mRNA and protein in a wide range of tumours (i.e. breast, liver, colon, stomach, esophagus, lung, uterus, brain, pancreas, ovary and kidney) derived from the Chinese population using semi-quantitative RT-PCR and Western blotting methods respectively. Results showed that the highest level of CYP2C19 mRNA was detected in hepatocarcinoma tissues and their adjacent normal tissue.

CYP2C19 protein was found only in hepatocarcinoma samples and their adjacent normal tissues, with no protein expression being detected in other types of tumour (Wu et al. 2006). These findings suggest that CYP2C19 may be a tumour-specific expression in human liver.

The CYP2D subfamily contains one functional gene CYP2D6 which is of great importance in the metabolism of several clinically used drugs (e.g. anti-arrhythmic, antidepressants and antipsychotic drugs) (Pelkonen et al. 1998). The CYP2D6 gene is located on chromosome 22q13.1 and contains nine exons (Gough et al. 1993). CYP2D6 primarily catalyses the 4-hydroxylation of tamoxifen which has potent antioestrogenic effects. Tamoxifen is commonly used to treat patients with oestrogen-dependent breast cancer (Ingelman-Sundberg 2005a). CYP2D6 mRNA and protein have been detected in the breast (Hellmold et al. 1998) and lung (Guidice et al. 1997). Furthermore, a study by Iscan and colleagues showed that CYP2D6 mRNA expression in breast tumour was equivalent to tumour-free breast tissue (Iscan et al. 2001).

Currently, more than eighty variant alleles of CYP2D6 have been identified. The CYP2D6 activity is extremely variable and interethnic variations in drug metabolism are well documented. Poor metabolisers (PMs) display low or absence of CYP2D6 activity and have been found in 7% of Caucasians, whereas ultra-rapid metabolisers (UMs) exhibit dramatically high CYP2D6 activity and have been observed in 29% of the population in North Africa (Ethiopians) and the Middle East (Saudi Arabians) (de Leon et al. 2006a). Recently, Schroth and colleagues investigated an association between breast cancer treatment outcome with adjuvant tamoxifen and genotypes of enzymes involved in tamoxifen metabolism. Findings demonstrated that CYP2D6 with low-activity alleles are unlikely to benefit from adjuvant tamoxifen treatment (Schroth et al. 2007). In light of this evidence, new clinical tools such as the AmpliChip CYP450 genotyping test are being used to analyse psychiatric patient genotypes for CYP2D6 prior to initiating treatment with tricyclic antidepressants and antipsychotics (de Leon et al. 2006b). This may be helpful for patients who take other CYP2D6 substrates including tamoxifen.

The CYP2E1 gene is located on chromosome 10q24.3-qter and contains nine exons (Kölble 1993). CYP2E1 is responsible for the oxidative metabolism of many drugs (e.g. acetaminophen, halothane and chlorzoxazone) as well as the activation of several low-molecular-weight toxicants and cancer suspect agents (e.g. ethanol, benzene, toluene and nitrosamines) (Tanaka et al. 2000). CYP2E1 is inducible by ethanol as well as pathophysiological conditions (e.g. obesity, starvation and diabetes) (Johnsrud et al. 2003). It is widely recognised that CYP2E1 produces free radicals causing tissue damage particularly the hepatotoxicity of acetaminophen (Lee et al. 1996). In addition, Reszka and colleagues examined a relationship between variant alleles of key enzymes implicated in dietary components and cancer susceptibility. Results showed that CYP2E1 high-activity alleles appear to be associated with colorectal cancer risk (Reszka et al. 2006).

The CYP2F1 gene is located on chromosome 19q13.1-13.2 and contains nine exons (Trask et al. 1993). CYP2F1 is highly expressed in the lung but at very low levels or not at all in the liver (Ding and Kaminsky 2003). A study by Thomas and co-workers showed a low level of CYP2F1 mRNA expression in sub-confluent cultured MCF10A cells but not in confluent cultures (Thomas et al. 2006). This enzyme is responsible for metabolism of the prototypical pneumotoxicants 3-methylindole and 1,1-dichloroethylene as well as the activation of carcinogens styrene, naphthalene and benzene which can produce carcinogenic epoxide in the lung tissues (Karthan and Yost 2008). In primary ovarian cancer, CYP2F1 protein has been found at higher levels than in adjacent normal ovary tissues (Downie et al. 2005).

The CYP2J2 gene is located on chromosome 1p31.2-p31.3 and contains nine exons (Ma et al. 1998). CYP2J2 is highly expressed in the coronary endothelial cells and cardiac myocytes but at lower levels in the liver, small intestine, colon and kidney (Wu et al. 1996). This enzyme is involved in the epoxidation of arachidonic acid in cardiac tissue,

forming epoxyeicosatrienoic acids (EETs) (Wu et al. 1996) which are potent endogenous vasodilators and inhibitors of vascular inflammation (Node et al. 1999, Scarborough et al. 1999). A study by Jiang and colleagues demonstrated that higher levels of CYP2J2 mRNA and protein were found in a variety of tumours (i.e breast, oesophagus, lung, stomach, liver and colon) than in their corresponding normal tissues (Jiang et al. 2005). Like CYP1B1, CYP2J2 is a tumour-associated cytochrome P450 enzyme which could be a new target for designing cancer therapeutic prodrugs activated by CYP2J2.

The CYP2R1 gene is located on chromosome 11p15.2 and contains four exons (Cheng et al. 2003). CYP2R1 (vitamin D₃ 25-hydroxylase) has been shown to play a crucial role in the synthesis of bioactive vitamin D₃. It has been long known that vitamin D₃ has potent anticancer properties because it can inhibit cell proliferation and induce cell apoptosis. Therefore, enzymes involved in vitamin D₃ signalling pathways have been developed and used as potential targets for anticancer therapeutics (Deeb et al. 2007). CYP2R1 is predominantly expressed in the testes, kidney and pancreas (Ingelman-Sundberg 2005b, Karlgren et al. 2005). Higher levels of CYP2R1 expression were detected in primary ovarian tumours than in their corresponding normal tissues (Downie et al. 2005). Like the oncogene CYP24A1 (Section 1.5.13), CYP2R1 may be of particular interest to maximise vitamin D₃ levels in tumour sites and could be used in cancer therapy.

The CYP2S1 gene is located on chromosome 19q13.1-13.2 and contains nine exons (Rylander-Rudqvist et al. 2003). CYP2S1 is highly expressed in human lung (Rivera et al. 2002, Smith et al. 2003) but at lower levels in the liver (Rylander et al. 2001). The expression of CYP2S1 mRNA and protein in human skin is induced by ultraviolet radiation, coal tar and all-*trans* retinoic acid (Smith et al. 2003). CYP2S1 is also inducible by dioxin and this induction is mediated by AhR/ARNT which is similar to a manner

typical of members of the CYP1 family, suggesting that CYP2S1 may be involved in the metabolism of carcinogens (i.e. PAHs and naphthalene) (Saarikoski et al. 2005).

In mouse models, the CYP2S1 gene is induced by hypoxia which is mediated via the HIF-1 α /ARNT heterodimer. A recent study demonstrated that induction of CYP2S1 by both dioxin and hypoxia is mediated in a large part by multiple overlapping xenobiotic and hypoxia response elements. In theory, AhR and HIF-1 α requires ARNT for dimerisation prior to binding with the DNA response element of their target genes. Therefore, an interaction between the AhR/ARNT and HIF-1 α /ARNT heterodimerisation may affect the transcriptional activity of CYP2S1 (Rivera et al. 2007). Higher levels of CYP2S1 protein expression was detected in ovarian cancer metastasis than in primary tumour cells (Downie et al. 2005). CYP2S1 protein has been shown to be associated with poor prognosis in primary colorectal cancer (Kumarakulasingham et al. 2005).

The CYP2U1 gene is located on chromosome 4q25 and contains five exons (Chuang et al. 2004). The human CYP2U1 mRNA and protein are predominantly expressed in the thymus and brain (Chuang et al. 2004, Karlgen et al. 2004). CYP2U1 converts arachidonic acid into 19- and 20-hydroxyeicosatetraenoic acid (HETE) which serves as an endogenous vasoconstrictor and plays a biologically important role in the modulation of blood flow in the brain (Chuang et al. 2004, Karlgren et al. 2005). The role of CYP2U1 in the metabolism of xenobiotic compounds has not been reported. In primary ovarian cancer, a markedly higher level of CYP2U1 protein was found compared with normal ovary samples (Downie et al. 2005).

The CYP2W1 gene is located on chromosome 7q22.3 and contains 9 exons (Nebert and Nelson 2005). CYP2W1 has been detected at very low levels or not at all in foetal and adult human normal tissues but with higher CYP2W1 mRNA levels occurring in human

tumours particularly in colon and adrenal tumours (Karlgrén et al. 2006). This may suggest that CYP2W1 is specifically expressed in tumours and that it could potentially be targeted by a novel anticancer drug (Karlgrén and Ingelman-Sundberg 2007). A study by Thomas and co-workers showed a low level of CYP2W1 mRNA in the human normal breast MCF10A cell line (Thomas et al. 2006) but no information on CYP2W1 expression in breast cancer. To date, little is known about the physiological function of CYP2W1 in humans.

1.5.3 CYP3 family

The human CYP3 family has a single subfamily CYP3A and four individual genes which account for approximately 30% of total liver cytochrome P450 content (Shimada et al. 1994). This family is the most important group of drug metabolising enzymes in humans, therefore, intra- and inter-individual variations of these enzymes may influence the efficacy of drug treatment. CYP3A4 (Inoue et al. 1992), CYP3A5 (Gellner et al. 2001), CYP3A7 (Gellner et al. 2001) and CYP3A43 (Gellner et al. 2001) genes are all located on chromosome 7q22.1 and contain thirteen exons.

CYP3A4 is the most abundant cytochrome P450s in human adult liver (Wrighton and Stevens 1992) and is also found in the small intestine (Kolars et al. 1992). CYP3A4 is responsible for the oxidative metabolism of a wide range of xenobiotic compounds including approximately 60% of all therapeutic drugs such as anticancer drugs paclitaxel, mitoxantrone, tamoxifen, cyclophosphamide and ifosfamide (Guengerich 1999). In addition, CYP3A4 is involved in the metabolism of several steroid hormones (i.e. oestrogen, progesterone and testosterone) (Wrighton and Stevens 1992) and activation of procarcinogens (e.g. aflatoxin B₁) to carcinogens (Forrester et al. 1990). The induction of CYP3A4 by these compounds is mainly mediated by pregnane X receptor (PXR, Section 1.7.4). Studies on CYP3A mRNA expression in normal human breast and breast tumour

samples appear to be generating inconsistent results. For example, Iscan and colleagues demonstrated that CYP3A mRNA was not detected in normal breast or breast tumours (Iscan et al. 2001), whereas Huang and co-workers reported that CYP3A mRNA was higher in non-tumour breast tissues than in breast tumour samples (Huang et al. 1996). In addition, Haas and colleagues reported that strong CYP3A4 protein expression was significantly associated with poor tumour differentiation (Haas et al. 2006).

CYP3A5 shares 84% amino acid sequence identity to CYP3A4 and its substrates are similar to those of CYP3A4. CYP3A5 is primarily expressed in the liver and extrahepatic tissues (e.g. kidney and intestine) (Blake et al. 2005). High levels of CYP3A5 protein appear to be associated with primary colorectal cancer (Kumarakulasingham et al. 2005) and ovarian cancer (Downie et al. 2005) compared with their corresponding normal tissue. Large inter-individual and ethnic differences exist in the expression of CYP3A5 (Gervasini et al. 2007). A recent study reported that no association between CYP3A5 polymorphisms, and cancer risk was observed in Caucasian patients with liver, gastric or colorectal cancer (Gervasini et al. 2007).

CYP3A7 is predominantly expressed in human foetal liver. CYP3A7 has similar catalytic properties to CYP3A4, and catalyses the 6 β -hydroxytestosterone and 16 α -hydroxylation of oestriol but not of oestradiol. This enzyme is involved in oestriol biosynthesis which it is an important enzyme for foetal growth and development (Blake et al. 2005). However, CYP3A7 has been shown to be involved in the formation of the procarcinogenic 16 α -hydroxyestrone in women (Lee et al. 2003). In addition, higher levels of CYP3A7 protein are found in primary ovarian cancer than in normal ovary tissue (Downie et al. 2005).

CYP3A43 is a novel cytochrome P450 belonging to subfamily CYP3A. CYP3A43 shares 76%, 76%, and 72% amino acid homology with CYP3A4, CYP3A5, and CYP3A7

respectively (Westlind et al. 2001). It is mainly expressed in the prostate (Domanski et al. 2001) but also at very low level in the liver which differs from other CYP3A subfamily enzymes (Westlind et al. 2001). Moreover, Zeigler-Johnson and colleagues examined an association between genotype of enzymes involved in testosterone metabolism and prostate cancer risk. Findings showed that a CYP3A43 variant seems to be associated with prostate cancer susceptibility (Zeigler-Johnson et al. 2004). In addition, CYP3A43 protein in primary ovarian cancer has been found to be higher than in normal ovary tissue (Downie et al. 2005).

1.5.4 CYP4 family

The CYP4 family consists of six subfamilies and twelve members (Table 1.4). The CYP4A11 (Bell et al. 1993) and CYP4A22 (Nebert and Nelson 2005) genes are located in a cluster of other cytochrome P450 genes (i.e. CYP4B1, CYP4Z1 and CYP4X1) on chromosome 1p33 (Bellamine et al. 2003) and contain twelve exons. CYP4A11 is predominantly expressed in the kidney and liver (Savas et al. 2003, Ito et al. 2007). It catalyses the ω -hydroxylation of fatty acids (i.e. lauric acid, palmitic acid and arachadonic acid) (Sharma et al. 1989, Palmer et al. 1993). CYP4A mRNA expression is induced by the synthetic glucocorticoid dexamethasone (Savas et al. 2003) and several peroxisome proliferators including hypolipidemic drugs clofibrate ciprofibrate and Wy14643 (or pirinixic acid) through a nuclear receptor called the peroxisome proliferator activated receptor alpha (PPAR α) (Okita and Okita 2001, Hsu et al. 2007) as detailed in Section 1.7.6.

CYP4A22 shares 96% amino acid sequence identity to CYP4A11. CYP4A22 mRNA is found to be expressed in the liver and kidney; however, the levels of CYP4A22 mRNA expression in human liver were lower than CYP4A11. Unlike CYP4A11, CYP4A22

mRNA is not induced by glucocorticoids and peroxisome proliferators (Savas et al. 2003).

The physiological role of human CYP4A22 is being investigated (Hsu et al. 2007).

Table 1.4: List of human CYP4 genes.

Subfamily of CYP4	Number of genes	Members
A	2	CYP4A11/22
B	1	CYP4B1
F	6	CYP4F2/3/8/11/12/22
V	1	CYP4V2
X	1	CYP4X1
Z	1	CYP4Z1

The CYP4B1 gene is located on chromosome 1p34-12 and contains twelve exons (Nhamburo et al. 1989). CYP4B1 is selectively expressed in human lung but at lower levels in the liver and gastrointestinal tissues (Poch et al. 2005). CYP4B1 is involved in the activation of the pulmonary toxicant 4-ipomeanol and the metabolism of various arylamines such as 2-aminofluorene, to more reactive intermediates which can cause bladder cancer (Baer and Rettie 2007), suggesting that CYP4B1 may be of interest for designing an anticancer prodrug activated by CYP4B1. Unlike other CYP4 enzymes, the role of CYP4B1 in the metabolism of endogenous compounds has not been reported.

There are six enzymes in the human CYP4F subfamily (Table 1.4) which are located on chromosome 19p13 (Nebert and Nelson 2005). CYP4F2 encodes for a leukotriene B4 (LTB4) ω -hydroxylase which inactivates LTB4 by ω -hydroxylation (Hsu et al. 2007). A study by Zhang and Hardwick demonstrated that this enzyme is highly expressed in human hepatoma HepG2 cells and the CYP4F2 gene is modulated by all-*trans* retinoic acid (Zhang and Hardwick 2000).

CYP4F3 consists of two forms, CYP4F3A and CYP4F3B, which arise from different promoters (Christmas et al. 1999). CYP4F3A preferentially catalyses the ω -hydroxylation of LTB₄ and is mainly expressed in human polymorphonuclear leukocytes whereas CYP4F3B mainly catalyses fatty acids especially long chain fatty acid (Hsu et al. 2007). CYP4F3B shares 92% amino acid identity with CYP4F2 and they are predominantly expressed in the liver and kidney (Christmas et al. 2001).

CYP4F8 (prostaglandin 19-hydroxylase) shares 81.2% and 76.7% amino acid similarity to CYP4F2 and CYP4F3 respectively (Bylund et al. 1999). CYP4F8 mRNA and protein are detected in various normal tissues (i.e. seminal vesicle epithelium, epidermis, corneal epithelium and proximal renal tubules) (Stark et al. 2003). CYP4F8 is closely related to CYP4F12 as they exhibit an overlapping catalytic profile. They convert arachidonic acid to 18-HETE, and metabolise prostaglandin H₁ and H₂ to 19-hydroxyl compounds (Stark et al. 2005, Hsu et al. 2007).

CYP4F11 and CYP4F12 mRNA are mainly expressed in human liver but also at lower levels in the kidney (Kalsotra et al. 2004). These two enzymes have different catalytic profiles. CYP4F11 efficiently metabolises xenobiotic compounds such as erythromycin but does not efficiently catalyse endogenous compounds particularly eicosanoids. In contrast, CYP4F12 efficiently hydroxylates arachidonic acids to 18-HETE (Bylund et al. 2001, Hsu et al. 2007). Their physiological roles have not been characterised.

CYP4F22 mRNA has been demonstrated to be expressed in human keratinocytes, testes, liver, kidney, small intestine and brain cell lines. Little is known about human CYP4F22. A recent study showed that mutations of the CYP4F22 gene are associated with lamellar ichthyosis type 3, suggesting that this enzyme may play a role in the pathway of 12R-

lipoygenase which is implicated in the regulation of barrier production in skin (Lefevre et al. 2006).

CYP4V2 is located on chromosome 4p35.1 and contains eleven exons (Li et al. 2004).

CYP4V2 mRNA is highly expressed in the retina but also found in a wide range of human tissues (i.e. heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas and lymphocytes) (Li et al. 2004). In human normal breast MCF10A cells, CYP4V2 mRNA was also detected (Thomas et al. 2006). In addition, CYP4V1 protein has been shown in colorectal cancer compared with adjacent normal colon samples. Results showed that higher levels of CYP4V1 were detected in colon cancer with lymph node metastasis than in their corresponding primary tumour cells (Kumarakulasingham et al. 2005). To date the catalytic properties and physiological function of CYP4V2 remain unknown.

The CYP4X1 gene is located on chromosome 1p33 and contains twelve exons (Nelson 2003). CYP4X1 mRNA is highly expressed in human trachea and aorta but at lower levels in the breast, ovary, uterus, liver and kidney (Savas et al. 2005). Recently, CYP4X1 protein has been shown to be specifically expressed in mouse brain (Al-Anizy et al. 2006). A study by Savas and co-workers indicated that CYP4X1 mRNA in human hepatoma HepG2 cells is regulated by PPAR α (Savas et al. 2005). A physiological role of CYP4X1 is currently unknown.

CYP4Z1 is located on chromosome 1p33 and contains twelve exons (Rieger et al. 2004).

CYP4Z1 mRNA is preferentially expressed in human mammary gland but lower levels in the liver, kidney, heart, brain, lung, prostate, testes and ovary. CYP4Z1 mRNA and protein are overexpressed in breast tumour compared to their corresponding normal tissue (Rieger et al. 2004). Higher levels of CYP4Z1 protein were found in primary ovarian cancer than in normal ovary tissue and its expression in primary ovarian cancer appears to

be an independent marker of prognosis (Downie et al. 2005). A study by Savas and colleagues demonstrated conditional regulation of the human CYP4Z1 gene in breast cancer cell lines T47D and MCF7 using quantitative RT-PCR. Results suggested that CYP4Z1 mRNA is induced by the glucocorticoid and progesterone receptor agonists but not by PPAR α agonist Wy14643 (Savas et al. 2005).

1.5.5 CYP5 family

The CYP5A1 gene is located on chromosome 7q34-q35 and contains thirteen exons (Chase et al. 1993). CYP5A1 (thromboxane A₂ synthase) has a role to play in the conversion of the prostaglandin endoperoxide into thromboxane A₂. Thromboxane A₂ is a potent vasoconstrictor and inducer of platelet aggregation which is important in the hemodynamic status in humans (Schuster and Bernhardt 2007). In addition, Nie and co-workers reported finding higher levels of CYP5A1 expression in prostate cancer cells than in normal prostate epithelial cells (Nie et al. 2004).

1.5.6 CYP7 family

Currently, the CYP7 family consists of two subfamilies, CYP7A1 and CYP7B1. CYP7A1 shares 40% amino acid sequence homology to CYP7B1. Both genes are located on chromosome 8q21.13. The CYP7A1 and CYP7B1 genes contain six and five exons respectively (Setchell et al. 1998). CYP7A1 (cholesterol 7 α -hydroxylase) directly converts cholesterol into 7 α -hydroxycholesterol, resulting in the production of deoxycholic acid (Beigneux et al. 2002). CYP7A1 has been found to be expressed only in the liver (Wu et al. 1999). The down-regulation and up-regulation of CYP7A1 are mediated by farnesoid X receptor (FXR, Section 1.7.8) and liver X receptor alpha (LXR α) respectively. A previous study showed that high levels of the deoxycholic acid in plasma are found in postmenopausal women with breast cancer. In addition, FXR has been found to be highly

expressed in breast tumours and involved in the induction of apoptosis (Swales et al. 2006).

CYP7B1 mRNA is found in the liver and extrahepatic tissues (i.e. brain, testes, prostate, ovary and kidney). CYP7B1 mainly catalyses 7α -hydroxylation of oxysterol (25- and 27-hydroxycholesterol) and neurosteroids [dehydroepiandrosterone (DHEA) and pregnenolone] (Wu et al. 1999). CYP7B1 expression is sexually dimorphic (male > female). A study by Martin and co-workers demonstrated that CYP7B1 generates 7α -hydroxy-DHEA to selectively activate oestrogen receptor beta in human prostate epithelium. This suggests that CYP7B1 may have a role in the regulation of androgen and/or oestrogen in prostate tissue (Martin et al. 2004).

1.5.7 CYP8 family

The human CYP8 family encodes two subfamilies, CYP8A and CYP8B (Nelson et al. 1996). The CYP8A1 gene resides on chromosome 20q13.11-13.13 and contains ten exons (Yokoyama et al. 1996). CYP8A1 (prostacyclin synthase) is widely expressed in human tissues (i.e. ovaries, heart, skeletal muscle, lung and prostate) (Miyata et al. 1994). It converts prostaglandin H_2 to prostacyclin (prostaglandin I_2) whose function is opposite to that of CYP5 (thromboxane A_2 synthase). Prostacyclin is a potent vasodilator and inhibitor of platelet aggregation. In addition, a stable prostacyclin analogue, cicaprost has been demonstrated to play a key role in the prevention of metastasis and reduce growth of breast tumours in rodent models (Schirner and Schneider 1997). Previous study showed that the levels of prostacyclin were reduced in colon cancer samples as compared with matched adjacent normal colon samples (Miyata et al. 1994). Recently, variants of CYP8A1 have been demonstrated to be associated with colorectal polyps risk (Poole et al. 2006).

The CYP8B1 gene is located on chromosome 3p21.3-22 and contains only one exon (Gafvels et al. 1999). CYP8B1 (sterol 12 α -hydroxylase) is found in the liver. It plays an important role in bile acid biosynthesis which is closely related to the CYP7 family (Eggertsen et al. 1996, Gafvels et al. 1999). CYP8B1 activity can be altered by fasting, cholestyramine, streptozotocin-induced diabetes mellitus and clofibrate (Eggertsen et al. 1996). It has been long known that soy isoflavones are promising agents for breast cancer prevention. Recently, Li and colleagues demonstrated that human hepatic CYP8B1 expression can be up-regulated by soy isoflavones, suggesting that these agents can modulate cholesterol metabolism through CYP8B1 (Li et al. 2007).

1.5.8 CYP11 family

The CYP11 family consists of two subfamilies containing three enzymes; CYP11A, CYP11B1 and CYP11B2 which are localised to the inner mitochondrial membrane. The CYP11A1 gene is located on chromosome 15q23-q24 and contains nine exons (Chung et al. 1986). This gene encodes the cholesterol side chain cleavage enzyme that catalyses the initial and rate-limiting step of steroid biosynthesis (Payne and Hales 2004). CYP11A1 is predominantly expressed in steroidogenic tissues such as adrenal cortex, ovary, testes, placenta and central/peripheral nervous systems (Compagnone et al. 1995). Yaspan and colleagues investigated the relationship between variation of genes involved in oestrogen biosynthesis and metabolism, in particular CYP11A1, and breast cancer susceptibility in a study of case-control from the Chinese population. Findings showed that the CYP11A1 polymorphisms may have a role in breast cancer (Yaspan et al. 2007).

The CYP11B1 gene shares 95% amino acid sequence homology with the CYP11B2 gene (Mornet et al. 1989) and these genes are mainly expressed in the adrenal cortex (Curnow et al. 1991). Both CYP11B1 and CYP11B2 genes reside on chromosome 8q21-q22 and contain nine exons (Mornet et al. 1989). CYP11B1 (11 β -hydroxylase) catalyses the last

steps in cortisol and corticosterone biosynthesis. CYP11B2 (aldosterone synthase) specifically catalyse aldosterone biosynthesis (Hakki and Bernhardt 2006). Inhibition of steroid biosynthesis by members of the CYP11 family is one of the therapy options for patients with inoperable adrenocorticoid cancer and hypercortisolism. However, CYP11B1 and CYP11B2 deficiencies can cause severe side effects such as Cushing's syndrome which leads to chronic glucocorticoid excess. Further development of selective inhibitors for distinct enzymes in this family is required (Schuster and Bernhardt 2007).

1.5.9 CYP17 family

The CYP17A1 gene is located on chromosome 10q24.3 which consists of eight exons (Fan et al. 1992). CYP17 (17 α -hydroxylase or 17,20-lyase) catalyses both 17 α -hydroxylation and 17,20-lyase conversion of C21 steroids (progesterone or pregnolone) to C19 precursors of sex steroids (dehydroepiandrosterone or androstenedione) (Payne and Hales 2004). CYP17A1 is closely related to CYP21 and it shares almost 30% amino acid sequence identical to CYP21, however; CYP17A1 has lost two exons which are present in CYP21 (Picado-Leonard and Miller 1987). CYP17A1 is predominantly expressed in steroidogenic tissues such as the adrenal cortex and ovary (Nagamani and Urban 2003, Olson et al. 2007).

CYP17A1 is a key enzyme in the androgen biosynthesis. Androgen is an important hormone in the development and maintenance of sexual characteristics in human males; however, it is believed to play a role in androgen-dependent diseases, in particular prostate cancer (Bruno and Njar 2007, Schuster and Bernhardt 2007). Thus the use of potent CYP17A1 inhibitors such as ketoconazole have been introduced to treat androgen-dependent prostate cancer patients (Bruno and Njar 2007, Moreira et al. 2007) and details of this treatment are provided in Section 1.8. Recently, the interactions between gene-gene and gene-environment which are involved in hormone biosynthesis and metabolism

pathways have been investigated by several research groups. One of these studies demonstrated that CYP17A1 polymorphism in combination with long-term hormone-replacement therapy use and high body mass index in postmenopausal women may contribute to breast cancer susceptibility (Chen et al. 2008).

1.5.10 CYP19 family

The CYP19A1 gene resides on chromosome 15q21.1 which contains eleven exons (Chen et al. 1988). CYP19A1 (aromatase or oestrogen synthase) has important physiological functions both in sex steroid hormone biosynthesis and growth/ differentiation in humans. It converts the C19 androgens, testosterone and 16 α -hydroxyandrostenedione into the C18 oestrogens, oestradiol and oestrone respectively. This enzyme is widely expressed in several tissues (i.e. ovary, Leydig cells, placenta, adipose tissue and bone), however; tissue- and sex-specific expression in cellular metabolism have been demonstrated in these tissues as reviewed by Payne and Hales (Payne and Hales 2004). CYP19A1 activity appears to be increased in breast cancer tissues, thus several potent CYP19A1 inhibitors have become a powerful tool in the treatment of oestrogen-dependent diseases, in particular breast cancer (Brueggemeier et al. 2005, Gibson et al. 2007, Jonat et al. 2007, Perez 2007). Details of CYP19 inhibitors in cancer therapy are provided in Section 1.8.

1.5.11 CYP20 family

The CYP20A1 gene is located on chromosome 2q33 which contains thirteen exons. Only one member has been found in this family and human CYP20A1 has been shown to be homologous in mouse, cow and fish but not in *Drosophila* or *Caenorhabditis elegans* (Nebert and Nelson 2005). A recent study suggests that a high level of CYP20A1 expression is found in immune system cell types (Thomas 2007) but its physiological function in humans is still unknown.

1.5.12 CYP21 family

The CYP21A2 is found on chromosome 6p21.3 which contains ten exons (Higashi et al. 1986). CYP21A2 (steroid 21-hydroxylase) is mainly expressed in the adrenal cortex (Wijesuriya et al. 1999) and converts the C21 of progesterone and 17α -hydroxyprogesterone into 11-deoxycorticosterone and 11-deoxycortisol respectively in the zona fasciculata of the adrenal cortex. CYP21A2 plays an important role in the biosynthesis of sex hormones, glucocorticoids and mineralocorticoids, and is closely related to CYP17A1 (Payne and Hales 2004, Dragan et al. 2006). Dragan and colleagues determined the effect of IC_{50} values of various known CYP17A1 inhibitors (i.e. ketoconazole, Sa40, YZ5ay and BW33) on CYP21A2 expression in a fission yeast strain expressing mammalian cytochrome P450 steroid hydroxylases. Results showed that CYP17A1 inhibitors appear to be moderate CYP21A2 inhibitors (Dragan et al. 2006).

1.5.13 CYP24 family

The CYP24A1 gene resides on chromosome 20q13.2-13.3 and contains twelve exons (Hahn et al. 1993). The mitochondrial enzyme CYP24A1 (1, 25-(OH) $_2$ D $_3$ -24-hydroxylase) plays a crucial role in the degradation of vitamin D $_3$ (1, 25-(OH) $_2$ D $_3$) (Masuda et al. 2006). CYP24A1 mRNA is regulated either by calcium ions or excess amounts of vitamin D $_3$ and is mediated through its nuclear receptor vitamin D receptor (VDR). CYP24A1 is expressed in several normal tissues such as kidney, liver and gastrointestinal tract (Jones et al. 1998). However, Anderson and colleagues detected overexpression of CYP24A1 in a wide range of tumour cells (i.e. breast, colon, ovary and lung) compared with their matched adjacent normal cells, suggesting that CYP24A1 may be an oncogene (Anderson et al. 2006). Inhibition of CYP24A1 has been developed as a new strategy for cancer therapy as it has an antiproliferative effect (Sundaram et al. 2006) (Section 1.8).

1.5.14 CYP26 family

Three CYP26 isoforms have been identified in humans, CYP26A1, CYP26B1 and CYP26C1 (Ray et al. 1997, Nelson 1999, Taimi et al. 2004). CYP26 is induced by all-*trans*-retinoic acid (ATRA) through its nuclear receptor (retinoic acid receptor, RAR) and retinoid X receptor (RXR), which then interacts with retinoic acid response elements within the promoter of CYP26 (Bastien and Rochette-Egly 2004). ATRA is an active metabolite of vitamin A or retinoid which play an important role in the cell differentiation and proliferation of epithelial cells, vision and reproduction (White et al. 2000). ATRA has been shown to induce apoptosis in various cancer cell lines, suggesting that it can be used as a chemopreventive agent (Osanai and Petkovich 2005)

The CYP26A1 gene is found on chromosome 10q23-q24 and contains seven exons (White et al. 1998). CYP26A1 is mainly expressed in the brain, liver and placenta (Ray et al. 1997). The metabolism of ATRA by CYP26A1 can produce two main metabolites, 4-hydroxy-ATRA and 4-oxo-ATRA which can bind to RAR but with low affinity, resulting in the inactivation of ATRA (Villani et al. 2004). This indicates that high levels of CYP26A1 decrease the effects of ATRA in target cells. A higher level of CYP26A1 protein was detected in primary ovarian cancer than in normal ovary tissues (Downie et al. 2005). In addition, overexpression of CYP26A1 has been shown to be associated with human breast and colon cancer cells (Sonneveld et al. 1998), suggesting that inhibition of CYP26A1 may be a new strategy to increase local ATRA and inhibit the catabolism of ATRA. CYP26A1 inhibitors (e.g. liarozole, R116010 and R115866) have entered clinical trials for the treatment of cancer patients (Schuster and Bernhardt 2007) and these are described in Section 1.8.

The CYP26B1 gene carries six exons located on chromosome 2p12 (Nebert and Nelson 2005). CYP26B1 is mainly expressed in the adult brain several regions (i.e. cerebellum

and pons) (White et al. 2000) and embryonic tissues (MacLean et al. 2007). CYP26B1 is involved in the metabolism of ATRA which is similar to CYP26A1. In animal models, it appears to have an essential role in the development of the embryo (MacLean et al. 2001).

The CYP26C1 is the newest member of the CYP26 family to be identified in humans. CYP26C1 expression is found in human keratinocyte cell lines. It can be induced by ATRA and 9-cis-retinoic acid which differs from CYP26A1 and CYP26B1 (Taimi et al. 2004, Njar et al. 2006). However, the physiological function of CYP26C1 relevant to diseases in humans remains unknown.

1.5.15 CYP27 family

The three subfamilies CYP27A1, CYP27B1 and CYP27C1 have been identified in the human CYP27 family. The CYP27A1 gene is located on chromosome 2q33-qter and contains eleven exons (Cali and Russell 1991). CYP27A1 is a multifunctional mitochondrial enzyme, sterol 27-hydroxylase, that is predominantly expressed in the liver and catalyses several oxidative reactions in the biosynthesis of bile acids and the bioactivation of vitamin D₃ (Gottfried et al. 2006). This enzyme is an initial enzyme in the pathway of bile acid biosynthesis which is related to CYP7A and CYP8B. Tang and co-workers demonstrated the regulation of CYP27A1 mRNA and activity in human hepatoma HepG2 and prostate cancer LNCaP cells. Results showed that CYP27A1 is regulated by hormones especially oestrogen and androgen compounds (Tang et al. 2007).

The CYP27B1 gene is located on chromosome 12q13.1-q13.3 and contains nine exons (St-Arnaud et al. 1997). CYP27B1 converts the vitamin D₃ precursor into the most active form of vitamin D₃ (1,25-(OH)₂D₃) in the kidney and is also called 25-OH-D₃-1 α -hydroxylase. This enzyme has a crucial role in physiological function such as cell differentiation and proliferation (Takeyama et al. 1997). In the proximal tubules,

CYP27B1 mRNA and protein have been shown to be repressed in the presence of $1,25\text{-(OH)}_2\text{D}_3$. CYP27B1 was found in non-renal tissues (i.e. skin, lymph nodes, colon, pancreas and brain) (Zehnder et al. 2001) including human mammary epithelial cells (Kemmis et al. 2006). In addition, several cancer cell lines have shown CYP27B1 expression (Diesel et al. 2005, Becker et al. 2007, Cordes et al. 2007, Turunen et al. 2007). A study by de Lyra and colleagues showed that decreased levels of $1,25\text{-(OH)}_2\text{D}_3$ are associated with breast cancer (de Lyra et al. 2006). In addition, maximising $1,25\text{-(OH)}_2\text{D}_3$ by using the combination of a CYP24A1 inhibitor (genetein) and a CYP27B1 inducer (trichostatin A) has been demonstrated to increase the antiproliferative effect of $1,25\text{-(OH)}_2\text{D}_3$ in prostate cancer cells (Farhan et al. 2003, Khorchide et al. 2005). Currently, the CYP27C1 gene has been sequenced and identified in humans but it was not detected in rodent (Nelson et al. 2004). The physiological function of CYP27C1 remains unknown.

1.5.16 CYP39 family

The CYP39A1 gene is located on chromosome 6p21-p11.2 and carries twelve exons (Li-Hawkins et al. 2000). CYP39A1 is predominantly found in the liver and its expression in females is higher than in males, which is opposite to that of CYP7B1 expression. In the liver, 7α -hydroxylation of cholesterol is the rate-limiting step of bile acid synthesis and metabolic elimination, and this is mediated by CYP7A1 and CYP39A1. CYP39A1 is specific for 24S -hydroxycholesterol as a substrate. Dietary cholesterol, bile acids or a bile acid-binding resin does not affect hepatic CYP39A1 mRNA level (Li-Hawkins et al. 2000). Higher levels of CYP39A1 protein were found in colorectal cancer patients with lymph node metastasis than in their corresponding primary tumours (Kumarakulasingham et al. 2005).

1.5.17 CYP46 family

The CYP46A1 gene resides on chromosome 14q32.1 and contains fifteen exons (Lund et al. 1999, Nebert and Nelson 2005). This gene encodes cholesterol 24S hydroxylase which is a rate-limiting enzyme for cholesterol removal from the brain. CYP46 mRNA and protein are selectively expressed in human brain particularly in neurons and some astrocytes. It modulates the turnover of cholesterol in the brain by converting excess cholesterol into 24S-hydroxycholesterol (Bjorkhem et al. 1998). High levels of CYP46A1 have been shown to be associated with degenerating neuritic plaques in Alzheimer's disease. These observations suggest that CYP46 expression may lead to cholesterol being abundant in plasma, resulting in greater loss of synapses in the brain and the development of Alzheimer's disease (Bogdanovic et al. 2001, Desai et al. 2002, Brown et al. 2004). Recently, Bretillon and colleagues examined CYP46A1 expression in mammalian neural retina using RT-PCR and immunohistochemical analysis. Results showed that CYP46A1 mRNA and protein were specifically expressed in bovine and rat samples (Bretillon et al. 2007).

1.5.18 CYP51 family

The CYP51A1 gene is located on chromosome 7q21.2-21.3 and contains ten exons (Nebert and Nelson 2005). CYP51A1 (lanosterol 14 α -demethylase) is found in both prokaryotes and eukaryotes (Aoyama et al. 1996, Waterman and Lipesheva 2005). CYP51A1 mRNA is expressed in most tissues in humans but the highest expression has been detected in Leydig cells in the testes (Stromstedt et al. 1998). CYP51A1 plays an important role in the key step of cholesterol biosynthesis as it catalyses the oxidative removal of the 14 α -methyl group from lanosterol (Rozman et al. 1996). Inhibitors of human CYP51A1 have been attracting interest in antimicrobial and anticholesterolemic drug research (Waterman and Lipesheva 2005, Schuster and Bernhardt 2007). A high expression of CYP51 protein has been shown to be associated with poor prognosis and is an independent marker of

prognosis in colorectal cancer (Kumarakulasingham et al. 2005). In addition, a higher level of CYP51A1 protein was detected in primary ovarian cancer than in normal ovary tissues (Downie et al. 2005).

1.6 Expression of cytochrome P450s in breast cancer cell lines

As mentioned in Section 1.3, *in vitro* cell culture models in breast cancer cells have been extensively studied in preclinical models with respect to the investigation of molecular mechanisms involved in breast carcinogenesis, mechanisms of drug resistance/sensitivity in tumour cells and the identification of novel targets for cancer therapeutics (Lacroix and Leclercq 2004). Knowledge of cytochrome P450 enzymes derived from preclinical models, including their expression profile can be representative of the malignant cells in relation to clinical findings. MCF7 is the most popular of the oestrogen-dependent breast cancer cells and this cell line has been shown to stably express cytochrome P450s (i.e. CYP1A1) in a similar manner to their tissue of origin (Rekha and Sladek 1997, Michael and Doherty 2005).

However, there remains controversy regarding mRNA/protein expression profile in breast cancer tissues and cell lines. For instance, expression of CYP1B1 mRNA and protein appeared to be high in human breast cancer tissues (McKay et al. 1995, Murray et al. 1997, McFadyen et al. 1999), but MCF7 cells expressed CYP1B1 mRNA but not protein (McFadyen et al. 2003). This may be due to the expression alteration of key transcription factors involved in the translation of cytochrome P450. It reflects individual variation in the expression profiles. Nevertheless, cell line models are useful tools, especially in human breast cancer research where animal models may not truly represent cytochrome P450 expression found in humans as species and strain differences in expression of these enzymes are well recognised (Miles et al. 1990, Michael and Doherty 2005). No model is perfect and it is important to keep these limitations in mind.

Previous studies have demonstrated the presence of cytochrome P450 mRNA in breast cancer cell lines. However, a very few of these models (i.e. MCF7, T47D and MDA-MB-231) have been widely used and only a limited number of individual cytochrome P450 enzymes are currently available in these models. Also, there appears to be a lack of data on the association between cytochrome P450 expression and breast tumour characteristics (i.e. oestrogen receptor status, invasiveness and ethnicity).

1.7 Nuclear receptors involved in regulation of cytochrome P450s

1.7.1 Introduction

Members of the nuclear receptor superfamily fundamentally function as ligand-regulated transcription factors by transforming the extracellular and intracellular signals, and controlling the transcription of their target genes. Nuclear receptors can be found within the nucleus and they are responsible for sensing the presence of certain ligands such as lipophilic hormones, vitamins, dietary lipids and other xenobiotic compounds (Zhang et al. 2004). These receptors are highly conserved across species and play a role in the growth and development of organisms. They are also important in several signalling pathways (i.e. cell differentiation, reproduction, metabolism of lipids, drugs and energy) (Sonoda et al. 2008). Currently, 48 nuclear receptors have been identified in the human genome (Zhang et al. 2004).

In principle, nuclear receptors share a common structural domain (Figure 1.4). The N-terminal regulatory domain contains the activation function-1 (AF-1) whose action is independent on the presence of bound ligand, and this domain is highly variable in sequence between various nuclear receptors. The DNA-binding domain (DBD) is the most conserved region which comprises of two zinc finger (ZF-1 and ZF-2) complexes and is involved in dimerisation (homo- or heterodimerisation) of nuclear receptors. The hinge region is responsible for connecting the DBD and ligand binding domain (LBD), and this

is a flexible domain containing the nuclear localisation signal. LBD, which is the largest domain, contributes to the dimerisation interface of the receptor and binds to coactivator/corepressor protein. The LBD contains the activation function-2 (AF-2) (Sonoda et al. 2008). Nuclear receptors bind to each other by either homo- or heterodimerisation, resulting in transcriptional activation or repression of the target genes, which mediates physiological significances (Figure 1.5).

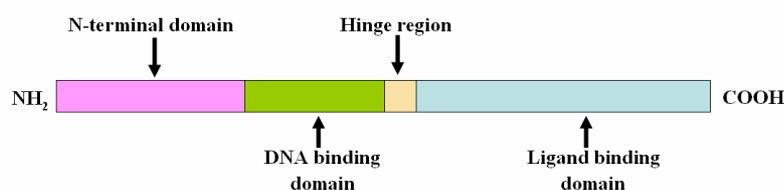


Figure 1.4: Schematic view of structural organisation of nuclear receptors (Adapted from Sonoda et al. 2008).

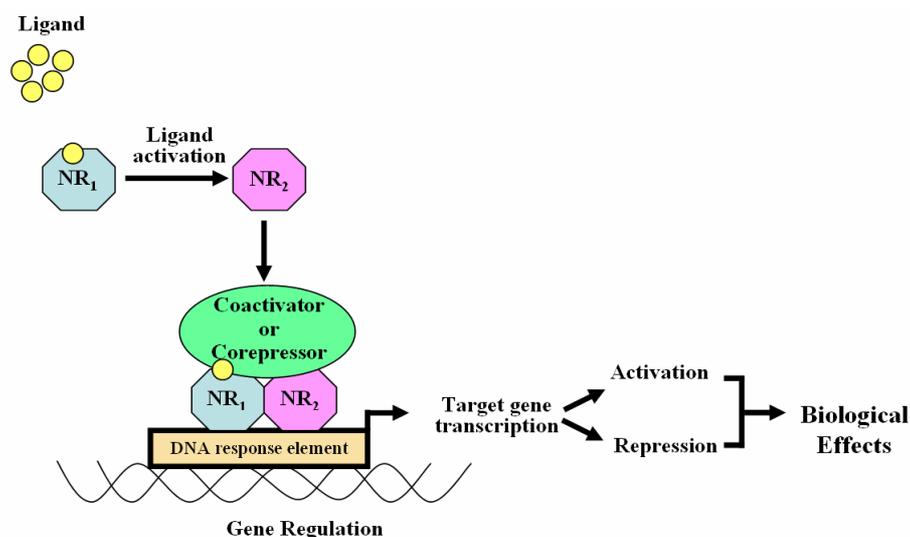


Figure 1.5: Schematic view of general mechanism for nuclear receptor activation and/or repression (Adapted from Xie and Evans 2001).

As mentioned above, nuclear receptors are usually activated by ligand binding, and many cytochrome P450 enzymes are involved in the metabolism of these ligands (Akiyama and Gonzalez 2003). The molecular mechanism of cytochrome P450 induction by its ligands was discovered almost 50 years ago, and this knowledge is essential for both the study of drug metabolism and the carcinogenic process in humans (Meyer 2007). Many ligand-activated nuclear receptors have been demonstrated to be key mediators in the induction of

cytochrome P450s. The transcriptional activation of some members of the cytochrome P450 family (i.e. families 1, 2, 3, 4, 7, 24 and 26) that are involved in the metabolism of endogenous and xenobiotic compounds have been identified (Honkakoski and Negishi 2000). The review in this section summarises the roles of cytochrome P450-associated nuclear receptors such as aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR), constitutive androstane receptor (CAR), PPAR α , vitamin D receptor (VDR), retinoic acid receptor (RAR) and farnesoid X receptor (FXR) including crosstalk in the regulation of cytochrome P450 expression. The regulation of cytochrome P450 genes by nuclear receptors and their heterodimerisation with a partner is shown in Figure 1.6.

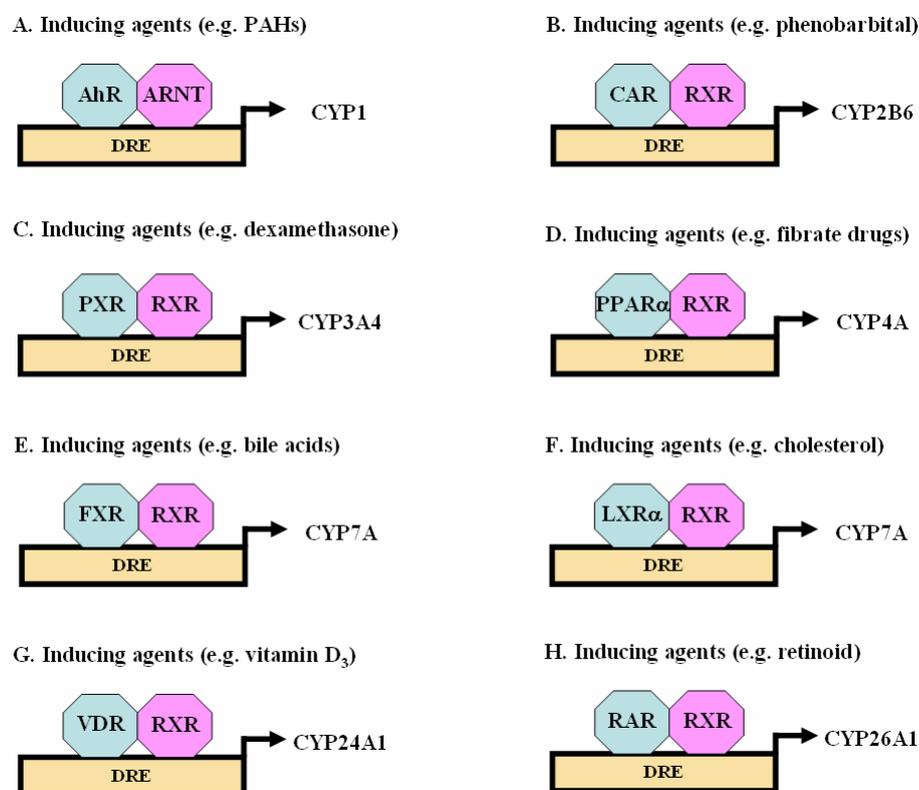


Figure 1.6: Schematic diagrams for activated-ligand nuclear receptors involved in the regulation of cytochrome P450s and their heterodimerisation with a partner.

Abbreviations: DRE; DNA response element, AhR; aryl hydrocarbon receptor, ARNT; AhR nuclear translocator, CAR; constitutive androstane receptor, RXR; retinoid X receptor, PXR; pregnane X receptor, PPAR α ; peroxisome proliferator-activated receptor alpha, FXR; farnesoid X receptor, LXR α ; liver X receptor, VDR; vitamin D receptor, RAR; retinoic acid receptor.

1.7.2 Aryl hydrocarbon receptor (AhR)

Aryl hydrocarbon receptor (AhR) is a member of the basic helix-loop-helix (bHLH) protein which belongs to the Per-Arnt-Sim (PAS) family of transcription factors. AhR plays a role in xenobiotic metabolism and in mediating the carcinogenic effects of dioxin-like compounds. A number of studies have demonstrated that the induction of CYP1 family member expression is controlled by AhR signalling pathway. AhR ligands include polycyclic aromatic hydrocarbons (PAHs, e.g. 3-methylcholanthrene) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Kim et al. 2005). In the absence of ligand, AhR is a cytoplasmic protein complex consisting of chaperone proteins such as heat shock protein 90 (Hsp90), p23 and ARA9. In the presence of ligand, AhR ligand-bound is dissociated from the chaperone proteins in the cytoplasm and is translocated into the nucleus where it dimerises with AhR nuclear translocator (ARNT, also called hypoxia inducible factor-1 beta; HIF-1 β) protein. This complex then binds to DNA response elements (DRE) in regulatory regions of AhR responsive genes (AhREs), leading to the induction of CYP1 genes in tissue-specific targets (Kawajiri and Fujii-Kuriyama 2007). In addition, Kim and co-workers demonstrated a significant positive correlation between hepatic AhR and CYP1A1 expression levels (Kim et al. 2005).

Another key protein in the PAS family is the AhR repressor (AhRR), which is constitutively expressed in the nucleus and is inducible in an AhR-dependent manner. AhRR needs to form a dimer with ARNT and the AhRR/ARNT complex binds to DREs. It was believed that AhRR can inhibit the AhR signalling pathway by inhibition of the AhR/ARNT complex and binding to DREs (Kawajiri and Fujii-Kuriyama 2007). However, Evans and colleagues have recently shown that repression of AhR activity by AhRR is through protein-protein interactions rather than the previously proposed mechanism (Evans et al. 2008). In addition to the induction of CYP1 genes by AhR, hypoxia-inducible factor-1alpha (HIF-1 α) requires dimerisation with ARNT to begin

transcription of its target genes which is similar to either AhR or AhRR expression. In the breast cancer ZR-75-1 cells grown under hypoxic conditions for 24 hours, AhR-mediated transactivation was significantly decreased and this contributed to loss of Ah-responsiveness, possibly due to decreased expression of AhR or competition for ARNT or their bindings with CYP1A1 promoter (Khan et al. 2007). These observations suggest that expression of AhR, ARNT, AhRR or HIF-1 α affects the regulation of CYP1 genes.

A novel chemotherapeutic prodrug aminoflavone (an AhR ligand) leads to a translocation of AhR from the cytoplasm to the nucleus, resulting in the interaction between AhR and DRE, and CYP1A1 transcription. This was observed in the aminoflavone-sensitive MCF7 cells but not in the resistant MDA-MB-435 cells, suggesting that the toxicity of aminoflavone prodrug was selectively activated through the engagement of AhR-mediated signal transduction only in a sensitive breast tumour cell line (Loaiza-Perez et al. 2004). This is an essential consideration for the use of aminoflavone in breast cancer patients in clinical studies.

1.7.3 Constitutive androstane receptor (CAR)

Constitutive androstane receptor (CAR), also called constitutive active receptor, is the most closely related to PXR. CAR is highly expressed in the liver and at lower levels in the intestine. It is called a 'constitutive' receptor because of its ability to transactivate in the absence of ligand-binding (Akiyama and Gonzalez 2003). CAR plays an important role in the transcription of CYP2B6, CYP3A4 and CYP2C (Pascussi et al. 2003), and its role in xenobiotic regulation of CYP2B6 genes has been widely studied in humans. The CYP2B6 gene is inducible by phenobarbital-like inducers (e.g. phynetoïn) through CAR, which bind to the phenobarbital-response element (PBRE) sites as a heterodimer with retinoid X receptor (RXR) in the nucleus. The CAR/RXR heterodimer results in the transcriptional activation of CYP2B6 mRNA (Wang et al. 2004). Chang and co-workers

showed a significant positive relationship between CYP2B6 and CAR expression in human liver (Chang et al. 2003).

In addition to CAR ligands, the PXR activator clotrimazole can bind to CAR and subsequently suppresses CYP2B6 transcription. The antifungal antibiotic clotrimazole and steroid hormone androstanol are classified as potent deactivators of CAR and inverse agonists, suggesting that CAR and PXR might share xenobiotic and steroid ligands (Moore et al. 2000). However, CAR has also been shown to be involved in the detoxification of some endogenous ligands (i.e. bile acids and bilirubin) (Moore et al. 2006) and biosynthesis of sterols and steroids in human liver (Meyer 2007) which alter the induction of CYP2B6 gene.

1.7.4 Pregnane X receptor (PXR)

Pregnane X receptor (PXR) also termed steroid X receptor (SXR) is predominantly expressed in the liver and small intestine (Moore et al. 2006) and mediates induction of CYP3A4, CYP2B6, CYP2C8 and CYP2C9 (Pascucci et al. 2003). PXR functions as a sensor for steroids (e.g. oestrogen, progesterone and corticosteroid) and xenobiotics (e.g. phenobarbital, clotrimazole, rifampicin and nifedipine). PXR mainly activates CYP3A4, which is a primary target gene of PXR. Therefore, compounds that activate PXR also induce transcriptional activity of CYP3A4. The ligand-activated PXR forms a heterodimer with RXR, and the heterodimer PXR/RXR binds with an everted repeat separated by six base pairs (ER6) from the CYP3A4 proximal promoter, resulting in an increase in CYP3A4 induction (Vyhlidal et al. 2004). PXR and CAR are closely related nuclear receptors, sharing ligands and sharing a heterodimer partner (RXR). In addition, CAR and PXR are also involved in biosynthesis and catabolism of steroid hormones, lipid metabolism, glucose homeostasis and inflammation. This could explain crosstalk between

these receptors (Section 1.7.9), which may affect cytochrome P450 expression and physiological functions (Moreau et al. 2007b).

Miki and colleagues investigated the expression of PXR mRNA and protein using real-time RT-PCR and immunohistochemical methods in 60 human breast cancer tissues. They reported that PXR was detected in breast tumours but not in matched adjacent normal breast cells. Positive correlations between PXR and both the histological grade and lymph node status of the tumours were noted in this study (Miki et al. 2006), suggesting that PXR may be involved in human breast carcinogenesis. These findings are in disagreement with a study by Dotzlaw and co-workers (Dotzlaw et al. 1999) though, who demonstrated PXR mRNA in both breast tumour and their corresponding normal breast tissues. Higher levels of PXR mRNA were detected in ER-negative tumours than in ER-positive tumours. They showed that PXR is expressed in breast cancer cell lines T47D, MCF7 and MDA-MB-231.

1.7.5 Vitamin D receptor (VDR)

1,25-(OH)₂D₃ and its analogues have been shown to inhibit proliferation, induce apoptosis and immunomodulation of human cancer cells mediated by vitamin D receptor (VDR). Ligand-activated VDR forms a heterodimer with RXR and binds to vitamin D-response elements (VDREs) on the target genes, causing either activation or repression of target gene expression (Moreau et al. 2007a). VDR is mainly expressed in the kidney, intestine and thyroid (Moore et al. 2006). CYP24A1 is one of the VDR target genes which is directly regulated by VDR, and plays a role in the inactivation of vitamin D in the kidney (Zhou et al. 2006). Anderson and colleagues demonstrated overexpression of CYP24A1 and down-regulation of VDR mRNA in various cancer cell lines including breast cancer MCF7 cells (Anderson et al. 2006), suggesting that the inverse relationship between CYP24A1 and VDR is associated with carcinogenesis. In addition, Yee and co-workers showed that a combination of ketoconazole and tetralone inhibits CYP24A1 mRNA and

enhances VDR signalling in androgen-independent prostate cancer DU-145 cells (Yee et al. 2006).

1.7.6 Peroxisome proliferator-activated receptor alpha (PPAR α)

Peroxisome proliferators (e.g. fibrate class of hypolipidemic drugs including WY-14643 or pirinic acid) are a group of chemicals that lead to an increase in size and number of hepatic peroxisomes when administered to rodents. PPAR α is highly expressed in the liver, kidney and vascular wall and is activated by a variety of fatty acids. PPAR α plays a role in the transcriptional regulation of enzymes involved in the ω - or β -fatty acid oxidative pathway (Bishop-Bailey and Wray 2003). In the presence of peroxisome proliferators, PPAR α can heterodimerise with RXR, and the PPAR α /RXR heterodimer complex binds to peroxisome proliferator-response elements (PPREs), leading to the transcriptional activity of target genes such as CYP4A (Ito et al. 2007). High levels of PPAR α mRNA have been demonstrated in MCF7 breast cancer cells, and this may suggest a crucial role for PPAR α in human breast carcinogenesis (Suchanek et al. 2002)

1.7.7 Retinoic acid receptor (RAR)

All-*trans*-retinoic acid (ATRA), the most active metabolite of retinoids (vitamin A) plays an important role in cell differentiation and proliferation of epithelial cells mediated by retinoic acid receptor alpha (RAR α) (Njar et al. 2006). The binding of ligand to RAR α results in a heterodimer with RXR, and the RAR α /RXR heterodimer binds to retinoic acid response elements (RAREs) or retinoid X response elements (RXREs), causing transcriptional suppression or activation of target genes such as CYP26A1. In human breast cancer, a positive relationship was found between the retinoid growth-inhibitory effects and RAR α expression (Fitzgerald et al. 1997). Expression of RAR and RXR has been demonstrated in breast cancer cells (Titcomb et al. 1994). In addition, Mu and

colleagues investigated the effects of various nuclear receptor ligands on CYP19A1 activity in the breast cancer MCF7 cells. Findings showed that RAR and RXR selective ligands increased CYP19A1 activity, suggesting that these compounds may be involved in the regulation of oestrogen production in breast cancer cells (Mu et al. 2000).

1.7.8 Farnesoid X receptor (FXR)

Bile acids are the main product of cholesterol metabolism in the liver and are important for lipid absorption in the intestine. Farnesoid X receptor (FXR) has been characterised as a bile acid receptor. In the presence of the most potent bile acid (chenodeoxycholic acid; CDCA), FXR forms a heterodimer with RXR and the FXR/RXR heterodimer binds to the bile acid response elements (BAREs), resulting in the suppressive effects of bile acid and a transcriptional repression of target genes such as CYP7A1 (Akiyama and Gonzalez 2003). FXR is found to be expressed in breast cancer tissue, suggesting that FXR expression in association with high levels of bile acid may play a role in breast carcinogenesis. This raised the possibility that FXR inhibitors could be used for breast cancer treatment (Swales et al. 2006).

1.7.9 Crosstalk between nuclear receptors

Crosstalk is defined as a functional interaction between two (or more) signalling pathways of members of the nuclear receptor superfamily. Crosstalk between nuclear receptors has been extensively studied (Keller et al. 1995, Zhou et al. 2006, Meyer 2007, Moreau et al. 2007b, Roth et al. 2008) and their interactions have been demonstrated to be involved in several intracellular signalling pathways (i.e. growth factors, hormones and cytokines) and xenobiotic metabolism (Figure 1.7). Regulation of target gene expression by nuclear receptors is complex. A better understanding of the associated molecular mechanism is likely to explain biological phenomena in the body.

As shown in Figure 1.5, transcriptional activation of nuclear receptors' target genes requires a number of different steps (i.e. ligand binding, coactivator/corepressor protein, dimerisation with a partner and DNA binding within their target genes) and each signalling pathway is usually connected with other pathways (Reményi et al. 2004). Crosstalk may lead to alterations in other genes' target gene expression or activity (Waxman 1999, Honkakoski and Negishi 2000). This section provides some examples of crosstalk between nuclear receptors which have been shown to affect cytochrome P450 expression.

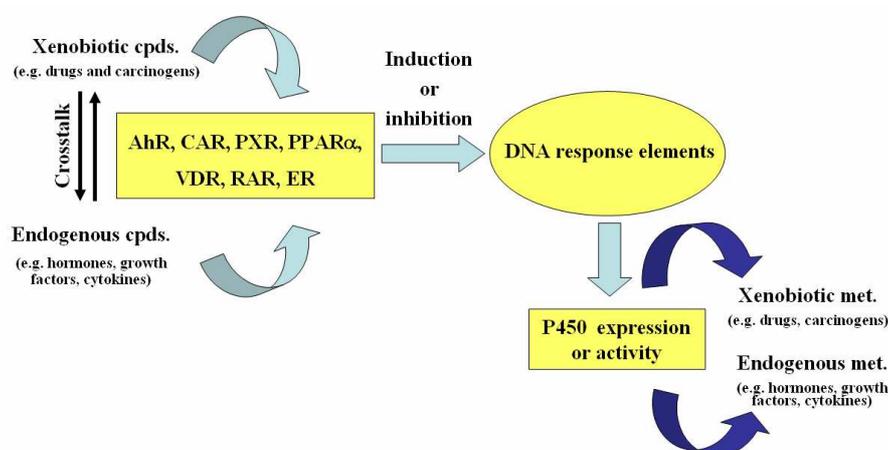


Figure 1.7: Crosstalk interactions between the regulation of xenobiotic and endogenous compounds.

Abbreviations: AhR; arylhydrocarbon receptor, PXR; pregnane X receptor, CAR; constitutive androstane receptor, PPAR α ; peroxisome proliferator-activated receptor alpha VDR; vitamin D receptor, RAR; retinoic acid receptor, ER; oestrogen receptor, P450; cytochrome P450, cpds; compounds, met; metabolism.

The negative crosstalk between ER and AhR signalling pathways has been well documented by several research groups using breast tumour tissue and cell lines (Safe et al. 1998, Safe et al. 2000, Safe and Wormke 2003, Matthews et al. 2005, Matthews and Gustafsson 2006). ER and AhR are ligand-activated transcription factors. As mentioned in Section 1.7.2, AhR mediates the toxic effects of dioxin-like compounds (e.g. TCDD) and is involved in the induction of CYP1 genes. However, the AhR signalling pathway can be linked to the ER signalling pathway as CYP1 genes play a key role in oestrogen metabolism. Recently, mechanisms of inhibitory cross talk between ER and AhR have been proposed by Matthews and Gustafsson (Matthews and Gustafsson 2006). There are at least five mechanisms that are involved in the role of AhR inhibiting ER activity (1)

direct inhibition via inhibitory xenobiotic response elements (iXREs), (2) squelching shared coactivators (i.e. ARNT), (3) synthesis of an inhibitory factor (unknown), (4) increased ER degradation and (5) increased expression of enzymes involved in oestrogen synthesis (i.e. CYP19) and metabolism (i.e. CYP1A1 and CYP1B1). In addition, these cytochrome P450s play an important role in the metabolism of anticancer drugs (Section 1.8), and this knowledge could improve the clinical approach to particular cancer therapies

Transcriptional crosstalk between ER and RAR has been demonstrated in human breast cancer cells (Rousseau et al. 2003). Retinoic acid has been shown to be associated with down-regulation of ER mRNA and inhibition of cell growth in oestrogen-dependent breast cancer MCF7 cells. By contrast, this was not observed in ER-negative breast cancer MDA-MB-231 cells. Higher levels of RAR were found in ER-positive breast cancer than in ER-negative breast cancer cell lines (Rubin et al. 1994), suggesting that expression of RAR and ER is essential for retinoid responsiveness. Furthermore, oestrogen has been shown to elevate RAR expression, whereas antioestrogen seems to be associated with down-regulation of RAR (Lu et al. 2005).

CAR and PXR are mediated by induction of CYP2B and CYP3A genes respectively, and share a common heterodimerisation partner i.e. RXR α , ligands and DNA response elements within their target genes (Handschin and Meyer 2003). The CAR and PXR genes are closely related members of the nuclear receptor family and have overlapping substrates. These nuclear receptors are involved in the regulation of various endogenous compounds such as steroid hormones, bile acids, eicosanoids and cholesterol (Echchgadda et al. 2007).

A recent study investigated CAR- and VDR-binding to specific response elements in human hepatocyte cells using electromobility shift assay, and determined CYP24

expression (Moreau et al. 2007a). Results showed that the CAR/RXR heterodimerisation binds to the promoter of CYP24, which is the same region as VDR/RXR, resulting in decreased CYP24 expression and response to vitamin D₃, suggesting that CAR ligands interfere with VDR target genes. These findings are similar to a previous study that investigated crosstalk between PXR and VDR and showed that the PXR/RXR heterodimer binds to the promoter of CYP24 and competes with VDR (Pascussi et al. 2005). This suggests that PXR, CAR and VDR share DNA response elements in the promoter of their target genes, leading to altered expression of CYP24A1, CYP2B6 and CYP3A4.

It is important to note that RXR is a common heterodimerisation partner for several nuclear receptors (i.e. PXR, VDR, RAR, PPAR α , FXR and LXR α , Figure 1.6) and this may also cause crosstalk between other signalling pathways, not only vitamin D₃ metabolism.

Crosstalk between PPAR α /RXR and ER has been reported by Keller and colleagues (Keller et al. 1995). A PPAR α /RXR heterodimer inhibits ER activity through competition for binding to oestrogen response elements.

1.8 Cancer treatment approaches based on cytochrome P450s

The role and importance of cytochrome P450s in cancer formation and progression has led to the development of cancer therapeutics-based on cytochrome P450 expression and metabolic pathways. Knowledge in the area of these enzymes' substrate and mechanism of action in tumour cells has become very useful in the design of new chemotherapeutics and/or development of more effective existing anticancer drugs. It can help reduce systemic toxicity and enhance specificity to tumour cells. To date, applications of cytochrome P450s in cancer therapy consist of (1) inhibiting enzymes involved in hormone-dependent cancer and vitamin metabolism, (2) designing prodrugs activated by the enzymes and (3) immunotherapy targeted at tumour-specific enzymes (McFadyen et al. 2004, Bruno and Njar 2007, Purnapatre et al. 2008) (Figure 1.8).

In steroid hormone biosynthesis pathways, CYP17 and CYP19 are key enzymes that regulate the biosynthesis of oestrogen. CYP17 plays an important role in the conversion of pregnenolone and progesterone to androgen and oestrogen precursors, whereas CYP19 or

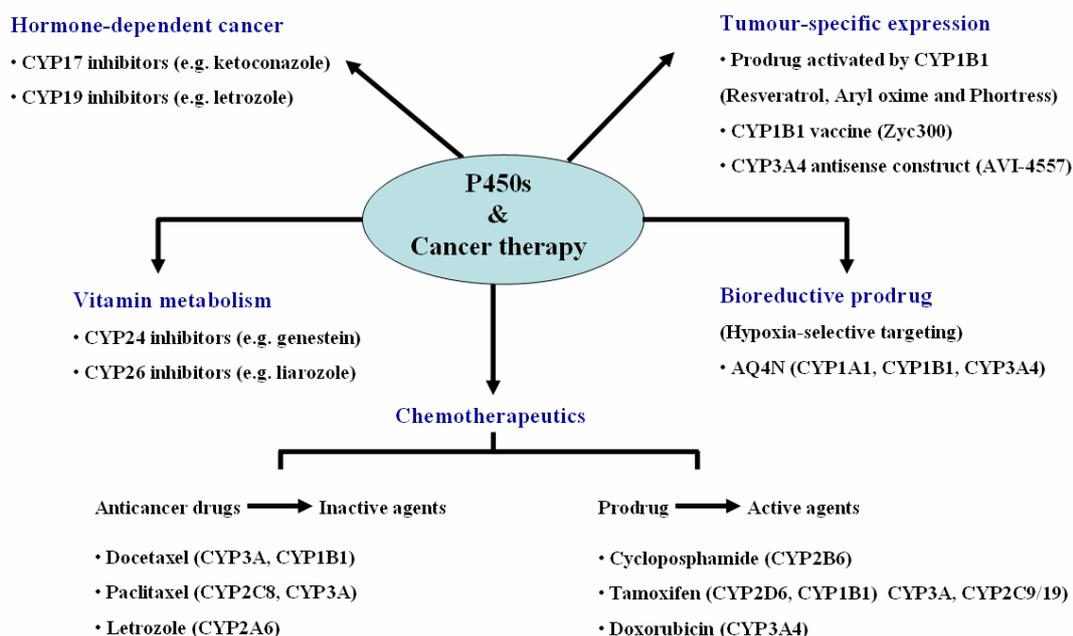


Figure 1.8: Implications of cytochrome P450s in cancer therapy.

aromatase is involved in the conversion of testosterone and androstenedione to oestradiol and oestrone. Several inhibitors of CYP19 such as letrozole, anastrozole and exemestane have been developed as anticancer agents for the management of breast cancer patients who have oestrogen-dependent tumours. These agents inhibit the production of oestrogen in target tissues and improve survival in patients with metastatic breast cancer (Gibson et al. 2007, Perez 2007a). Inhibitors of CYP17 such as ketoconazole and abiraterone are also used as anticancer drugs for the treatment of androgen-dependent cancers particularly prostate cancer, and these compounds inhibit androgen production and are more effective in advanced prostate cancer treatment (Moreira et al. 2007).

Vitamins A and D are believed to have a crucial role in cancer prevention and treatment. All-*trans*-retinoic acid (ATRA) is the most active metabolite of vitamin A, it plays a role in cell differentiation and proliferation of epithelial cells, and has been shown to be associated

with tumour growth inhibition in various solid tumours (Njar 2002). CYP26A1 is involved in the metabolic inactivation of ATRA and it is related to the rapid ATRA metabolism in cancer patients. Inhibitors of CYP26A1 (e.g. liarozole, R115866 and R116010) inhibit both ATRA metabolism and CYP19 activity. However, liarozole lacks specificity, and has a relatively weak potency for CYP26A1, and is no longer being developed as an anticancer agent. R116010 is more potent and a selective inhibitor of CYP26A1 (Njar et al. 2006) which is currently in preclinical trial for patients with hormone-independent prostate and breast tumours (Njar 2002, Njar et al. 2006).

The biologically active metabolite of vitamin D₃ has been shown to play a role in the inhibition of cell proliferation, promotion of differentiation and induction of apoptosis and it has potential as a cancer therapeutic (González-Sancho et al. 2006, Deeb et al. 2007).

CYP24A1 is the key enzyme involved in the catabolism of 1,25-(OH)₂D₃ to the less active vitamin D₃ metabolite. Overexpression of CYP24A1 has been shown in a wide range of tumours (Mimori et al. 2004, Anderson et al. 2006, González-Sancho et al. 2006) and is known as an oncogene (Mimori et al. 2004). In addition, Townsend and colleagues demonstrated that higher levels of CYP24A1 were detected in breast tumour compared to normal breast tissue (Townsend et al. 2005). Inhibitors of CYP24 such as genestein, QW-1624F2-2 and EB1089 are vitamin D₃ analogues and have ability to inhibit CYP24A1 expression (Bruno and Njar 2007). Recently, Sundaram and co-workers showed that QW-1624F2-2 inhibits cell proliferation and invasion of breast tumour and has been proposed for preclinical studies as an effective chemotherapeutic agent in breast cancer treatment (Sundaram et al. 2006).

As mentioned previously (Section 1.2.2), hypoxic conditions existing in the tumour microenvironment are associated with resistance to chemotherapy and radiotherapy in malignant cells. However, tumour hypoxia appears to be a specific property of cells in

many solid tumours, and it is a potential target for tumour-specific prodrugs activated by oxygen-sensitive reductase (bioreductive drugs) including cytochrome P450s (Denny 2004). The topoisomerase II inhibitor prodrug AQ4N (Banoxantrone) is designed to have anti-tumour activity following activation by CYP1A1, CYP1B1 and CYP3A4. Under hypoxic conditions, AQ4N is metabolised to the potent topoisomerase II inhibitor AQ4 this process is inhibited by oxygen.. AQ4N is currently in phase Ib/IIa clinical trials, and it has been reported that it is well tolerated and less toxic than another bioreductive prodrug tirapazamine (McKeown et al. 2007).

Recently, Albertella and colleagues demonstrated clinical results derived from a phase I study of AQ4N in thirty-two patients with advanced solid tumours (i.e. breast, cervix, glioblastoma, bladder, and head and neck). These patients received a single dose of AQ4N prior to surgery and results showed that AQ4N penetrated well within tumour cells, as high concentrations of the activated form AQ4 were detected in tumour samples (Albertella et al. 2008). This data indicates that the effect AQ4N has on solid tumours is hypoxia-selective and further evaluation in clinical trials is required.

A number of cytochrome P450 enzymes are involved in the activation and/or inactivation of several anticancer drugs. Paclitaxel which is widely used in cancer chemotherapy is inactivated in human liver by CYP2C8 forming a 6 α -hydroxylation derivative with 30-fold less than the parent drug (Taniguchi et al. 2005). Another example of a taxane anticancer drug or stabilising-microtubule agent that is inactivated in the liver by CYP3A is docetaxel (Scripture et al. 2005). In addition, the tumour response to docetaxel in human breast tumours can be predicted by CYP3A4 (Miyoshi et al. 2005). Patients with CYP3A4 negative tumours showed a higher response rate to docetaxel than those with CYP3A4 positive tumours. However, a previous study reported that the coadministration of CYP3A4 inhibitors (e.g. ketoconazole and midazolam) with docetaxel may cause a

significant drug interaction, resulting in toxic effects such as febrile neutropenia (Engels et al. 2004). These findings are in agreement with a study by Alexandre and colleagues (Alexandre et al. 2007) who investigated the relationship between CYP3A activity, inflammatory status and risk of docetaxel-induced febrile neutropenia in patients with advanced cancer.

The anticancer prodrugs cyclophosphamide, doxorubicin and tamoxifen are bioactivated to active agents mainly by cytochrome P450s. Cyclophosphamide is an alkylating agent derived from the prodrug oxazaphosphorine which is primarily activated in the liver by CYP2B6 to generate a cytotoxic activity of phosphoramidate mustard and acrolein, then delivered to tumour cells via blood circulation (Stoff-Khalili et al. 2006). To prevent deleterious secondary effects and minimise damage to normal tissue, cytochrome P450 mediated gene-directed enzyme and prodrug therapy (GDEPT) have recently been developed with CYP2B6 for the treatment of cyclophosphamide by delivery of exogenous cytochrome P450s to the target site (Roy and Waxman 2006). MetXia encodes human CYP2B6, it activates cyclophosphamide in tumour cells is designed to sensitise malignant cells and can be directly injected into tumours prior to cyclophosphamide treatment. MetXia is currently in phase I/II clinical trials for advanced breast cancer patients (Braybrooke et al. 2005).

The anthracycline antibiotic prodrug doxorubicin is one of the most widely used anticancer drugs. Doxorubicin is activated in human liver by CYP3A4 to form a potent active metabolite methoxymorpholinyl doxorubicin (MMDX) which interacts with DNA by intercalation and inhibition of topoisomerase II (Mathijssen and van Schaik 2006). In addition, a study by Lu and colleagues investigated anti-tumour activity of MMDX in various tumour cell lines (i.e. breast, lung, brain and colon). Results showed that very low levels of CYP3A4 expression and low anti-tumour activity of MMDX were found in these

cell lines (Lu and Waxman 2005), suggesting that increasing the content of CYP3A4 in the tumour site using gene-therapy vectors may be helpful when treating patients with doxorubicin.

Tamoxifen is currently used for the treatment of oestrogen-dependent breast cancer patients and is activated in the liver by several cytochrome P450s (i.e. CYP2D6, CYP3A, CYP2C9, CYP2C19 and CYP2B6). It is mainly activated by CYP2D6, forming 4-hydroxytamoxifen which has 50-100-fold more potent antioestrogenic effects than tamoxifen (Dehal and Kupfer 1997). CYP2D6 is a highly polymorphic gene and this may contribute to variability of treatment outcome between individuals and/or ethnicities (Zanger et al. 2007). Other cytochrome P450s appear to have less important roles in the metabolism of tamoxifen (Scripture et al. 2005).

Novel synthetic prodrugs selectively designed to be activated by cytochrome P450s, especially CYP1A1 and CYP1B1, have been developed and show promise as new options for cancer therapeutics. The phytoestrogen resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) is derived from natural sources (i.e. red grape skin). Resveratrol is mainly metabolised by CYP1B1 within tumour cells to form the anticancer agent piceatannol (Piver et al. 2004) which has antioestrogen effects. It has antioxidant and anti-inflammatory effects which may induce apoptosis and modulate the function of ER in breast cancer cells (Athar et al. 2007). Phase I and pharmacokinetic evaluations of oral resveratrol in healthy volunteers have recently been demonstrated through investigation of six metabolites of resveratrol in urine (Boocock et al. 2007). Results suggested that the highest dose of resveratrol did not cause severe adverse drug reaction and cancer chemopreventive effects of resveratrol metabolites in this study warrant further investigation by clinical studies.

Another novel synthetic prodrug involving cytochrome P450s is a water-soluble fluorinated benzothiazole prodrug phortress that is a potent AhR agonist and can induce CYP1A1 expression. CYP1A1 converts phortress prodrug into an electrophilic reactive intermediate, that induces formation of DNA adducts resulting in DNA damage and cell death. Phortress is rapidly hydrolysed in the presence of cells to its lipophilic parent drug, which is then selectively taken up into sensitive cells, followed by AhR binding and translocation into the nucleus, leading to induction of CYP1A1 (Leong et al. 2003). Phortress has recently entered phase I clinical studies with breast cancer patients in the UK (Bradshaw and Westwell 2004). However, Brockdorff and colleagues demonstrated that increased expression of CYP1A1 and CYP1B1 is associated with antioestrogen resistance in breast cancer cell lines (Brockdorff et al. 2000), and further clinical studies are required to investigate CYP1A1 and CYP1B1 expression in tumour cells and avoid this problem in patients.

Gene expression of tumour-specific cytochrome P450s has introduced the concept of cancer immunotherapy using a DNA vaccine. CYP1B1 has been shown to be a tumour-associated antigen that is overexpressed in a wide range of tumour tissues and cell lines (e.g. breast, uterus and ovary) and therefore represents an attractive target for immune-based therapy. The CYP1B1-based DNA vaccine Zyc300 is designed to induce the immune system anti-CYP1B1-specific CD8⁺ T-cell against CYP1B1-expressing tumour cells. Zyc300 is currently in phase I evaluation (Maecker et al. 2003) and has been shown to improve survival in cancer patients who have developed immunity to CYP1B1. In addition, a study by Gribben and co-workers has shown that increased anti-CYP1B1 immunity in patients with metastatic tumours (i.e. ovarian, colorectal, renal, prostate and breast cancer) dramatically improved their response to next salvage therapy (Gribben et al. 2005). Though unexpected, it was associated with a good clinical outcome and low toxicity.

A CYP3A4-based therapeutic antisense phosphorodiamidate morpholino oligomer AVI-4457 has been developed as CYP3A4 plays an important role in the deactivation of paclitaxel and cyclophosphamide which are important chemotherapeutic agents, but it is not a tumour-specific expression enzyme (McFadyen et al. 2004). A previous study demonstrated that inhibition of CYP3A4 expression was observed in primary human hepatocytes after 24 hours exposure to AVI-4557. Findings showed that the cytotoxic activity of cyclophosphamide and paclitaxel are inhibited and induced with AVI-4557 treatment in these cells respectively, but no effect on the cytotoxic activity of cisplatin (Arora et al. 2002). However, further information on clinical trials of this agent has not been reported.

1.9 Aims

Cytochrome P450 enzymes are involved in the carcinogenic process as well as cancer treatment. The current study aimed to detect the presence of these enzymes and their regulatory nuclear receptors in breast cancer cell lines as models, and to identify potential novel targets for breast cancer treatment based upon the expression profiles obtained. This study selected 24 cytochrome P450 genes and 10 P450-regulatory nuclear receptors for investigation (Table 1.5). Characterisation and regulation of CYP4Z1 expression were of particular interest.

Table 1.5: *List of candidate genes in this study.*

Cytochrome P450 enzymes	Cytochrome P450-regulatory nuclear receptors
Family 1: CYP1A1, CYP1A2 and CYP1B1 Family 2: CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2F1, CYP2J2, CYP2R1, CYP2S1 and CYP2U1 Family 3: CYP3A4, CYP3A5, CYP3A7, CYP3A43 Family 4: CYP4X1 and CYP4Z1 Family 24: CYP24A1 Family 26: CYP26A1 and CYP26B1	AhR, ARNT, AhRR, HIF-1 α , CAR, PXR, VDR, RAR, RXR and ER

Chapter 2

Materials and Methods

2.1 Introduction

As previously detailed (Section 1.3), the use of cell culture as an *in vitro* model system has become increasingly important in several aspects of biomedical sciences at the molecular and cellular levels in order to provide a better understanding of cell biology *in vivo* particularly in disease stages. Established human breast cancer cell lines representing characteristics of the primary breast tumours (e.g. ethnicity, ER α status, epithelial-mesenchymal transition (EMT) and invasive phenotype) have been extensively used to determine target gene expression (Burdall et al. 2003).

Microbial contamination in cell culture is one of the most common problems that can lead to incorrect interpretation of gene expression as it may result in serious effects (i.e. alteration of cell growth pattern, cell morphology, and synthesis of DNA or RNA or protein). To minimise contamination, all cell culture procedures (e.g. maintenance of cell lines, resuscitation of frozen cells, trypsinisation and freezing cell cultures) in this study were carried out in a Class II safety cabinet (BioAir Instruments S.r.l., Sizzano, Italy). Pipette tips and glass containers were autoclaved at 121°C and 15 lbs/in² for 15 minutes, and glassware was heated at 160°C for 2 hours. Penicillin/streptomycin solution is the most widely used combination of antibiotics in mammalian cell culture to prevent bacterial growth. In this study, a final concentration of 100 U/ml of penicillin and 100 μ g/ml of streptomycin was used in growth media (Freshney 2005).

In addition, all cell lines, growth media and sterile solutions of reagent were tested for microbial contamination prior to use. Brain heart infusion (BHI) broth was used to test for

bacterial contamination by incubating at 37°C for 1-2 days. Sabouraud (SAB) broth was used to detect fungal and yeast contamination by incubating at 32°C for 7-10 days (Freshney 2005).

Over the last 50 years, molecular biologists have made advances in the isolation, manipulation, and characterisation of the genetic material in cells. The genetic material is transcribed into RNA and then translated into protein. Total RNA is isolated from the cell of interest, and a complementary DNA (cDNA) library is synthesised from messenger RNA (mRNA) using an oligonucleotide deoxythymidylic acid (oligo dT) primer which is annealed onto the polyadenylated (poly A) tail of the mRNA template. Thus, single stranded mRNA is converted into a cDNA copy of the message using a reverse transcriptase enzyme and deoxynucleotide triphosphates (dNTPs). The reverse transcription-polymerase chain reaction (RT-PCR) is commonly used in the determination of the abundance of mRNA within a cell as a measure of gene expression. RT-PCR is used to amplify specific regions of the cDNA template using sequence specific primers. The molecular weight size of the DNA fragment is estimated by semi-logarithmic plotting and compared to size prediction from known sequence. Restriction digestion and DNA sequencing are widely used to identify the gene products.

Figure 2.1 represents a flowchart of the process used in this thesis for the detection of gene-specific expression. The following sections in this chapter provide information on molecular biology techniques. A list of chemicals and reagents including their suppliers are presented in Appendix A.

2.2 Cell culture and cell lines

The growth media, cell lines and culture techniques employed in starting growth and maintaining cell lines, resuscitation of frozen cells, trypsinisation, freezing cell cultures

and cell viability determination are described below. All working steps in cell culture procedure were performed in a Class II safety cabinet.

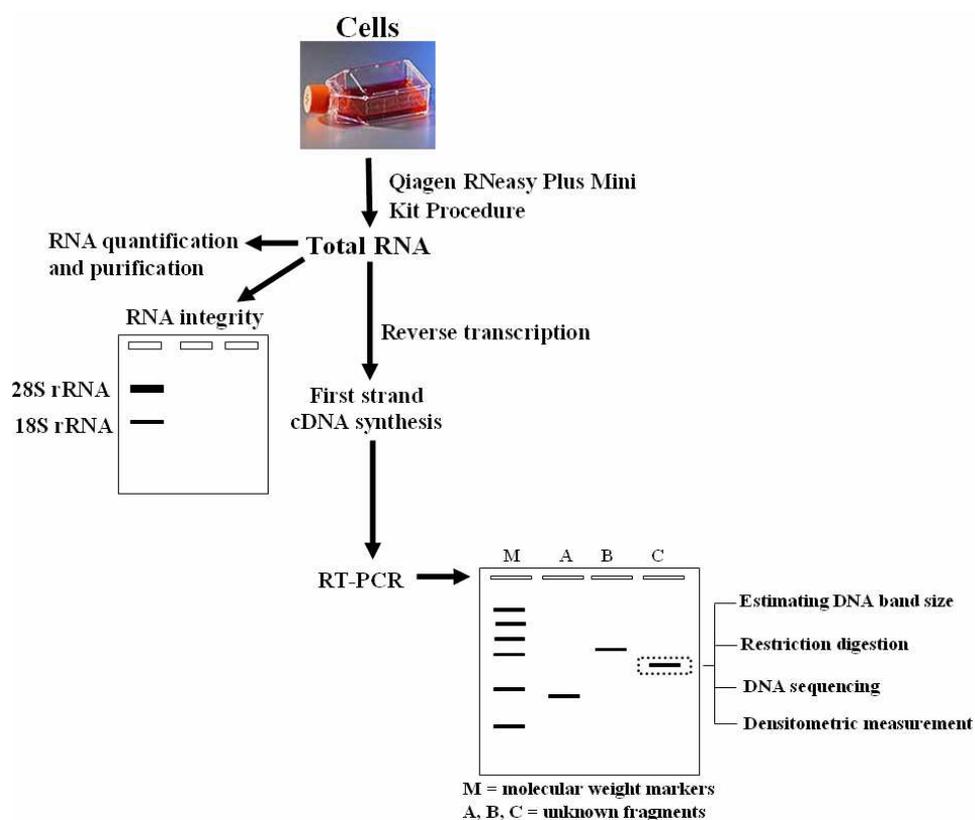


Figure 2.1: Flowchart of procedure for the detection of gene-specific expression.

2.2.1 Growth media

2.2.1.1 GlutaMaxTMI

L-glutamine is an unstable essential amino acid required in most cell culture media formulations. It is used for energy production, protein synthesis and nucleic acid synthesis. However, L-glutamine spontaneously degrades in cell culture media or aqueous solution to the ammonium product. High ammonia concentrations are toxic to cells, lowering cell viability and protein production. Therefore, several cell culture media which can be purchased are now supplemented with L-glutamine in dipeptide forms, such as alanyl-L-glutamine and glycyl-L-glutamine.

GlutaMax™I formulation is a dipeptide which is cleaved by aminopeptidase releasing, L-glutamine and L-alanine. GlutaMax™I contains a stabilized form of L-glutamine, in equal molar concentrations as glutamine-containing media. Therefore, GlutaMax™I-containing cell culture media can increase media stability, minimise toxic ammonia build-up and maximize cell performance (Christie and Butler 1994). Media formulations RPMI 1640, L15, DMEM and DMEM/F12 supplemented with GlutaMax™I were employed for all cell culture experiments.

2.2.1.2 Roswell Park Memorial Institute medium (RPMI1640)

This was developed by Moore et al. (Moore et al. 1967) at Roswell Park Memorial Institute (RPMI). The formulation utilizes a bicarbonate buffering system, and is based on the RPMI1630 series with alterations in the amounts of amino acids and vitamins. RPMI1640 medium has extensive applications for culturing growth of several types of cells, such as human normal and neoplastic leukocyte cell lines.

2.2.1.3 Leibovitz's medium (L15)

This medium was developed by Leibovitz (Leibovitz 1963) in 1963 and was originally formulated for use in the culture of tumour cell lines in the absence of carbon dioxide (CO₂) and without a sodium bicarbonate buffer system. L15 medium is buffered by a particularly high concentration of free base amino acids (L-arginine primarily, but also L-histidine and L-cysteine) and sodium pyruvate. The sodium pyruvate can be utilized as a more readily accessible energy source for cell metabolism and can produce endogenous carbon dioxide. Production of acidic metabolites is reduced by replacing glucose with galactose in order to maintain physiological pH (Barngrover et al. 1985).

2.2.1.4 Dulbecco's Modified Eagle's Medium (DMEM)

DMEM is one of the most commonly used variations of Basal Medium Eagle (BSE). This medium contains twice the amino acid concentrations of MEM and four times the vitamin concentrations, as well as additional components to facilitate better buffering (e.g. hydrogencarbonate and carbon dioxide concentrations). High and low glucose forms are available containing 4.5 g/L and 1 g/L of glucose respectively. This formulation is well suited for culturing a wide range of mammalian cell lines (Dulbecco and Freeman 1959).

2.2.1.5 DMEM/Ham's F12 medium

Ham's F12 media was developed to clone Chinese Hamster Ovary (CHO) cells in low serum concentrations (Ham 1965) and is now extensively used to culture different kinds of mammalian cells. The mixture of DMEM and Ham's F12 (1:1 v/v) is suitable for some epithelial, endothelial, and granulose cell types.

2.2.2 Cell lines

A database of the seven breast cancer cell lines used in this study is shown in Table 1.1. MDA-MB-157, MDA-MB-231, T47D, ZR-75-1 and ZR-75-30 cells were obtained from the European Collection of Cell Cultures (ECACC; Salisbury, Wiltshire, UK). MDA-MB-468 cells were a generous gift from Professor Andreas Gescher (Department of Cancer Studies and Molecular Medicine, Leicester Medical School, University of Leicester, UK). MCF7 cells were an established cell line within the School of Pharmacy and Life Sciences, The Robert Gordon University, Aberdeen, UK.

MCF10A, a non-transformed human breast epithelial cell line is derived from breast tissue of a 36 year old patient with fibrocystic disease (Soule and McGrath 1986). A previous study demonstrated that MCF10A contains a number of features of normal breast epithelium, for example, lack of carcinogenicity in nude mice, and dependence on

hormones and growth factors for cell proliferation (Soule et al. 1990). In the present study, MCF10A was obtained from the American Type Culture Collection (ATCC; LGC Promochem, Middlesex, UK).

2.2.3 Growth and maintenance of human normal breast and breast cancer cells

All the media used (i.e. DMEM, RPMI1640, L15 and DMEM/Ham's F12) were supplemented with GlutaMax™I and all cell lines were cultured under the recommended conditions. Stock cultures of MCF7, T47D and MDA-MB-157 cells were grown in DMEM. Cell stocks of ZR-75-30 and MDA-MB-468 cells were propagated in RPMI1640. Stock cultures of ZR-75-1 cells were grown in RPMI1640 containing 1 mM sodium pyruvate. The media above were supplemented with 10% (v/v) foetal bovine serum (FBS). Stock cultures of MDA-MB-231 cells were cultured in L15 supplemented with 15% (v/v) FBS. Cell stocks of MCF10A cells were grown in DMEM: Ham's F12 medium (1:1) supplemented with 20 ng/mL epidermal growth factor, 0.01 mg/mL insulin, 500 ng/mL hydrocortisone and 5% (v/v) horse serum (Thomas et al. 2006). All growth media were prepared containing penicillin (100 U/mL) and streptomycin (100 µg/mL) in order to reduce the risk of microbial contamination. Cells were seeded at $2-5 \times 10^4$ cells/cm² and grown as a monolayer at 37°C in 5% CO₂ and 95% air. The cell culture media was changed regularly every 2-3 days to prevent a build-up of waste products as the cells grew and divided. Cells reached 70-80% confluence within 5-7 days.

As mentioned above, L15 medium (Section 2.2.1.3) must be used without carbon dioxide, therefore, MDA-MB-231 cells were cultured in flasks without vented filter caps and the flask caps were opened in the cell culture cabinet for 30 minutes daily to allow gaseous exchange to occur with atmospheric air and the replenishment of oxygen content as recommended by ECACC.

MDA-MB-157 cells were grown in L15 with GlutaMax™I supplemented with 15% (v/v) FBS as recommended (ECACC), but they were extremely slow growing. Subsequently, ECACC's technical support suggested changing their growth media to DMEM with GlutaMax™I supplemented with 10% (v/v) FBS. The MDA-MB-157 cells clearly grew better in DMEM medium than L15 medium. However, there was no difference in the morphological appearance between the cells cultured in L15 and in DMEM growth medium. The morphological appearance of all cell lines in this study is shown in Figures 2.2 and 2.3. All cells were photographed as a living monolayer (at high and low density) using an inverted microscope Leica AF6000 system (Germany) with Leica DFC300FX camera (20x magnification).

2.2.4 Resuscitation of frozen cells

Gentle handling and immediate removal of cryopreservative medium was required for the recovery of cells, as cryopreserved cells are extremely fragile. A cryovial containing frozen cells was transferred from liquid nitrogen storage to a Class II safety cabinet. The outside of the cryovial was wiped with 70% (v/v) ethanol to avoid microbial contamination, and the screw top was opened slightly for 2 seconds to reduce the pressure within the vial. The bottom half of the vial was then immediately immersed in a 37°C water bath until thawed. The cell suspension was gently transferred to a sterile universal tube containing 10 mL of an appropriate pre-warmed growth medium in order to remove or dilute residual dimethyl sulphoxide (DMSO), prior to centrifugation at 500 x g (Heraeus Biofuge Primo centrifuge, USA) for 5 minutes at room temperature. Increased speed and prolonged spin time has been suggested as decreasing viability of cells (Freshney 2005). The cell pellet was then re-suspended in 10 mL growth medium and the resulting suspension was transferred into a 25 cm² flask, before being placed in the incubator at 37°C in an atmosphere of 5% CO₂ and 95% air.

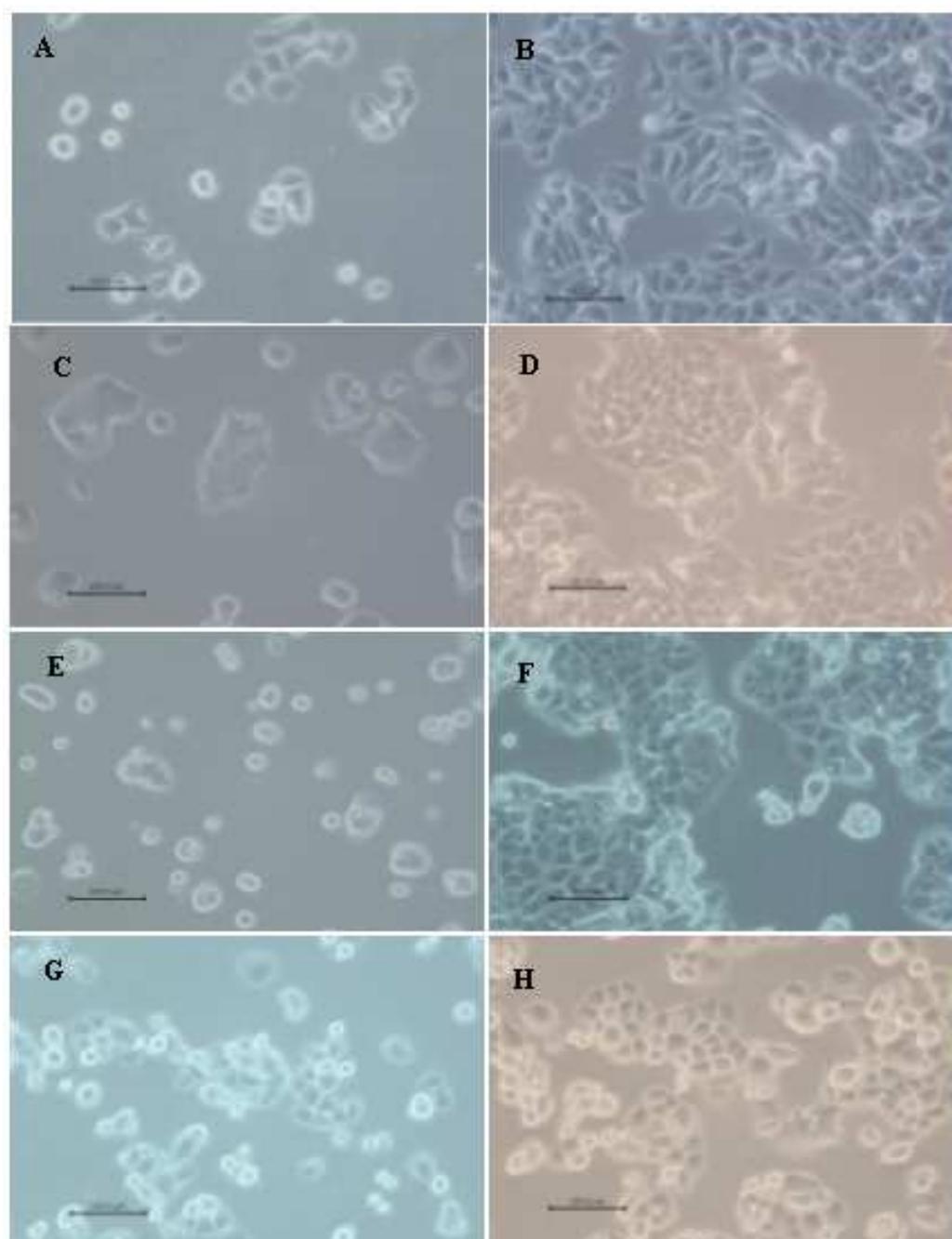


Figure 2.2: Morphology of oestrogen positive receptor breast cancer cell lines. Living monolayer, low and high density of MCF7 (A, B), T47D (C, D), ZR-75-1 (E, F) and ZR-75-30 (G, H) cells. Scale bar = 200 μm .

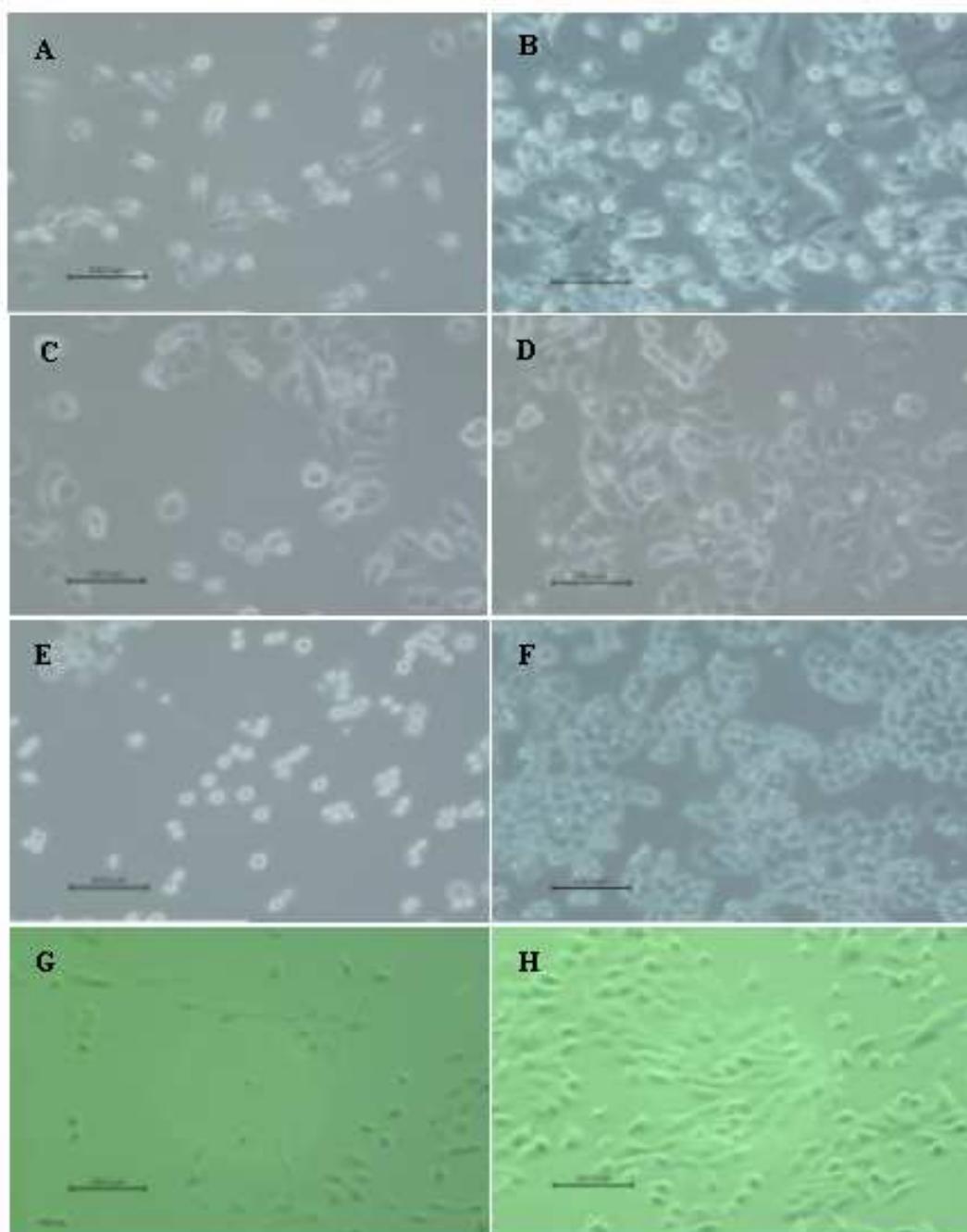


Figure 2.3: Morphology of oestrogen negative receptor breast cancer cell lines. Living monolayer, low and high density of MDA-MB-231 (A, B), MDA-MB-157 (C, D), MDA-MB-468 (E, F), and normal breast cell line MCF10A (G, H) cells. Scale bar = 200 μm .

2.2.5 Trypsinisation

Trypsin is a proteolytic enzyme that cleaves proteins at the carboxyl side of the basic amino acids lysine and arginine. In a cell culture laboratory, trypsin solutions are commonly used to dissociate adherent cell monolayers during the process of harvesting cells. In this study, cells were harvested using trypsin-ethylenediaminetetraacetic acid (EDTA) (0.05% w/v trypsin with 0.53 mM EDTA.4Na) which had been derived from porcine pancreas. This solution was prepared in Hanks' Balanced Salt Solution (HBSS) without calcium and magnesium. EDTA promotes disaggregation of cells and is a chelator that binds divalent cations such as calcium and magnesium.

Cells were harvested at 70-80% confluence. Spent culture medium was aspirated from the flask, and cells were washed twice with 10 mL of calcium-magnesium free-phosphate buffered saline (pH 7.4) to remove residual medium containing foetal bovine serum, which is a rich source of trypsin inhibitors. Calcium and magnesium also inhibit trypsinisation, as these two cations play a critical role in cell-to-cell and cell-to-substrate attachment. Depending on the size of flask either 2 mL (for a 25 cm² flask) or 3 mL (for a 75 cm² flask) of trypsin-EDTA was added, and incubated at 37°C in 5% CO₂ and 95% air. The progress of cell detachment was checked briefly under the inverted microscope every 5 minutes. When the cells were completely detached, trypsin was inactivated and EDTA removed by adding 10 mL of growth medium containing foetal bovine serum. The resulting cell suspension was collected and centrifuged at 500 x g for 5 minutes at room temperature. The cell pellet was re-suspended in 10 mL growth medium, and cell density and viability were determined using the trypan blue stain (Section 2.2.7). Cells were seeded at 2-5x10⁴ cells/cm² into a new flask. A total volume of 10 mL and 15 mL growth medium was used for 25 cm² and 75 cm² flasks respectively. The cell cultures were incubated at 37°C in an atmosphere of 5% CO₂ and 95% air.

2.2.6 Freezing cell cultures

Freezing cells or cryopreservation is a technique where cells are stored at very low temperatures. Cells become metabolically inert and genetically stable and ice crystal formation is minimised. DMSO solution is the most commonly used cryoprotectant for the protection of cells from mechanical and physical stress, and reduction of water content within cells. The reagents are made into a solution with media or serum; cells are suspended in this solution, and are stored in liquid nitrogen at -196°C (Greene et al. 1967, Freshney 2005).

As indicated earlier, cells cultured following a high passage are more likely to change in cell characteristics (i.e. morphology, growth pattern and genetic stability) than cells derived from a low passage (Davis 2002). The procedure of freezing cells allows cells to be stored for long periods without alterations in viability or other characteristics (Freshney 2005). For this reason, the present study kept a stock of cells at a low passage level in liquid nitrogen with freezing medium. Cells that had been passaged between 5 and 15 times were used for experiments.

After trypsinisation (Section 2.2.5), the cell pellet was re-suspended in 10 mL growth medium, cell samples were counted and viability determined using trypan blue (Section 2.2.7). The suspended cells were centrifuged at $500 \times g$ for 5 minutes at room temperature. The cell pellet was re-suspended in an appropriate volume of freezing medium containing 10% (v/v) DMSO and 90% (v/v) FBS (ECACC recommended) to achieve a cell density of 1×10^6 cells/mL and placed in cryovials. These cells were frozen by a controlled rate freezing device (isopropanol bath at -80°C) to slow the freezing process to $\sim -1^{\circ}\text{C}/\text{minute}$. A slow cooling rate is important as it provides adequate time for proper adjustment of cells to the frozen environment and this limits cell death. Cells were placed in a -80°C freezer for 24 hours, prior to transfer to liquid nitrogen for storage.

2.2.7 Cell viability determination by trypan blue stain

Trypan blue stain which is used to determine the viability of cells does not pass across the plasma membrane unless the cell is damaged or dead. Therefore, viable cells with intact cell membranes are unstained and non-viable cells are stained blue. Cells were counted using a haemocytometer and an inverted microscope (Freshney 2005).

After trypsinisation (Section 2.2.5), the cell pellet was re-suspended in 10 mL growth medium, and 20 μ L of the cell suspension was mixed with 20 μ L 0.4% (w/v) trypan blue stain in a sterile 1.5 mL microcentrifuge tube (giving a dilution factor of 2). This mixture was placed on a haemocytometer for cell counting. The hemacytometer has 2 chambers (Figure 2.4A) and there are 9 large squares with grid in each chamber (Figure 2.4B). Each outer quadrant is divided into 16 smaller squares and Figure 2.4B shows one chamber of a haemocytometer slide under 10x magnification. Only cells that overlap the top and left borders of squares should be counted to avoid overestimating the cell concentration (Davis 2002).

The four outer quadrants in the grid were used in both chambers to count the non-viable and viable cells at 10x magnification. The total count was divided by eight to determine the average number of cells in one quadrant, and this number was multiplied by 2 (the dilution factor) and then 10^4 in order to calculate the number of cells in one millilitre of suspension (the number 10^4 is derived from the total volume of each quadrant; 0.1 mm^3 or $1 \times 10^{-4} \text{ mL}$). The percentage of viable cells was determined by dividing the number of unstained cells by the total number of cells and multiplying by 100.

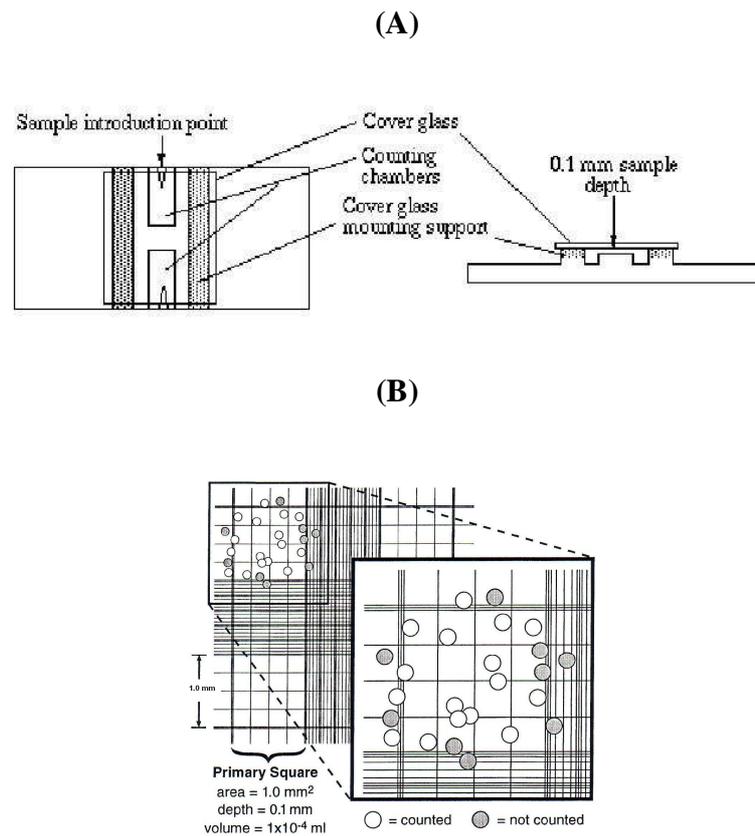


Figure 2.4: Cell counting using a haemocytometer. (A) A haemocytometer slide. (B) Improved Neubauer pattern ruled haemocytometer (Davis 2002).

2.3 Purification of total RNA

Monolayer cells were washed twice with 10 mL sterile phosphate buffered saline. Total RNA was isolated from the cells using a Qiagen RNeasy Plus Mini kit according to the manufacturer's instructions as follows (Figure 2.5). In brief, lysis buffer which contained guanidine thiocyanate and 1% (v/v) beta-mercaptoethanol as a reducing reagent was added to the cells in order to disrupt the cell walls, and release the RNA contained in the sample. The lysed cells were manually scraped and transferred to a new 1.5 mL microcentrifuge tube. Cell lysate was passed through a 23-gauge needle attached to a sterile plastic syringe 20 times manually to reduce the viscosity of the cell lysates, and shear the high-molecular weight genomic DNA. The lysate was then transferred to a genomic DNA Eliminator spin column and centrifuged at 14,500 x g for 30 seconds (Eppendorf microcentrifuge 5415D, Germany). The column was discarded, and one volume of 70% (v/v) ethanol was added to

the flow-through and mixed well. The cell lysate was then placed in an RNeasy mini column and centrifuged at 14,500 x g for 15 seconds. The flow-through was discarded and this process repeated. An aliquot (700 μ L) of RW1 Buffer (provided in the Kit) was added to the RNeasy column and centrifuged at 14,500 x g for 15 seconds; the flow-through was then discarded. The RNeasy column was washed with 500 μ L RPE Buffer (provided in the Kit) and centrifuged at 14,500 x g for 15 seconds; again the flow-through was discarded. The RNeasy silica-gel membrane was dried by adding an additional 500 μ L of RPE Buffer prior to centrifugation at 14,500 x g for 2 minutes, the resulting flow-through was discarded and the tube recentrifuged once more at 25,000 x g for 1 minute. The RNeasy column was then placed on a new sterile 1.5 mL microcentrifuge collection tube. Total RNA was eluted from the column by the addition of 2 x 50 μ L RNase-free water, and the column was then centrifuged twice at 14,500 x g for 1 minute, using the same collection tube. All centrifugation steps were performed at room temperature. The RNA isolation procedures were performed in a Class II safety cabinet.

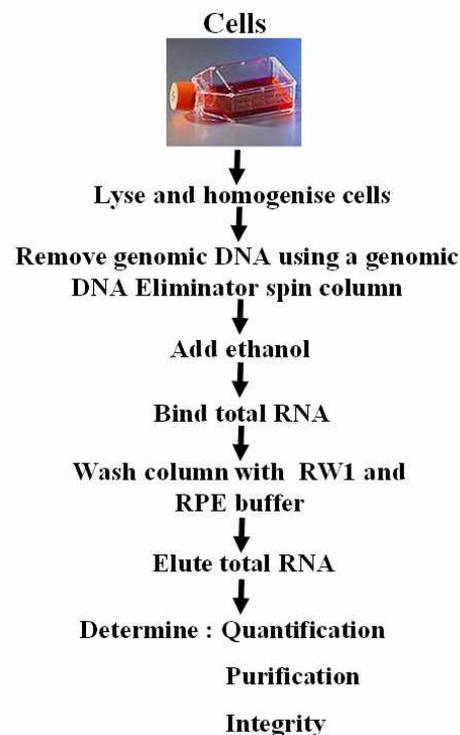


Figure 2.5: Flowchart of isolation of RNA using RNeasy Plus procedure.

The total RNA was precipitated by the addition of 0.1x volume of 3.0 M sodium acetate (pH 5.2) and 3x volume of ice cold ethanol. The RNA sample was subsequently mixed gently and stored at -20°C overnight. RNA was recovered by centrifugation at 15,000 x g (Heraeus Biofuge 28RS centrifuge, Germany) for 5 minutes at 4°C. The pellet was washed with 0.5 mL of 70% (v/v) ethanol and then centrifuged at 15,000 x g for 5 minutes at 4°C and this process repeated in order to remove residual salt. Evaporation of residual ethanol from the RNA pellet was performed by leaving the open tube on the lab bench at room temperature for 20 minutes. Care was taken not to overdry the RNA pellet, as this makes it more difficult to solubilise the pellet. The RNA pellet was re-suspended in 20 µL 0.1% (v/v) diethyl pyrocarbonate (DEPC)-treated water (Appendix B) and stored at -80°C until required (Sambrook and Russell 2001). RNA samples solubilised in RNase-free water can be stored at -80°C without degradation for more than one year (Chomczynski 1992).

RNA concentration and purity were determined by spectrophotometry (Thermo Electron Spectrometry Biomate 5TM, UK). Ten µL of the total RNA was diluted with 490 µL of 0.1% (v/v) DEPC-treated water (a dilution factor of 50). The RNA concentration of a diluted sample was determined by absorbance at 260 nm (A_{260}), with one absorbance unit of A_{260} corresponding to 40 µg of RNA per mL. The A_{260}/A_{280} ratio was calculated to give an indication of sample purity, with ranges between 1.8 and 2.1 considered acceptable for good quality RNA (Sambrook and Russell 2001).

Agarose gel electrophoresis is commonly used to judge the quality of the isolated total RNA sample by inspection of the 28S and 18S ribosomal RNA (rRNA) bands. A sample loading of 1 µg RNA was verified visually by electrophoresis on a 1.0% (w/v) agarose gel dissolved in Tris-Borate-EDTA (TBE) buffer and containing a final concentration of 0.5 µg/mL ethidium bromide. Electrophoresis was carried out for 60 minutes at 100 V in a Minicell EC370M (EC Apparatus Corporation, USA). With intact total RNA, the upper

rRNA (28S, Mw 5.0 kb) band should be approximately twice as intense as the lower (18S, Mw 1.9 kb) rRNA band, indicating non-degraded high quality RNA (Jones et al. 1994). Additional information on agarose gel electrophoresis is provided in Section 2.8.

2.4 Reverse transcription and first strand cDNA synthesis

Since RNA does not serve as a template for DNA polymerase, a total RNA sample must firstly be reverse-transcribed into complementary DNA (cDNA) using a reverse transcriptase (RT) reaction. The subsequent cDNA can then be amplified by the polymerase chain reaction (PCR) method to detect the expression of target genes. The RT reaction consists of RNA template, reverse transcriptase (e.g. moloney murine leukemia virus; M-MLV or avian myeloblastosis virus; AMV), specific primers (e.g. oligo(dT)_n, anchored oligo(dT)_n, random hexamers, sequence-specific) and deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP) (Figure 2.6).

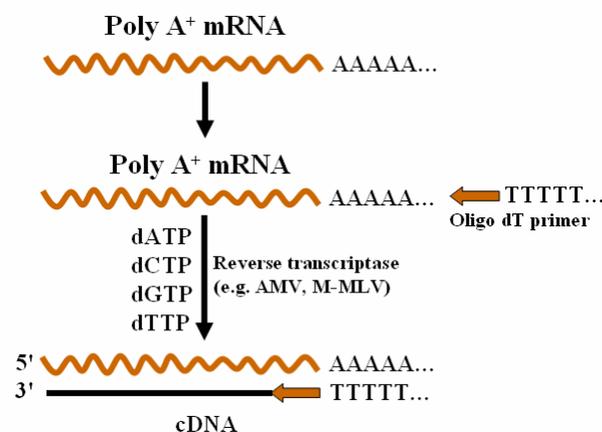


Figure 2.6: Synthesis of the first strand cDNA.

Abbreviations: cDNA; complementary deoxyribonucleic acid, A⁺; adenine dT; deoxythymidylic acid dATP; deoxyadenosine triphosphate, dCTP; deoxycytidine triphosphate, dGTP; deoxyguanosine triphosphate, dTTP; deoxythymidine triphosphate, mRNA; messenger ribonucleic acid, M-MLV; moloney murine leukemia virus; or AMV; avian myeloblastosis virus

M-MLV reverse transcriptase is most commonly used to synthesise full-length copies of RNA molecules which can generate high copies of cDNA in samples containing low amounts of RNA (Newton and Graham 1997). At the 3' end of mRNA, there is a long sequence of adenine nucleotides known as 'the 3' poly (A) tail' which can be hybridised

with a short complementary synthetic oligo (dT) primer. Oligo (dT)_n is an oligomer (n usually between 12 and 18 nucleotides) that is mainly used to synthesise full-length cDNAs from poly (A)_n mRNA (Sambrook and Russell 2001).

In this study, 5 µg of total RNA was added to a 5 µM suspension of oligo (dT)₁₃ primers, and then made up to a reaction volume of 12 µL with 0.1% (v/v) DEPC-treated water, gently mixed, and followed by a brief centrifuge at 4000 x g for 2 seconds to bring all components to bottom of tube. The reaction was incubated at 70°C for 10 minutes in the Biometra T3000 thermocycler (Thistle scientific Ltd., UK) to allow annealing of the oligo dT to the RNA template; the tubes were then placed on ice for 15 minutes to stop the reaction. Eight µL of RT reaction mixture consisted of 2 µL 10x RT reaction buffer, 1 µL 200 U M-MLV reverse transcriptase, 1 µL 10 mM dNTPs and 0.1% (v/v) DEPC-treated water to make up a total volume of 8 µL. The RT reaction was prepared as presented in Table 2.1. The reaction mixture was incubated at 42°C for 50 minutes, prior to terminating the reaction at 95°C for 5 minutes and chilling at 4°C for 30 minutes in the thermocycler. The thermal profile for the first strand synthesis was set as shown in Table 2.2. Following the thermocycling reaction 80 µL 0.1% (v/v) DEPC-treated water was added to the samples. In order to standardise all subsequent procedures, the concentration of this is then represented as an equivalent cDNA concentration of 50 ng cDNA/µL which was stored at -80°C until further use.

Table 2.1: *Components of first strand (RT) reaction.*

Component	Concentration in reaction
Template RNA	5 µg
Oligo(dT) ₁₃	5 µM
10x RT reaction buffer	1x (containing 50 mM Tris-HCl, pH 8.3, with 50 mM KCl, 3 mM MgCl ₂ , and 5 mM DTT)
dNTPs (10 mM)	0.5 mM each of dATP, dCTP, dGTP, dTTP
M-MLV RT	10 U
0.1% (v/v) DEPC-treated water	To make final volume 20 µL

Table 2.2: Thermocycling conditions for first strand (RT) cDNA synthesis.

Phase	Temperature	Duration
Oligo (dT) priming	70°C	10 minutes
Reverse transcription (RT)	42°C	50 minutes
RT inactivation	95°C	5 minutes

2.5 Qualitative reverse transcription-polymerase chain reaction

(RT-PCR) analysis

Polymerase chain reaction (PCR), a technique originally invented by Kary Mullis in 1985, is an *in vitro* enzymatic reaction capable of amplifying the number of copies of a target DNA sequence. This method can be used for a variety of analyses in biomedical research (Newton and Graham 1997). Over two decades, PCR has been modified and developed for different applications. Reverse transcription-polymerase chain reaction (RT-PCR) is a highly sensitive technique and widely used in the study of gene expression at the RNA level, as a low copy number of RNA molecule can be detected. The first strand cDNA is synthesised (Section 2.4), followed by amplification of the specific parts of double-stranded DNA within cells using the PCR. PCR is carried out in a thin walled tube contained within a thermocycler using a specified programme that can control the precise temperature requirements for each step of the reaction.

The PCR requires basic components containing *Taq* DNA polymerase, reaction buffer (including magnesium chloride; MgCl₂), dNTPs, primers, template DNA and water in a single tube. Determination of the optimal concentration of each component in a PCR reaction is important when DNA synthesis fidelity is critical. *Taq* DNA polymerase is a thermostable polymerase originally isolated from the thermophilic bacterium *Thermus aquaticus* and is the most widely used in PCR as it allows application of high annealing and extension temperatures during the amplification process. Under optimal conditions this enzyme can synthesise a new DNA strand at a rate of 35-100 nucleotides/second at 70-80°C and is stable during incubation at 95°C, allowing denaturation steps in the

amplification cycling (Newton and Graham 1997). The enzyme has exonuclease activity 5'→3' but not on the 3'→5' direction. The concentrations between 1.0 and 2.5 U of *Taq* DNA polymerase are usually used in 50 µL of PCR reaction mixture. An optimal concentration of *Taq* DNA polymerase is necessary to obtain a better yield of PCR products as a higher concentration of enzyme may result in non-specific products and lower amounts of *Taq* DNA polymerase may result in low yield of the desired product (Toranzos 1997).

The MgCl₂ concentration in the reaction is one of the most important factors for successful amplification of cDNA as it functions as a cofactor for DNA polymerase. However, Mg²⁺ ions can interact with dNTPs, primers and DNA templates to form complexes so the MgCl₂ concentration has to be optimal for each experiment. Excessive Mg²⁺ ions can lead to high levels of non-specific products, whereas inadequate Mg²⁺ ions will inhibit the reaction, resulting in low yield of PCR products. The recommended range of MgCl₂ concentration in the final reaction mixture is between 0.5 and 5.0 mM for the standard reaction conditions (Newton and Graham 1997). All four dNTPs (i.e. dATP, dCTP, dGTP and dTTP) are required for the synthesis of DNA. Usually, the final concentration of each dNTP in the PCR reaction mixture is between 0.15 mM and 0.2 mM and these should be used at equal concentration to minimise misincorporation (Toranzos 1997).

The design of good synthetic oligonucleotide primers to anneal to target DNA is essential for the specificity of amplification product in order to have a successful PCR. Guidelines for primer selection in the current study are detailed in Section 2.6. In the PCR reaction mixture, the final concentration of each primer is in the range of 0.1-0.5 µM and must be used in equal concentrations. Higher concentrations of primer usually generate non-specific products and form primer-dimers (Toranzos 1997).

The working concentration of template DNA should be between 100 and 500 ng in a total PCR reaction volume of 50 μ L. Insufficient template DNA can lead to low yield of products while excessive template DNA usually may generate non-specific products and reduce efficiency (John and Stirling 2003).

Regarding the cycling conditions, a series of twenty-five to thirty-five cycles of PCR are usually carried out with each cycle of the PCR consisting of three steps (i.e. denaturation, annealing and extension of the DNA template, Figures 2.7A and 2.7B) (Newton and Graham 1997). During repeated cycles of PCR, the copy of new synthesised DNA strands increases exponentially and the target DNA replicates several million-fold. It is essential that the target DNA is analysed within the exponential phase of the PCR reaction, before the plateau phase when the reaction components are insufficient, leading to the low efficiency of amplification (Kainz 2000).

Initially, the double-stranded DNA is heated to 94-96°C in order to break apart the hydrogen bonds between the two DNA strands. The first denaturing step requires an extended time period to ensure that the template DNA is completely separated into single-strands. Denaturation for 0.5-2 minutes is usually sufficient. Following denaturation, the temperature is lowered to allow the primers to anneal to specific sequences on the single-stranded DNA in a step known as primer annealing. Identifying the appropriate annealing temperature (T_a) for each primer pair is a critical factor in the PCR reaction and is based on the composition of the individual primer pairs (i.e. melting temperature; T_m) in order to minimise non-specific amplification products. T_a should be set approximately 5°C below T_m (Newton and Graham 1997). Annealing of the oligonucleotides to the sequence of interest usually takes between 1 and 2 minutes. In the final elongation step the temperature is raised to the optimal temperature for the thermostable enzyme *Taq* DNA polymerase (70-80°C) as mentioned above.

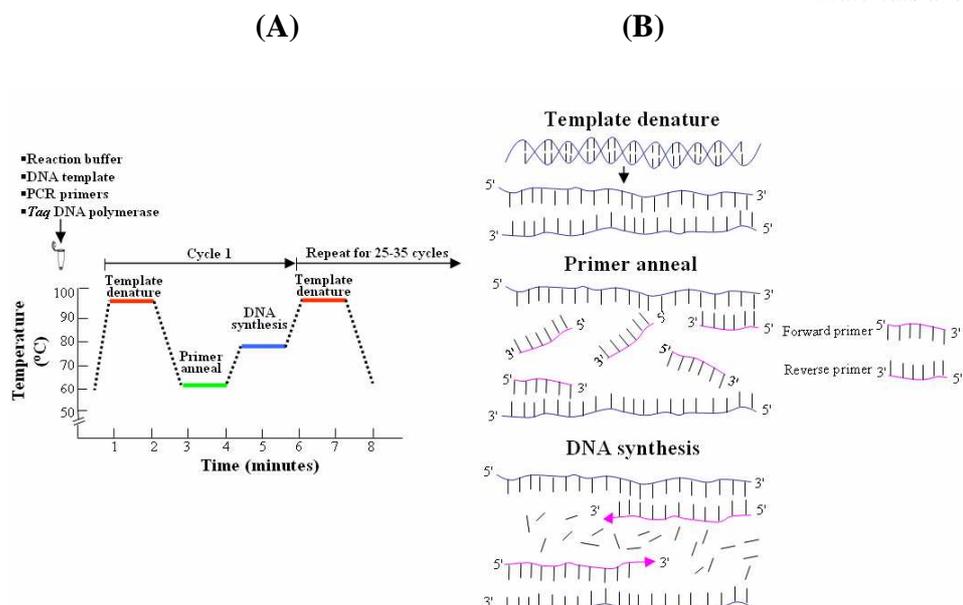


Figure 2.7: The polymerase chain reaction (PCR). (A) The PCR temperature cycling profile (B) The cycling reaction (Adapted from Newton and Graham 1997).

Housekeeping genes are constitutively expressed in all cells and can be used to ensure the efficiency of amplification reaction. Several housekeeping genes have been used as control genes in different model systems such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β -actin and β_2 -microglobulin (Schmittgen and Zakrajsek 2000, Lupberger et al. 2002). β_2 -microglobulin is commonly used as an internal standard gene in gene expression studies as it shows low variation between different cell origins (Lupberger et al. 2002). This gene was used in the present study to verify the expression of target genes.

In this study, the standard PCR was performed in 50 μ L reaction mixtures. The PCR reaction mixture contained 5 μ L of 10x PCR reaction buffer, 1 μ L of dNTP mixture, 0.5 μ L of *Taq* DNA polymerase (5 U/ μ L), 5 μ L of the mixture of forward and reverse primers, template cDNA (50 ng/ μ L) and 0.1% (v/v) DEPC-treated water to make up a total volume of 50 μ L. A negative control (no template cDNA) was included in each PCR reaction. Initially, the standard PCR reaction was prepared as shown in Table 2.3. All components were mixed gently and followed by a brief centrifuge at 4000 x g for 2 seconds to collect them at the bottom of the microfuge tube. The reaction mixture was incubated in a

Biometra T3000 thermocycler (Germany) which was set as shown in Table 2.4.

Optimisation of the PCR procedure involved identification of the appropriate annealing temperature, the concentration of template cDNA and the appropriate primer sets in the PCR reaction as described in Chapter 3.

Table 2.3: *Components of second strand (PCR) reaction.*

Component	Concentration in reaction
10x reaction buffer	1x (containing 10 mM Tris-HCl, 1.5 mM, MgCl ₂ , 50 mM KCl, pH 8.3)
Template cDNA	100 ng
Mixture of forward and reverse primers	0.5 μM each of forward and reverse primers
dNTPs (10 mM)	0.2 mM each of dATP, dCTP, dGTP, dTTP
<i>Taq</i> DNA polymerase	2.5 U
0.1% (v/v) DEPC-treated water	To make final volume 50 μL

Table 2.4: *Thermocycling conditions for second strand (PCR) cDNA synthesis.*

Phase	Temperature	Duration	Number of cycle
Initial denaturation	94°C	2 minutes	1
Template denaturation	94°C	50 seconds	30
Primer annealing	5°C below T _m of primers	1 minute	
DNA synthesis	72°C	1 minute	
Elongation	72°C	5 minutes	1

2.6 Design of synthesis of oligonucleotide primers

A primer is a short synthetic oligonucleotide which is used to determine the DNA fragment to be amplified in applications of PCR or DNA sequencing. The primers are designed to be complementary to the target sequence of template DNA, which serves as a starting point for synthesising a new DNA strand or DNA replication by a DNA polymerase. Primer design is a critical issue in PCR-based detection methods as an inappropriate primer may produce internal secondary structures and primer-dimers. Internal secondary structure and primer-dimer formation occurs, if the forward and reverse primer can anneal to each other or have internal hybridisation. This reduces the available amount of primers, resulting in low effective amplification. Poor primer design may also cause non-specific products which appear as an incorrect product (Newton and Graham 1997, Lodge et al. 2007).

Selection of efficient and specific primers remains empirical. There is no firm rule for the synthesis of effective or specific primer pairs for an amplification reaction but the primer should be designed according to some general guidelines. Usually, a suitable primer for the PCR process should be between 20 and 30 nucleotides in length with a melting temperature (T_m) between 55°C and 65°C, and the PCR reaction works well if primer pairs have similar T_m in order to set the same annealing temperature. Primers that are too short may lead to non-specific copies as they can anneal at many points on a DNA template. However, if primers are too long they are limited by high annealing temperature (Newton and Graham 1997). Currently, there are several free and commercially available web-based services or software provided for primer design, with Primer3 being the most widely used. The web-based application Primer3 programme is hosted by the Whitehead Institute for Biomedical Research (Rozen and Skaletsky 2000). Initially, primers in this study were designed to cross an exon-exon boundary in order to prevent co-amplification of genomic DNA. The designation was performed using design criteria as shown in Table 2.5.

Table 2.5: *Criteria for primer picking conditions using the web-based Primer3 programme.*

Primer condition	Minimum	Optimum	Maximum
Primer size (bp)	18	20	27
Primer T_m (°C)	57	60	63
Primer GC content (%)	20	50	80

Resulting primer sequences were then analysed for any cross-matching with the non-redundant database of GenBank using the standard nucleotide-nucleotide BLAST (The Basic Local Alignment Search Tool) search of the National Centre for Biotechnology Information (NCBI) available at <http://www.ncbi.nlm.nih.gov/blast/>. The Genbank accession number of each P450 and P450-NR genes used for primer design is shown in Tables 2.6 and 2.7. Table 2.6 lists the primers for the candidate genes which were designed using programme Primer3. Table 2.7 shows the list of published primers which were considered for PCR experiments depending on particular objectives, and details will be provided in the subsequent chapters.

Table 2.6(1): List of designed primers.

Gene name	Accession No	Primer	Sequence (5' to 3')	Length	T _m (°C)	T _a (°C)	Product size (bp)
β ₂ -microglobulin	NM_004048	Forward	²⁴ CCTTGAGGCTATCCAGCGTACTCCAAA ⁵⁰	27	60.7	58	319
		Reverse	³⁴² TGATGCTGCTTACATGTCTC ³²³	20	55.2		
CYP1A1	K03191	Forward	¹⁰¹⁹ CTTGGACCTCTTTGGAGCTG ¹⁰³⁸	20	63.8	58	212
		Reverse	¹²³⁰ CGAAGGAAGAGTGTCCGAAG ¹²¹¹	20	63.8		
CYP1A2	Z00036	Forward	⁵⁷⁵ CAATCAGGTGGTGGTGTTCAG ⁵⁹⁴	20	64.2	58	245
		Reverse	⁸¹⁹ GCTCCTGGACTGTTTTCTGC ⁸⁰⁰	20	63.9		
CYP1B1	U03688	Forward	¹¹³² GCAGCTCAACCGCAACTTCA ¹¹⁵¹	20	68.8	60	397
		Reverse	¹⁵²⁸ AAAGCTGGAGAAGCGCATGG ¹⁵⁰⁹	20	68.6		
CYP2A6	X13930	Forward	¹⁰⁰¹ TTGACAGAGTGATCGGCAAG ¹¹²⁰	20	64.1	58	186
		Reverse	¹¹⁸⁶ GGTACACTTCGGTGCCCTTA ¹¹⁶⁷	20	63.7		
CYP2B6	M29874	Forward	²⁴⁰² CCCCATACCCCATCTCTTT ²⁴²¹	20	63.6	58	194
		Reverse	²⁵⁹⁵ GCAGAGGTTGCAGTGAACAA ²⁵⁷⁶	20	64.1		
CYP2C8	Y00498	Forward	⁹⁶⁴ TATGGACTCCTGCTCCTGCT ⁹⁸³	20	63.9	58	230
		Reverse	¹¹⁹³ CCCTTGGGGATGAGGTAGTT ¹¹⁷⁴	20	63.9		
CYP2C9	M61855	Forward	⁹⁰⁵ GGACAGAGACGACAAGCACA ⁹²⁴	20	64.2	58	156
		Reverse	¹⁰⁶⁰ CATCTGTGTAGGGCATGTGG ¹⁰⁴¹	20	64.1		
CYP2C18	M61856	Forward	¹⁵²² CCGCATGGAGCTGTTTTTAT ¹⁵⁴¹	20	63.7	58	199
		Reverse	¹⁷²⁰ GAGAATTGCAGGTGACAGCA ¹⁷⁰¹	20	64.1		
CYP2C19	M61854	Forward	⁸⁸⁷ ACTTGGAGCTGGGACAGAGA ⁹⁰⁶	20	64.0	58	167
		Reverse	¹⁰⁵³ CATCTGTGTAGGGCATGTGG ¹⁰³⁴	20	64.1		

Table 2.6(2): List of designed primers.

Gene name	Accession No	Primer	Sequence (5' to 3')	Length	T _m (°C)	T _a (°C)	Product size (bp)
CYP2D6	X08006	Forward	⁸³⁰ CAGAGATGGAGAAGGCCAAG ⁸⁴⁹	20	63.8	58	191
		Reverse	¹⁰²⁰ CCCTATCACGTCGTCGATCT ¹⁰⁰¹	20	64.0		
CYP2S1	AK027605	Forward	³³¹ AGGTCAGGCTGAGGAGTTCA ³⁵⁰	20	64.0	58	216
		Reverse	⁵⁴⁶ TCTGTCCCCTGGAATGTCTC ⁵²⁷	20	64.1		
CYP3A4	J04449	Forward	⁶⁶⁶ CAAGACCCCTTTGTGAAAA ⁶⁸⁵	20	63.7	58	184
		Reverse	⁸⁴⁹ CGAGGCGACTTTCTTTCATC ⁸³⁰	20	63.7		
CYP3A5	J04813	Forward	⁸⁵⁶ AAGAAAAGTCGCCTCAACGA ⁸⁷⁵	20	63.7	58	227
		Reverse	¹⁰⁸² TGCAGTTTCTGCTGGACATC ¹⁰⁶³	20	64.1		
CYP3A7	D00408	Forward	⁷⁷⁶ AAGGTCGCCTCAAAGAGACA ⁷⁹⁵	20	63.9	58	223
		Reverse	⁹⁹⁸ TGCACTTTCTGCTGGACATC ⁹⁷⁹	20	64.1		
CYP3A43	AA417369	Forward	⁹⁷ AGGGAAAGTCAGGGTCCACT ¹¹⁶	20	63.8	58	190
		Reverse	²⁸⁶ GCATGAGGTTTGCTCTCACA ²⁶⁷	20	64.1		
CYP4Z1	AY262056	Forward	⁵⁷⁵ TCATGAAGTGTGCCTTCAGC ⁵⁹⁴	20	64.1	58	159
		Reverse	⁷³³ GGCCTTGAGAGCTGAATTTG ⁷¹⁴	20	63.7		
CYP24A1	L13286	Forward	¹⁵⁹⁰ GGCAACAGTTCTGGGTGAAT ¹⁶⁰⁹	20	63.9	58	249
		Reverse	¹⁸³⁸ TATTTGCGGACAATCCAACA ¹⁸¹⁹	20	63.8		
CYP26A1	AF005418	Forward	¹¹⁵⁹ TTCGGGTTGCTCTGAAGACT ¹¹⁷⁸	20	63.9	58	191
		Reverse	¹³⁴⁹ TCCTCAAATGGAATGAAGC ¹³³⁰	20	63.8		
CYP26B1	AF252297	Forward	³⁸³ ACACGGTGTCCAATTCCATT ⁴⁰²	20	64.0	58	172
		Reverse	⁵⁵⁴ GCCTCCTGGTACACGTTGAT ⁵³⁵	20	63.9		

Table 2.6(3): List of designed primers.

Gene name	Accession No	Primer	Sequence (5' to 3')	Length	T _m (°C)	T _a (°C)	Product size (bp)
HIF-1 α	U29165	Forward	²⁰³⁵ GAAAGCGCAAGTCCTCAAAG ²⁰⁵⁴	20	63.8	58	167
		Reverse	²²⁰¹ TGGGTAGGAGATGGAGATGC ²¹⁸²	20	64.0		
AhR	NM_001621	Forward	¹¹¹⁷ GGACAGAAAAAGAAAGGGAAAG ¹¹³⁸	22	61.8	55	262
		Reverse	¹³⁷⁸ CGGCACAATAAAGCATATCAG ¹³⁵⁸	21	61.9		
ARNT	NM_178427	Forward	²⁰⁷⁵ ATCCTCCTTCAGCTCCATGTCC ²⁰⁹⁶	22	67.6	60	387
		Reverse	²⁴⁶¹ TTTCTCCCCACCCCTTATC ²⁴⁴²	20	65.9		
AhRR	BM682431	Forward	¹²⁰ TTCAGGATAGGCTCCTGCAT ¹³⁹	20	63.6	58	171
		Reverse	²⁹⁰ AGTGACCACCATCCAAGCTC ²⁷¹	20	64.1		
CAR*	Z30425	Forward	⁸⁶¹ GCAGCTGTGGAAATCTGTCA ⁸⁸⁰	20	64.1	58	232
		Reverse	¹⁰⁹² CAGGTCGGTCAGGAGAGAAG ¹⁰⁷³	20	64.0		
PXR	AJ009936	Forward	³⁹⁵⁸ CTGTGTGGATGCTGAGCTGT ³⁹⁷⁷	20	64.3	58	214
		Reverse	⁴¹⁷¹ TGATTGTCAGCGTAGCCTTG ⁴¹⁵²	20	63.9		
VDR	J03258	Forward	¹⁵⁹⁷ GAGACCTCAGCCATGAGGAG ¹⁶¹⁶	20	64.0	58	250
		Reverse	¹⁸⁴⁶ CGTGAGTAAGGCAGGAGAGG ¹⁸²⁷	20	63.8		
ER	X03635	Forward	¹⁶²⁵ TGGAGATCTTCGACATGCTG ¹⁶⁴⁴	20	64.1	58	208
		Reverse	¹⁸³² GCCATCAGGTGGATCAAAGT ¹⁸¹³	20	63.9		

Note: *; This primer set was also used as nested primer for the published CAR.

Table 2.7(1): List of published primers.

Name	Accession No	Primer	Sequence (5' to 3')	Length	T _m (°C)	T _a (°C)	Product size (bp)	Reference
CYP1A1	K03091	Forward	⁹⁶⁷ TGGATGAGAACGCCAATGTC ⁹⁸⁶	20	66.0	58	394	Huang et al. 1996
		Reverse	¹³⁵⁸ TGGGTTGACCCATAGCTTCT ¹³³⁹	20	63.3			
CYP2D6	M20403	Forward	¹⁰³³ CCAGAGATGGGTGACCAG ¹⁰⁵⁰	18	61.8	55	261	Thomas et al. 2006
		Reverse	¹²⁹³ CTCCGGCTTCACAAAGTG ¹²⁷⁶	18	62.1			
CYP2F1	NM_000774	Forward	⁶⁴⁶ CCATTATCCGCCTTATCAAT ⁶⁶⁵	20	60.1	55	305	Thomas et al. 2006
		Reverse	⁹⁵⁰ GCCAAAGAGCAGGTTATGT ⁹³²	19	59.3			
CYP2J2	NM_000775	Forward	⁴⁶⁶ GCTTAGAGGAACGCATTCA ⁴⁸⁴	19	60.5	55	714	Thomas et al. 2006
		Reverse	¹¹⁷⁹ CAGCCAAAGTGGTATCAACT ¹¹⁶⁰	20	59.2			
CYP2R1	NM_024514	Forward	²⁶⁰ TGGTTCTAAATGGCTATGATG ²⁸⁰	21	59.0	52	308	Thomas et al. 2006
		Reverse	⁵⁶⁷ GTTTGAAACAGCATTTCGTTA ⁵⁴⁸	20	57.8			
CYP2U1	NM_183075	Forward	⁵⁴⁵ GTCATCTCCATCGTGAC ⁵⁶²	18	59.2	55	320	Thomas et al. 2006
		Reverse	⁸⁶⁴ TTTCTAGGCCTCGTGACATA ⁸⁴⁵	20	59.8			
CYP3A4	M14096	Forward	⁷⁸² CTGTGTGTTTCCAAGAGAAGTTAC ⁸⁰⁵	24	60.6	55	742	Huang et al. 1996
		Reverse	¹⁵²³ TGGTTGAAGAAGTCCTCCTAAG ¹⁵⁰²	22	61.2			
CYP3A5	J04813	Forward	⁷⁹⁹ GTCTCTGTGTTTCCAAAAGATACC ⁸²²	24	60.9	55	737	Huang et al. 1996
		Reverse	¹⁵³⁶ TGAAGAAGTCCTTGCGTGTC ¹⁵¹⁶	20	63.0			
CYP4X1	NM_178033	Forward	⁶⁵⁵ GCATCGTCGCCTACTAACTC ⁶⁷⁴	20	61.4	55	364	Thomas et al. 2006
		Reverse	¹⁰¹⁸ CTGATTCAACACTCGGCTTA ⁹⁹⁹	20	60.4			
CYP4Z1	NM_178134	Forward	¹¹⁴⁷ ATATCCCGGTTACTCGACAA ¹¹⁶⁶	20	60.7	55	257	Thomas et al. 2006
		Reverse	¹⁴⁰³ GCCACTGCCACTTTACT ¹³⁸⁵	19	60.3			

Table 2.7(2): List of published primers.

Name	Accession No	Primer	Sequence (5' to 3')	Length	T _m (°C)	T _a (°C)	Product size (bp)	Reference
CAR	Z30425	Forward	⁶⁸⁴ CCAGCTCATCTGTTCATCCA ⁷⁰³	20	63.9	58	417	Chang et al. 2003
		Reverse	¹¹¹⁰ GGTAACTCCAGGTCGGTCAG ¹⁰⁸¹	20	63.5			
PXR	AF061056	Forward	⁶⁷⁸ CAAGCGGAAGAAAAGTGAACG ⁶⁹⁸	21	65.7	60	246	Chang et al. 2003
		Reverse	⁹²³ CACAGATCTTTCCGGACCTG ⁹⁰⁴	20	64.6			
PGR	M15716	Forward	¹⁸¹⁷ GATTCAGAAGCCAGCCAGAG ¹⁸³⁶	20	64.0	58	533	Lau et al. 1999
		Reverse	²³⁴⁹ TGCCTCTCGCCTAGTTGATT ²³³⁰	20	63.7			
GCR	X03225	Forward	²⁰³⁸ CTACCCTGCATGTACGACC ²⁰⁵⁶	19	60.6	55	360	Gupta and Wagner 2003
		Reverse	²³⁹⁶ TCAGCTAACATCTCGGGG ²³⁷⁸	18	61.4			
PPAR α	L02932	Forward	⁹¹³ GCCAGTAACAATCCACCTTTT ⁹³³	21	61.7	55	735	Saidi et al. 2006
		Reverse	¹⁶⁴⁷ AAGGTGTGGCTGATCTGAAGG ¹⁶²⁷	21	65.4			
RXR	NM_002957	Forward	⁴⁹⁰ GCTCCTCAGGCAAGCACTATGGAGTGTA ⁵¹⁷	28	73.1	68	521	Wang and Yen 2004
		Reverse	¹⁰¹⁰ GGAGAAGGAGGCGATGAGCAGCTCATT ⁹⁸⁴	27	75.9			
RAR	NM_000964	Forward	⁹⁰⁸ GGGCATGTCCAAGGAGTCTGTGAGAA ⁹³³	26	73.8	68	756	Wang and Yen 2004
		Reverse	¹⁶⁶³ GGGATCTCCATCTTCAGCGTGATCA ¹⁶³⁸	25	73.0			

Oligonucleotide primers were synthesised by Sigma-Genosys Ltd. Lyophilised primers were diluted to a stock concentration of 100 μM by adding 0.1% (v/v) DEPC-treated water according to the manufacturers supplied OD readings, then adjusting this to a working concentration of 5 μM .

2.7 Nested PCR

Nested PCR is used to enhance sensitivity and specificity of amplification as it can eliminate the occurrence of non-specific products generated from unexpected primer binding sites. Nested PCR technique is performed with two sets of primers one internal to the other. The primary amplification product is used as template in the nested PCR reaction with another primer set (Figure 2.8). The components for this reaction are listed in Table 2.8.

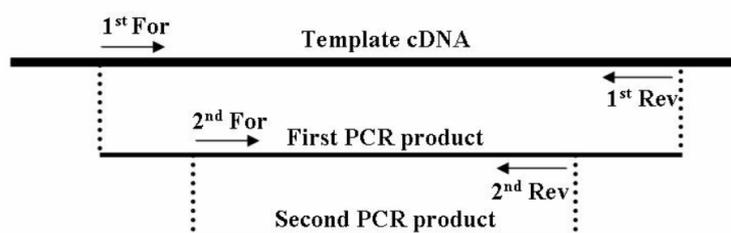


Figure 2.8: Illustration of nested PCR.

Table 2.8: Components of the nested PCR.

Components	Concentration in reaction
10x reaction buffer	1x (1.5 mM MgCl_2)
RT-PCR product from the primary amplification used as a template	5 μL
Mixture of forward and reverse nested primers	0.5 μM each of forward and reverse nested primers
dNTPs (10 mM)	0.2 mM each of dATP, dCTP, dGTP, dTTP
<i>Taq</i> DNA polymerase	0.025 U
0.1% (v/v) DEPC-treated water	To make final volume 50 μL

2.8 Agarose gel electrophoresis of DNA

Electrophoresis is a technique used to separate DNA, RNA or protein molecule by size. Nucleic acids (DNA and RNA) have a negative charge and when an electric current is applied they move through the agarose matrix migrating towards the positive electrode. Larger DNA fragments move at a slower rate than smaller fragments. Ethidium bromide is a fluorescent dye that intercalates between bases of nucleic acids. On exposure to UV light, ethidium bromide transmits the energy as visible orange light and allows detection of DNA fragments. A final concentration of 0.5 µg/mL ethidium bromide is usually used and this can be added into the gel either before or after electrophoresis. The gel is prepared by dissolving the agarose in an appropriate volume of buffer following heating in a microwave oven. The dissolved agarose solution is then placed into a horizontal gel manifold and allowed to cool. Once the gel is set (approximately 30 minutes to 1 hour) it is then placed into the electrophoresis tank. The same buffer used to prepare the gel is then used to fill the tank. Samples (PCR products) are prepared as follows. Gel loading buffer must always be added to the samples prior to loading to increase the density of the samples and ensure that they sink into the well, and to add colour to the samples so they can be easily seen when loading. The gel is generally run at 1-10 V/cm until the dyes have migrated an appropriate distance which depends on the size of DNA to be analysed. Each gel electrophoresis must include at least one lane of molecular weight markers to enable analysis of DNA fragment size in the samples. The most commonly used buffers for DNA are TAE (Tris-acetate-EDTA) and TBE (Tris-borate-EDTA). TBE buffer is recommended for analysing fragments less than 1 kb as it gives better resolution and sharper bands. The concentration of agarose used is dependent on the size of product being determined and is varied accordingly for different fragment ranges (Table 2.9) in order to achieve good separation (Martin 1996, Sambrook and Russell 2001).

Table 2.9: Agarose concentration used for separating different DNA fragment sizes (Sambrook and Russell 2001).

Agarose concentration [% (w/v)]	DNA fragment ranges (kb)
0.3	5-60
0.6	1-20
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-3
2.0	0.1-2

For analysing the PCR product in this study, a 2.5 μL aliquot of 6x loading dye (10mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF and 60% glycerol, 60mM EDTA) was added to 10 μL of the PCR product and mixed well. A 1 μL aliquot of 6x loading dye was added to 5 μL of PCR markers (1000, 750, 500, 300, 150 and 50 bp) and this mixture was loaded beside the samples as a reference for relative qualification of PCR products. The PCR marker and sample mixture were separated by gel electrophoresis using a 1.5% (w/v) agarose gel dissolved in TBE buffer (89 mM Tris-HCl pH 7.8, 89 mM Boric acid and 2 mM EDTA) containing ethidium bromide. Electrophoresis was carried out for 70 minutes at 150 V in a Sub-Cell GT system (BioRad, USA) at room temperature. The UV visualised photographs of gels were taken using a Polaroid camera (Hoya, Japan) in the roof of the transilluminator (UVI tech, Cambridge, England). To photograph a gel, the aperture (F-stop) at 5.6 was set with an exposure time (shutter speed) of 2 seconds.

Following visualisation of a band in a PCR product, the size of DNA fragments was determined using semi-logarithmic plotting to estimate molecular weight. To predict an unknown fragment, a logarithmic scale on the vertical (y axis) was plotted for all the molecular weight marker sizes in base pairs against the distance each of those bands had migrated from the wells in millimetres (horizontal; x-axis). The log-linear relationship between migration on the gel and fragment size of the markers provided a standard curve to determine the size of unknown fragments by measuring distance migrated. Figure 2.9

shows how DNA fragment size was estimated by semi-logarithm plotting using an example from an experiment in the current study. The semi-logarithm plot of distance migration based on the DNA markers used allows the size of an unknown fragment that migrated 43 mm to be estimated as approximately 275 bp in size.

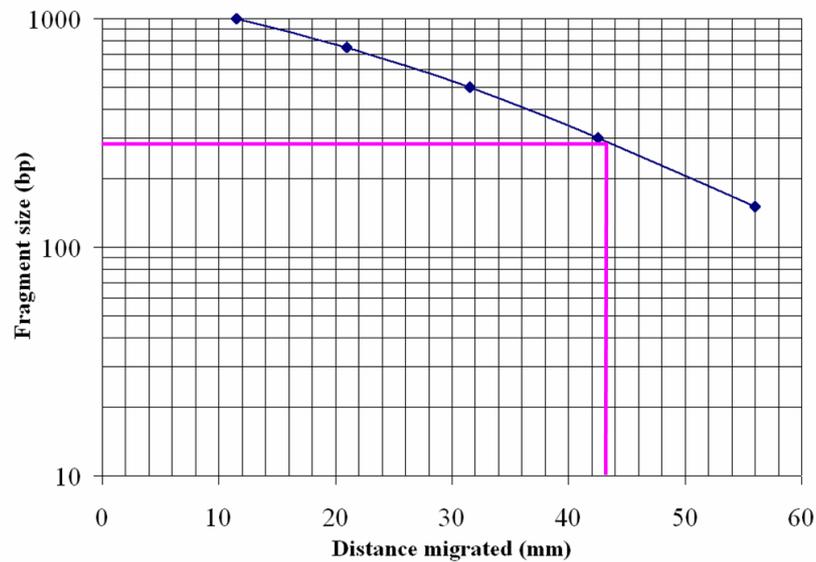


Figure 2.9: Example of semi-logarithm plot of distance migrate the DNA markers used (scanned from one of the results in this study).

2.9 Restriction digestion

Restriction digestion is the method of cutting double-stranded DNA molecules at specific sequences (known as recognition sites) into smaller fragments using restriction enzymes.

This process is useful in molecular biology applications as it provides high sequence specificity and precision cutting. Figure 2.10 shows an example of the DNA nucleotide sequences recognised by the restriction enzyme known as *EcoRI*.

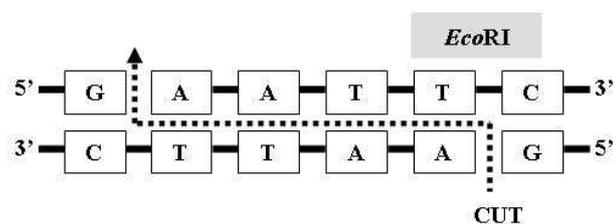


Figure 2.10: Recognition sequence of the restriction endonuclease *EcoRI* (Adapted from Albert et al 2002).

The expression of an individual gene can be identified by restriction digestion. In this study, an appropriate restriction enzyme for each individual (presence) candidate gene was

selected and information on these enzymes is presented in Table 2.10. Table 2.11 highlights the conditions used for restriction digests in this research. All components were mixed gently by pipetting and followed by a brief centrifuge at 4000 x g for 2 seconds to collect them at the bottom of the tube. This mixture was kept on ice prior to the reaction. Restriction digests were performed in a thermocycler at 37°C for 1 hour to complete digestion, and then incubated at 70°C for 10 minutes to inactivate the restriction enzyme. The digested and undigested products were further analysed by 2% (w/v) agarose gel electrophoresis to separate the fragments by size or alternatively stored at -20°C until required.

2.10 Purification of DNA and DNA sequencing

DNA sequencing is used to determine nucleotide sequences of a specific DNA molecule in order to identify the gene product and predict its physiological functions. This requires the purification of DNA fragments from agarose gels or amplification reactions. Purification of DNA from low-melting point (LMP) agarose gel is commonly used as it is a quick procedure and produces high purity DNA. LMP agarose gel can be melted at 65-75°C and this will not dissociate the double-stranded nature of duplex DNA, whereas a standard agarose gel can only be melted at 80-90°C and this will disrupt duplex DNA molecules (Martin 1996).

A high Pure PCR product purification kit (Roche Diagnostics Ltd., Lewes, UK) was used for isolation of DNA, according to the manufacturer's instructions (Figure 2.11). In brief, PCR product was loaded on a 1.8% (w/v) low melting point (LMP) agarose gel containing a final concentration of 0.5 µg/mL ethidium bromide and TAE (40 mM Tris acetate, 2 mM EDTA; pH 8.5) was used as running buffer. Electrophoresis was carried out for 60 minutes in a Minicell EC370M (EC Apparatus Corporation) at 100 V. To handle LMP

Table 2.10: Recognition sequences for the restriction endonucleases used.

Name	Restriction enzyme	Recognition site	Size of fragment (bp)	Buffer**
β_2 -microglobulin	<i>EcoRI</i>	G/AATTC	215, 104	SH
CYP1B1	<i>RsaI</i>	GT/AC	174, 223	SL
CYP2A6	<i>HaeIII</i>	GG/CC	48, 72, 66	SM
CYP2B6	<i>HaeIII</i>	GG/CC	30, 164	SM
CYP2C8	<i>MspI</i>	C/CGG	170, 60	SL
CYP2C18	<i>RsaI</i>	GT/AC	125, 74	SL
CYP2S1	<i>AluI</i>	AG/CT	103, 63, 50	SA
CYP3A7	<i>EcoRI</i>	G/AATTC	65, 158	SH
CYP4Z1	<i>RsaI</i>	GT/AC	47, 112	SL
CYP24A1	<i>BamHI</i>	G/GATCC	68, 181	SB
CYP26A1	<i>AluI</i>	AG/CT	172, 19	SA
CYP26B1	<i>AluI</i>	AG/CT	104, 68	SA
HIF-1 α	<i>SspI</i>	AAT/ATT	140, 27	SH
AhR	<i>MspI</i>	C/CGG	99, 163	SL
ARNT	<i>PstI</i>	CTGCA/G	118, 269	SH
AhRR	<i>HaeIII</i>	GG/CC	92, 79	SM
VDR	<i>PstI</i>	CTGCA/G	198, 52	SH
ER	<i>RsaI</i>	GT/AC	111, 97	SL
Nested CAR	<i>PstI</i>	CTGCA/G	170, 62	SH
CYP1A1*	<i>HaeIII</i>	GG/CC	188, 40, 164	SM
CYP2D6*	<i>RsaI</i>	GT/AC	119, 142	SL
CYP2F1*	<i>MspI</i>	C/CGG	195, 110	SL
CYP2J2*	<i>AluI</i>	AG/CT	331,170,159,54	SA
CYP2R1*	<i>HaeIII</i>	GG/CC	130, 118, 60	SM
CYP2U1*	<i>HaeIII</i>	GG/CC	252, 60, 8	SM
CYP4X1*	<i>RsaI</i>	GT/AC	278, 86	SL
CYP4Z1*	<i>AluI</i>	AG/CT	196, 61	SA
PXR*	<i>MspI</i>	C/CGG	142, 90, 14	SL
PGR*	<i>AluI</i>	AG/CT	32, 84, 417	SA
GCR*	<i>AluI</i>	AG/CT	148, 36, 172, 4	SA
PPAR α *	<i>RsaI</i>	GT/AC	518, 188, 29	SL
RXR*	<i>BamHI</i>	G/GATCC	280,153,88	SB
RAR*	<i>HaeIII</i>	GG/CC	456, 193, 107	SM

Note: *; Previously published primers, ** ; The digestion buffer was varied according to the enzyme of choice and was supplied along with the restriction enzyme from supplier. Composition of the 1x buffer is shown in Appendix C.

Table 2.11: Components of the restriction digest.

Stock solution	Undigested sample (μL)	Digested sample (μL)
Re-amplified RT-PCR product	2	2
10x digestion buffer	2	2
Restriction enzyme (10 U/ μL)	0	1
0.1% (v/v) DEPC-treated water	16	15

agarose gel, these procedures were performed on ice due to the fragile nature of gels at higher temperatures (Martin 1996).

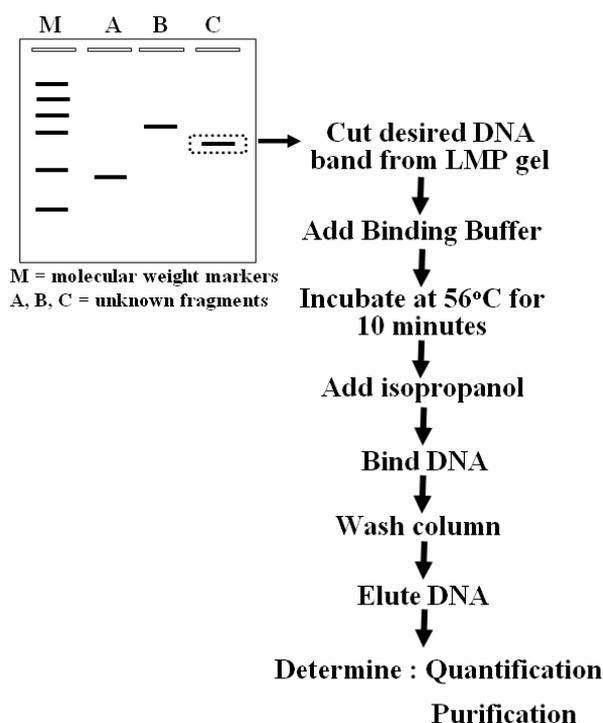


Figure 2.11: Flowchart of purification of DNA using Roche High Pure PCR product Purification procedure.

The desired DNA band was excised from the gel using a sterile scalpel blade and the DNA gel slice dissected into small fragments under UV light. A 100 mg portion of the agarose gel slice was then placed into a pre-weighed 1.5 mL microcentrifuge tube containing 300 μL Binding Buffer provided by the manufacturer (3 M guanidine-thiocyanate, 10 mM Tris-HCl, 5% ethanol (v/v), pH 6.6) and vortexed for 30 seconds. To release the DNA, the agarose gel slice was dissolved in the binding buffer, incubated at 65°C for 10 minutes and vortexed for 15 seconds every 2 minutes during incubation. Isopropanol (150 μL) was added to the dissolved gel and vortexed for 15 seconds prior to transfer to a High Pure

Filter Tube. The tube was centrifuged at 25,000 x g for 1 minute at room temperature and the flow-through was discarded. A 500 μ L aliquot of Washing Buffer (20 mM NaCl, 2 mM Tris-HCl, pH 7.5) was added to the filter tube and centrifuged at 25,000 x g for 1 minute with flow-through again being discarded. To ensure optimal purity of the sample, an additional wash with 200 μ L of Wash buffer provided by the manufacturer centrifuged at 25,000 x g for 1 minute was carried out. The filter tube was then placed into a new sterile 1.5 mL microcentrifuge tube and a 50 μ L aliquot of Elution Buffer provided by the manufacturer (10 mM Tris-HCl, pH 8.5) was added to the tube and centrifuged at 25,000 x g for 1 minute.

The purified DNA was precipitated by the addition of 0.1x volume of 3 M sodium acetate (pH 5.2) and 2x volume of ice cold ethanol to the DNA sample which was gently mixed, before storing at -20°C overnight. The DNA was recovered by centrifugation at 15,000 x g (Heraeus Biofuge 28RS centrifuge, Germany) for 5 minutes at 4°C. The pellet was washed with 0.5 mL of 70% (v/v) ethanol and then centrifuged twice at 15,000 x g for 5 minutes at 4°C to remove residual salt. Evaporation of residual ethanol from the DNA pellet was performed by leaving the open tube on the lab bench for 20 minutes. The DNA pellet was re-suspended in 20 μ L 0.1% (v/v) DEPC-treated water and stored at -80°C until required.

DNA amount and purity was determined by spectrophotometry (Thermo Electron Spectrometry Biomate 5TM, UK). Ten microlitres of the purified DNA was diluted with 490 μ L of 0.1% (v/v) DEPC-treated water. Absorbance at 260 nm was measured to determine DNA concentration, with each unit of A_{260} corresponding to 50 μ g/mL double stranded DNA. The A_{260}/A_{280} ratio provided an estimate of the DNA purity. Pure preparations of DNA should have a value of 1.8 and 2.1. All purified DNA of interest was sent to the Rowett Research Institute (Bucksburn, Aberdeen UK) to identify the sequences of DNA. The method used was automated dideoxy DNA sequencing. The results of

sequencing were analysed using the standard nucleotide-nucleotide BLAST search and comparing with the non-redundant database of GenBank.

2.11 Summary

This chapter encompasses the methodological approach used in this thesis. Section 2.2 covers cell biology techniques, while molecular biology techniques are described in Sections 2.3 to 2.10. In Chapters 3, 4, 5 and 6, the techniques are described in more detailed terms and in relation to the particular objectives of the thesis. Chapter 3 describes validation of RT-PCR analysis. In Chapter 4, the conditions for RT-PCR analysis were optimised for MCF7 and MDA-MB-468 cell lines and these conditions were used to profile P450s in a panel of breast cancer cell lines compared to a normal breast cell line. Chapter 5 is concerned with characterisation of CYP4Z1 in PCR products, while Chapter 6 identifies the receptors that regulate the expression of the CYP4Z1 gene and evaluates whether the transcription or translation process regulates CYP4Z1 expression.

Chapter 3

Validation of RT-PCR analysis in breast cancer cell lines

3.1 Introduction

RT-PCR is a highly sensitive method so the risk of contamination and false-positive results is of concern. To reduce the risk of RT-PCR contamination, the preparation of total RNA including pre-PCR work was performed in a Class II safety cabinet and away from the PCR area which was set up in a separate designated laboratory. The PCR workstation was always cleaned with 70% (v/v) ethanol before use. Pipette tips and pipettors used in the amplification procedures were pre-treated with UV crosslinker for 15 minutes, and water used for the PCR reactions was treated with diethylpyrocar-bonate (DEPC) prior to use in order to inactivate RNase (Appendix B, Sambrook and Russell 2001). A set of dedicated pipettes was used for each separate laboratory procedure (i.e. pre-PCR, PCR and post-PCR work) and storage of all PCR reagents isolated from samples.

To detect DNA carryover contamination and false-positive products, the use of negative controls (no DNA template) is important and these must be routinely used when conducting PCR. No product formation should be seen (Hamadeh and Afshari 2004). In addition, a known housekeeping gene such as β_2 -microglobulin which is expressed in most cells should be employed to verify the expression of DNA template (Section 2.5).

The validation process identifies aspects of a procedure that are critical and must be carefully controlled. To validate an RT-PCR assay, a demonstration of the accuracy, reliability and reproducibility of PCR-based technologies is required and realisation of the limitations of the method. As detailed in Sections 2.4 and 2.5, there are a number of considerations involved in amplifying a specific region of cDNA such as the quality of

RNA sample and the PCR reaction conditions which are detailed in the following sections.

Quality of total RNA obtained is essential for the successful amplification of the gene of interest and this can be checked on a gel by examination of the 28S and 18S rRNA bands (Section 2.3).

As described in Section 2.8, agarose gel electrophoresis of DNA is most commonly used to determine the size of products obtained from PCR reaction. The appearance of desired DNA fragments under UV light is vital for identification of the PCR products which can be further confirmed by restriction digestion or purified for various purposes such as DNA sequencing. In the current study, both restriction digestion and DNA sequencing were used to identify the sequence specificity of PCR products. However, if PCR products appear as multiple fragments or non-specific products, nested PCR can be used to improve specificity of the PCR reaction.

The main aim of this chapter was to optimise the conditions for RT-PCR analysis for the target cytochrome P450 and P450-regulatory nuclear receptor genes using MCF7 and MDA-MB-468 cells as representative oestrogen receptor (ER) protein positive and negative breast cancer cell lines respectively.

3.2 Materials and Methods

3.2.1 RNA isolation

As discussed in Section 2.3, total RNA was isolated with the Qiagen RNeasy Plus Mini kit. The absorbance at 260 nm (A_{260}) of a diluted RNA sample was used to assess RNA concentration and the A_{260}/A_{280} ratio was employed to determine RNA purity. Quality of the purified RNA was determined by 1% (w/v) agarose gel electrophoresis. Total RNA was precipitated by ethanol and sodium acetate to ensure stable and concentrated storage

of the starting material. The resulting pellet was washed and re-centrifuged prior to storage or use.

3.2.2 Reverse transcription (RT) and cDNA synthesis

As described in Section 2.4, first strand cDNA was synthesised from 5 µg of purified RNA samples using M-MLV reverse transcriptase. The components of the RT-reaction are summarised in Table 2.1. All reagents were thawed on ice prior to centrifugation. The reverse transcriptase assay was initiated by addition of M-MLV reverse transcriptase. The reaction conditions were as previously described in Table 2.2. Following first strand synthesis the RT product was placed on ice to inactivate the reaction before it was diluted to a concentration of 50 ng of template cDNA per mL and stored at -80°C until use.

3.2.3 Optimisation of PCR analysis

3.2.3.1 Initial PCR amplification

Optimisation of each component (i.e. concentration of primer, amount of template cDNA, dNTPs, *Taq* DNA polymerase, MgCl₂ and thermocycling parameters) including thermocycling conditions in a PCR reaction is important to obtain a better yield of amplification products (Section 2.5). Using cDNA template derived from the MCF7 and MDA-MB-468 cells as representative of the test cell lines, it was possible to detect the presence/absence and optimise the RT-PCR conditions for the determination of the PCR products of 19 cytochrome P450 and 8 P450-regulatory nuclear receptor genes (Table 2.6). The routine amplification was developed as shown in Tables 2.3 and 2.4. In each PCR reaction in this investigation, negative controls and β₂-microglobulin were always included. Where a visible PCR product is evident, this is subsequently referred to as mRNA by extrapolation from the method described.

3.2.3.2 Optimisation of template cDNA and primer concentrations

In this research, when the routine amplification resulted in no product formation the concentration of template cDNA was first varied (i.e. 150, 200 and 250 ng) and the concentration of primers was kept constant at 0.5 μM (Figure 3.1, stage 2A). If PCR product was still not seen, a high range of primer concentrations (i.e. 1.0 and 1.5 μM) was then used (Figure 3.1, stage 2B). A low range of primer concentrations (i.e. 0.1, 0.2, 0.3 and 0.4 μM) was employed when the presence of a desired band as well as non-specific products were detected (Figure 3.1, stage 2C). Optimisation of primer concentration, both low and high ranges, was performed with a fixed quantity of 100 ng template DNA. In addition, the recommended concentrations of *Taq* DNA polymerase, MgCl_2 and dNTPs were used for the RT-PCR assay (Table 2.3).

3.2.3.3 Oligonucleotide primer design

All designed primers (Table 2.6) were optimised using the procedures described in Sections 3.2.3.1 and 3.2.3.2. However, there were twelve primer sets used that failed to detect mRNA expression in the MCF7 and MDA-MB-468 cells. This may be because mRNA expression was below the limit of detection, and details will be provided in the result section. Five out of the twelve undetectable genes (i.e. CYP1A1, CYP3A4, CYP3A5, CAR and PXR) were considered for PCR experiments using previously published primer sequences (Table 2.7) as previous studies have provided evidence of these genes in breast cancer research. These primers were checked for specificity by BLAST analysis.

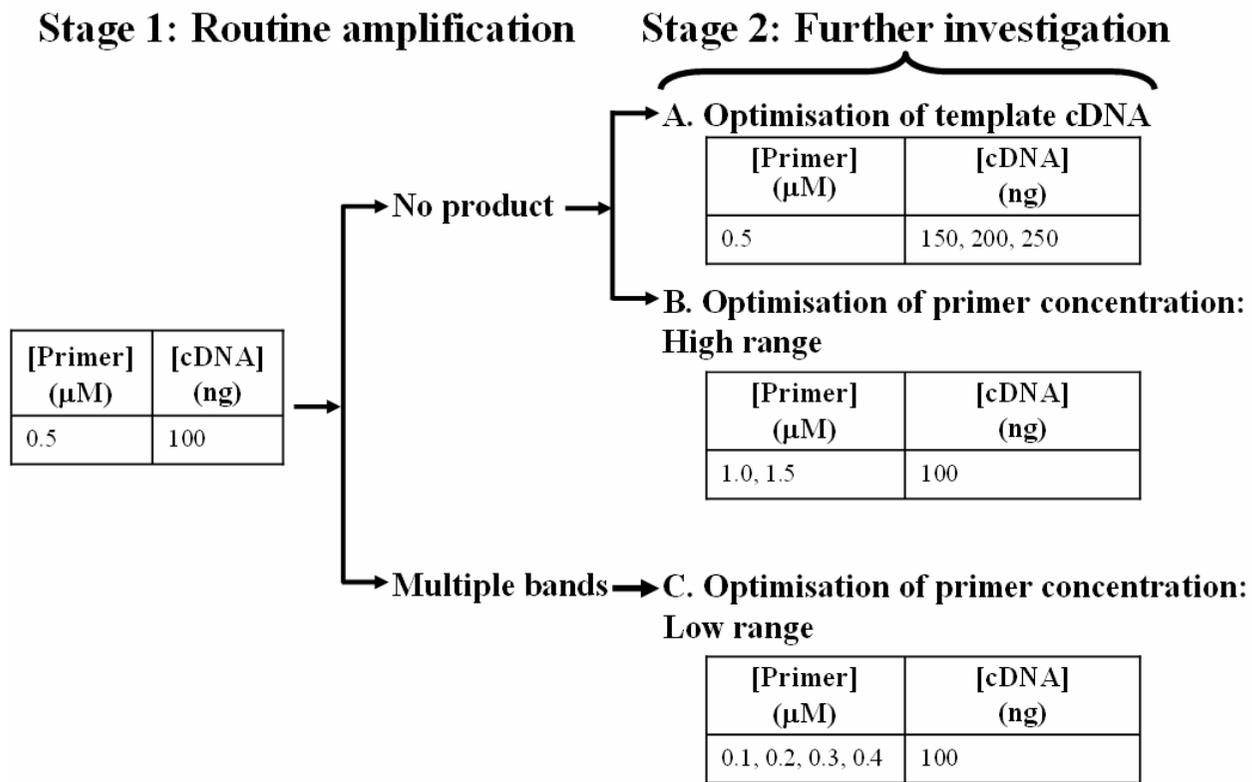


Figure 3.1: General guidelines used for optimisation of template cDNA and primer concentration.

3.2.4 Nested PCR

As mentioned in Section 2.7, nested PCR is a simple technique which can be employed to improve specificity of multiple fragment products using the first PCR product as a template for the second PCR reaction. In this research, design of the internal primer sequences for the CAR used in the nested PCR was performed by importing the known sequence of the primary PCR product (417 bp in size) into the Primer3 programme. BLAST analysis confirmed the specificity of the primer used. Information on these primers is shown in Table 2.6 and the nested PCR reaction was performed under the conditions described in Table 2.8.

3.2.5 Restriction enzyme digestion of DNA

As previously described (Section 2.9), digestion of PCR products was carried out with specific restriction enzymes for each individual PCR product (Table 2.10). All restriction digests were performed in the same manner as the example presented in Table 2.11.

3.2.6 DNA purification and sequencing

The desired DNA fragment was purified with the High Pure PCR product purification kit as detailed in Section 2.10. DNA concentration and purity were assessed by spectrophotometry and the acceptable A_{260}/A_{280} ratio was between 1.8 and 2.1. The purified DNA was sequenced by the method of automated dideoxy DNA sequencing.

3.3 Results

3.3.1 RNA isolation

Initial purity of total RNA in this study was poor with a calculated RNA A_{260}/A_{280} ratio of between 1.3 and 1.6. On investigation, the number of lysed cells loaded onto the RNeasy spin column was shown to play a major role in determining the purity of RNA collected. The number of cells loaded onto individual columns was reduced from $> 2 \times 10^6$ cells per

purification to an optimal loading of between 5×10^5 and 2×10^6 cells. Additional measures were also employed to increase purity and recovery as explained in Section 2.3. Following these measures the recovery of total RNA was between 25 and 60 μg per purification. In addition, the ratio of RNA obtained (A_{260}/A_{280}) was between 1.8 and 2.1. The quality of the purified RNA was confirmed by inspecting the rRNA bands on a 1% (w/v) agarose gel containing ethidium bromide. No smear or band below the lower 18S rRNA band, a common indication of degraded low-quality RNA, was found in any sample (Figure 3.2).

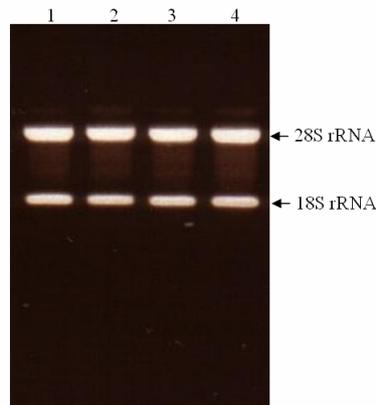


Figure 3.2: *Quality of RNA isolated from four independent RNA preparations. Lanes 1-2: RNA isolated from the MCF, Lanes 3-4: RNA isolated from the MDA-MB-468.*

3.3.2 β_2 -microglobulin RT-PCR

β_2 -microglobulin was identified as a band of 319 bp corresponding to the β_2 -microglobulin cDNA product size. The intensity of β_2 -microglobulin amplification product increased between 15 and 30 cycles. The optimal cycle number for β_2 -microglobulin amplification was identified as 20 cycles because under these conditions the product appeared as a sharp band (Figure 3.3). This cycle number was used for all β_2 -microglobulin RT-PCR experiments.

Little variation was observed between β_2 -microglobulin mRNA in the MCF7 and MDA-MB-468 cells. Figure 3.4 highlights the level of reproducibility found in β_2 -microglobulin expression in replicate studies of this research. Restriction digestion using *EcoRI*

confirmed the specificity of β_2 -microglobulin with a total digest of the PCR product resulting in fragments of the expected sizes 215 bp and 104 bp (Figure 3.5).

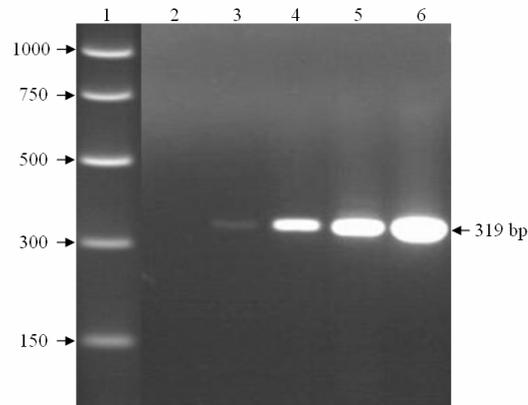


Figure 3.3: Determination of the optimal cycle number for amplification of β_2 -microglobulin. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: 15 cycles, Lane 4: 20 cycles, Lane 5: 25 cycles, Lane 6: 30 cycles.

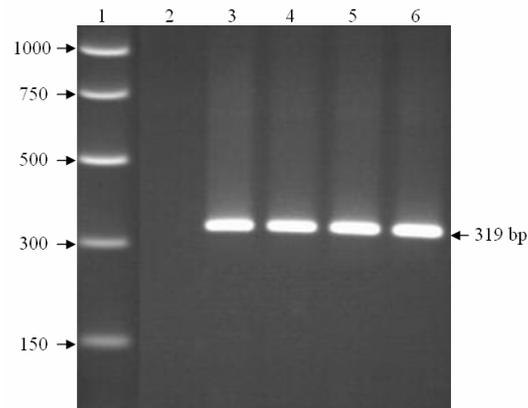


Figure 3.4: β_2 -microglobulin RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lanes 3&4: cDNA samples from the MCF7, Lanes 5&6: cDNA samples from the MDA-MB-468.

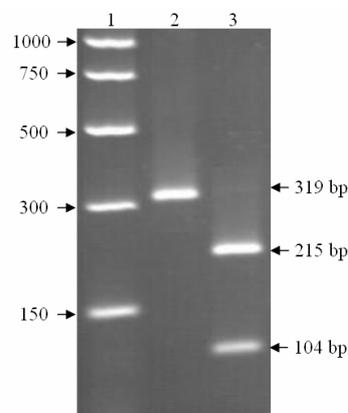


Figure 3.5: Restriction digestion of β_2 -microglobulin RT-PCR using *EcoRI*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

3.3.3 Cytochrome P450 enzyme RT-PCR

3.3.3.1 CYP1 family RT-PCR

Initially, the primer sequence designed for CYP1A1 by Primer3 (Table 2.6) failed to detect expression of CYP1A1 mRNA in either the MCF7 or MDA-MB-468 cells. These results did not correlate with the findings of earlier investigations which showed CYP1A1 mRNA to be present in MCF7 cells (Spink et al. 1998, Brockdorff et al. 2000, McFadyen et al. 2003). To further confirm the expression of CYP1A1 mRNA in the MCF7 cells, a published CYP1A1 primer set (Table 2.7) was used in this experiment. A band of 392 bp was observed for CYP1A1 mRNA expression. The amount of template cDNA derived from the MCF7 cells was varied (i.e. 100, 150, 200 and 250 ng) with 0.5 μ M of CYP1A1 primer (Figure 3.6). One hundred and fifty ng of template cDNA was selected for CYP1A1 amplification in subsequent experiments as this was the minimum concentration of cDNA that generated a sharp and clear band. Expression of CYP1A1 mRNA in the MCF7 cells was confirmed following observation of 188 bp and 164 bp fragments in restriction enzyme *Hae*III digested samples (Figure 3.7). However, no CYP1A1 mRNA was detected in the MDA-MB-468 cells using these methods.

The CYP1A2 primer sequence designed by Primer3 (Table 2.6) showed the expected amplified product (245 bp) with bands representing non-specific amplification in either the MCF7 or MDA-MB-468 cells (Figure 3.8) and the images were inverted from positive to negative images for better visualisation. As detailed in section 3.2.3.2, optimisation of primer concentration in a low range (Figure 3.1, stage 2C) was performed but no PCR product of the correct size was seen. This experiment failed to confirm the specificity of CYP1A2. However, further experiments screening for the presence/absence of CYP1A2 mRNA in the remaining cell lines were performed and results are presented in Chapter 4.

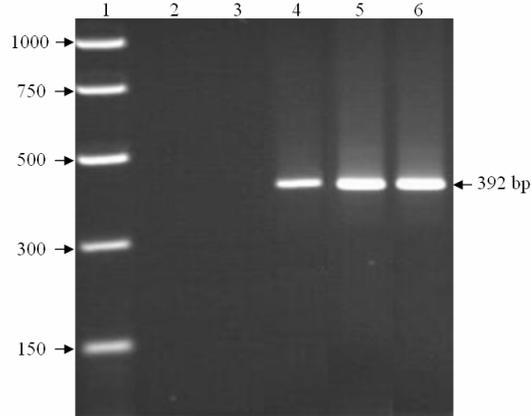


Figure 3.6: *CYP1A1* RT-PCR optimisation of template cDNA in MCF7. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: 100 ng, Lane 4: 150 ng, Lane 5: 200 ng, Lane 6: 250 ng.

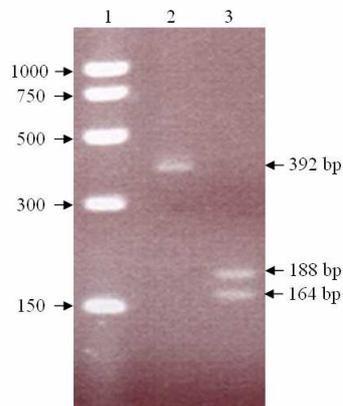


Figure 3.7: Restriction digestion of *CYP1A1* RT-PCR with *HaeIII*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

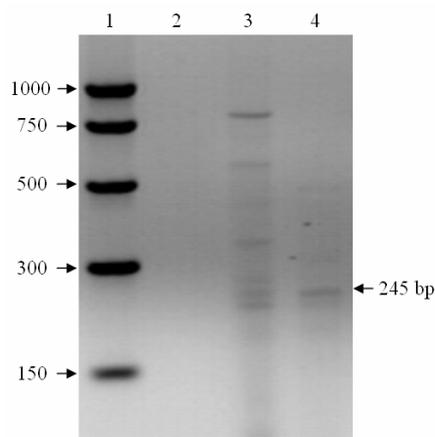


Figure 3.8: *CYP1A2* RT-PCR (negative image). Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF7, Lane 4: MDA-MB-468.

The routine amplification by RT-PCR using CYP1B1 primers did not show a 397 bp cDNA product corresponding to CYP1B1 mRNA. To further optimise the PCR

conditions, the primer concentration was set at 0.5 μ M and the amount of template cDNA varied (i.e. 100, 150, 200 and 250 ng). The optimal concentration of template cDNA was identified as 150 ng because this was the minimum concentration that appeared as a sharp band (Figure 3.9). CYP1B1 mRNA was found in both the MCF7 and MDA-MB-468 cells. The presence of CYP1B1 mRNA was confirmed by restriction digest with *RsaI*, which generated a digested fragment of the correct size (174 bp and 223 bp) as shown in Figure 3.10.

3.3.3.2 CYP2 family RT-PCR

Initial studies failed to demonstrate the presence of CYP2A6 or CYP2D6 mRNA in either the MCF7 or MDA-MB-468 cell lines. Further experiments to verify the presence or absence of the CYP2A6 and CYP2D6 PCR product were performed. Figure 3.1 (stage 2A and 2B) indicates the primer and cDNA template concentrations used. CYP2A6 and CYP2D6 mRNA were below the levels of detection in both the MCF7 and MDA-MB-468 cells under the conditions used.

A faint amplification product of 194 bp was correctly determined for CYP2B6 RT-PCR in the MDA-MB-468 cells, though no corresponding product was observed in the MCF7 cells when analysis was performed using 250 ng of template cDNA (Figure 3.11). CYP2B6 mRNA expression in the MDA-MB-468 cells was confirmed by restriction digestion with *HaeIII* which generated a fragment of 164 bp (Figure 3.12).

Differential expression of the CYP2C subfamily mRNA was observed in the MCF7 and MDA-MB-468 cell lines. CYP2C9 and CYP2C19 mRNA were below detectable limits in both cell lines under the conditions used although template cDNA and primer (a high range) concentrations have been optimised (Figure 3.1, stages 2A and 2B).

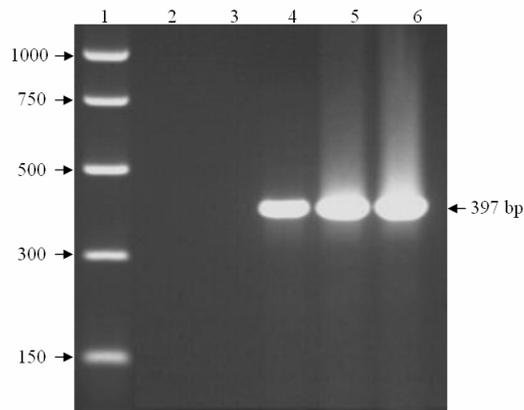


Figure 3.9: *CYP1B1* RT-PCR optimisation of template cDNA in MCF7. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: 100 ng, Lane 4: 150 ng, Lane 5: 200 ng, Lane 6: 250 ng.

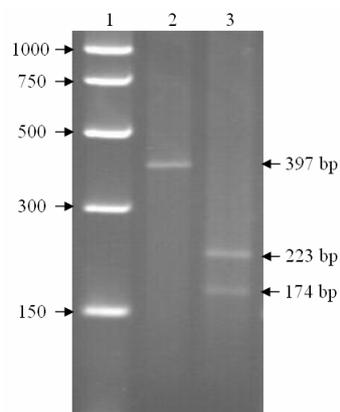


Figure 3.10: Restriction digestion of *CYP1B1* RT-PCR using *RsaI*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

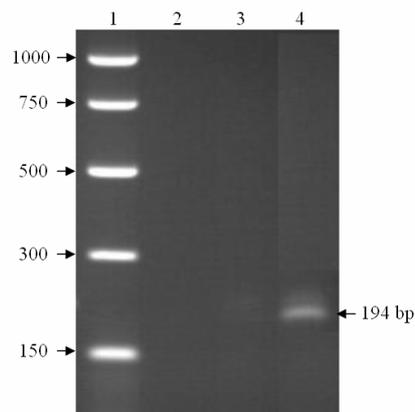


Figure 3.11: *CYP2B6* RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF7, Lane 4: MDA-MB-468.

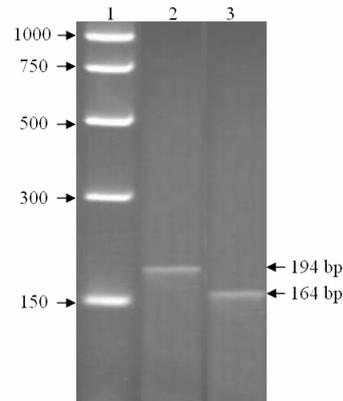


Figure 3.12: Restriction digestion of CYP2B6 RT-PCR using *HaeIII*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

Expression of CYP2C8 mRNA was only detectable in the MCF7 cells as a faint product at the correct size of 230 bp and a negative image of gel is presented in Figure 3.13 in order to improve the visualisation. To confirm CYP2C8 mRNA expression, the PCR product was digested with *MspI* and the predicted digested fragments of 170 bp and 60 bp were observed (Figure 3.14).

CYP2C18 mRNA was detected in both cell lines at the correct cDNA product size of 199 bp. Similar levels of expression were observed in both the MCF7 and MDA-MB-468 cells (Figure 3.15). Restriction digestion of CYP2C18 mRNA by the enzyme *RsaI* generated fragments (125 bp and 74 bp) of the correct size, though a partial digest product was seen (Figure 3.16) and this will be discussed in Section 3.4.

A band of 216 bp was observed at the correct size for CYP2S1 mRNA in both the MCF7 and MDA-MB-468 cells. It was noticed that a slightly higher level of CYP2S1 mRNA expression occurred in the MDA-MB-468 cells compared to the MCF7 cells (Figure 3.17). To confirm the CYP2S1 mRNA expression, the PCR product was digested with restriction enzyme *AluI* and the correct fragments were found at 103 bp and 63 bp (Figure 3.18). The purified DNA from the 216 bp fragment was confirmed by DNA sequencing (Section

2.10). The results of sequencing were analysed using the standard nucleotide-nucleotide BLAST search and compared with the non-redundant GenBank database. The findings were 100% matched (183/183) to *Homo sapiens* CYP2S1 mRNA (GenBank accession number: NM_030622) (Appendix D, Figure D.1).

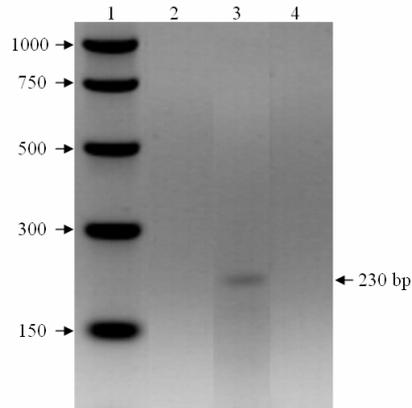


Figure 3.13: *CYP2C8* RT-PCR (negative image). Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF7, Lane 4: MDA-MB-468.

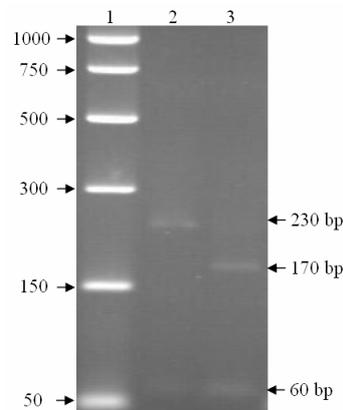


Figure 3.14: Restriction digestion of *CYP2C8* RT-PCR using *MspI*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

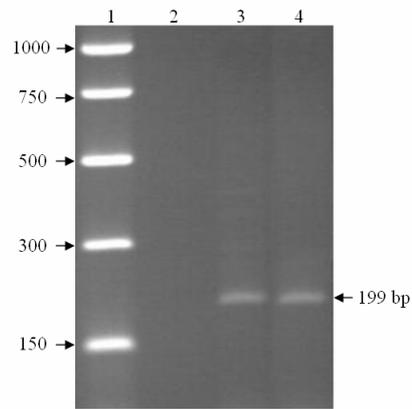


Figure 3.15: *CYP2C18* RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF7, Lane 4: MDA-MB-468.

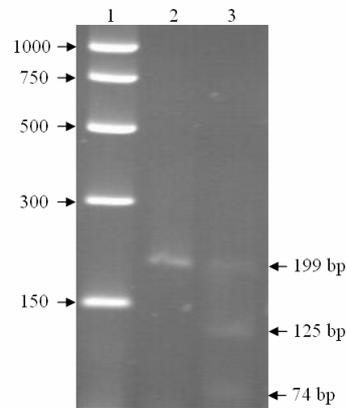


Figure 3.16: Restriction digestion of *CYP2C18* RT-PCR using *RsaI*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

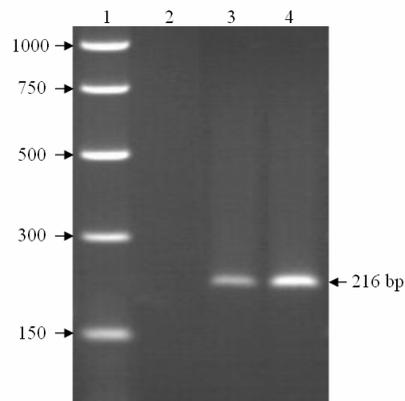


Figure 3.17: *CYP2S1* RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF7, Lane 4: MDA-MB-468.

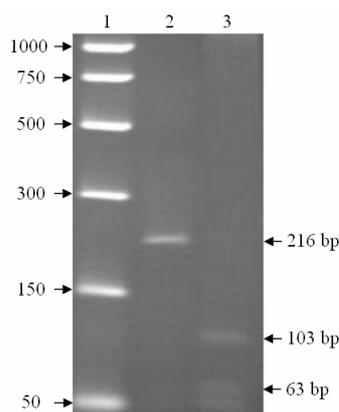


Figure 3.18: Restriction digestion of CYP2S1 RT-PCR using AluI. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

3.3.3.3 CYP3A subfamily RT-PCR

Initially, the primer sequence designed for CYP3A4/5/7/43 by Primer3 (Table 2.6) failed to detect the presence of any of the four candidate genes in either the MCF7 or MDA-MB-468 cells. From the literature, it appears that many research groups have demonstrated the expression of CYP3A in human breast tumours, but these results are inconsistent (Albin et al. 1993, Huang et al. 1996, Iscan et al. 2001, Modugno et al. 2003, Miki et al. 2006).

These inconsistencies could have arisen in several ways, for example the use of samples from non-matched individuals and method choices. However, CYP3A expression has not been previously reported in breast cancer cell lines. Furthermore, CYP3A plays a major role in the metabolism of oestrogen and several anticancer drugs such as tamoxifen, ifosphamide, taxol and vinblastine (Scripture and Figg 2006). In the present study, CYP3A4 and CYP3A5 mRNA expression were therefore examined using published primer sets (Table 2.7) which were analysed by the BLAST search. Optimisation of template cDNA and primer concentration was performed as described in Section 3.2.3 (Figure 3.1), but the expression of CYP3A4 mRNA and CYP3A5 mRNA were below detectable limits under the conditions used. In addition neither CYP3A7 nor CYP3A43 mRNA was detected in this study.

3.3.3.4 CYP4 family RT-PCR

Expression of CYP4Z1 mRNA was detectable in the MDA-MB-468 cells as a product at the correct size of 159 bp but no corresponding product was observed in the MCF7 cells. Analysis was performed using 100 ng of template cDNA with 0.5 μ M of CYP4Z1 primer set. The result obtained from the MCF7 cells was compared with data from previous studies (Rieger et al. 2004, Savas et al. 2005) which detected CYP4Z1 mRNA in the MCF7 cells. Template cDNA and primer concentrations were therefore optimised (Figure 3.1, stages 2A and 2B) but these studies failed to demonstrate the expression of CYP4Z1 mRNA in the MCF7 cells. Regarding the presence of CYP4Z1 mRNA in a cDNA product of MDA-MB-468 cells, it should be noted that an additional band of approximately 250 bp (~250 bp) was observed (Figure 3.19).

Template cDNA samples from the MDA-MB-468 cells were amplified at different annealing temperatures (i.e. 55, 56, 57, 58, 59, 60, 61, 62 and 63°C) under the same PCR conditions. The purpose of this was to investigate the effect of annealing temperature on the specificity of primers and the yield of PCR product of CYP4Z1 mRNA, and to understand the presence of the additional band. After varied annealing temperature, products of 159 bp and ~250 bp were still visualised though DNA bands appeared sharp and clear at annealing temperatures of 58 and 59°C (Figure 3.20). Therefore, an annealing temperature of 58°C was selected for CYP4Z1 amplification in all subsequent experiments.

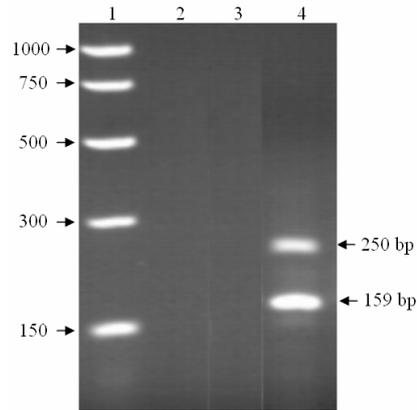


Figure 3.19: *CYP4Z1* RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF7, Lane 4: MDA-MB-468.

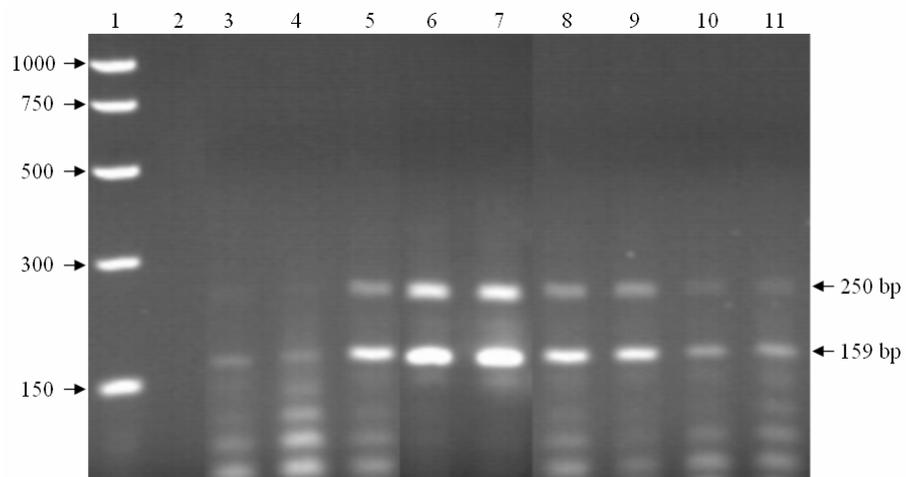


Figure 3.20: Illustration of the effect of annealing temperature on *CYP4Z1* RT-PCR products. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: 55°C, Lane 4: 56°C, Lane 5: 57°C, Lane 6: 58°C, Lane 7: 59°C, Lane 8: 60°C, Lane 9: 61°C, Lane 10: 62°C, Lane 11: 63°C.

The 159 bp DNA fragments and the additional band of ~250 bp obtained from the MDA-MB-468 cells were purified by the method explained in Section 2.10, and the resulting DNA products were digested with *RsaI*. Restriction digestion of the purified DNA product of the 159 bp fragment generated a fragment of the 112 bp (Figure 3.21A). By comparison, the purified DNA product of the ~250 bp band generated a fragment of 112 bp and an additional band at 120 bp (Figure 3.21B).

The purified DNA products from the 159 bp and ~250 bp fragments were further investigated by DNA sequencing. The standard nucleotide-nucleotide BLAST was used to analyse the results of DNA sequencing and compared with the non-redundant GenBank database. This indicated that purified DNA of the 159 bp and ~250 bp fragments were 94% (118/125) (Appendix D, Figure D.2) and 98% (77/78) (Appendix D, Figure D.3) identical to *Homo sapiens* CYP4Z1 mRNA (GenBank accession number: NM_178134) respectively. The expression of CYP4Z1 mRNA in breast cancer cell lines will be further investigated in Chapter 4 and characterised in Chapter 5.

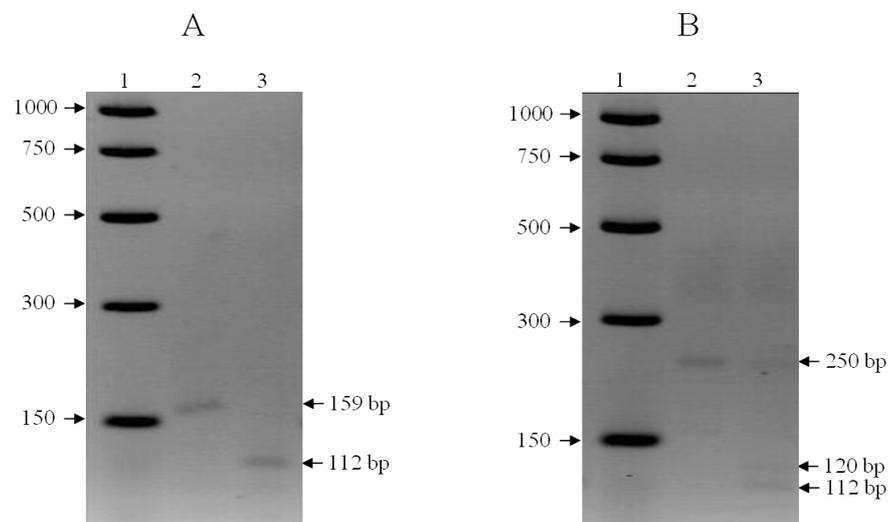


Figure 3.21: Restriction digestion of purified DNA products from CYP4Z1 RT-PCR using *RsaI* (negative images). (A) Purified DNA product of 159 bp fragment. (B) Purified DNA product of 250 bp fragment. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

3.3.3.5 CYP24 family RT-PCR

A band of the correct size at 249 bp was not observed for CYP24A1 mRNA expression after routine amplification. When the amount of template cDNA was varied (i.e. 100, 150, 200 and 250) with 0.5 μ M of CYP24A1 primer, expression of CYP24A1 mRNA was found in the MCF7 cells but no amplification product was detected in the MDA-MB-468 cells. The optimal amount of template cDNA was identified as 150 ng for CYP24A1 amplification because this was the lowest amount that produced a sharp DNA band (Figure 3.22). This amount would be utilised in subsequent experiments. Expression of CYP24A1

mRNA in the MCF7 cells was confirmed by restriction digestion with *Bam*HI and observation of the correct digested fragments of 68 bp and 181 bp (Figure 3.23).

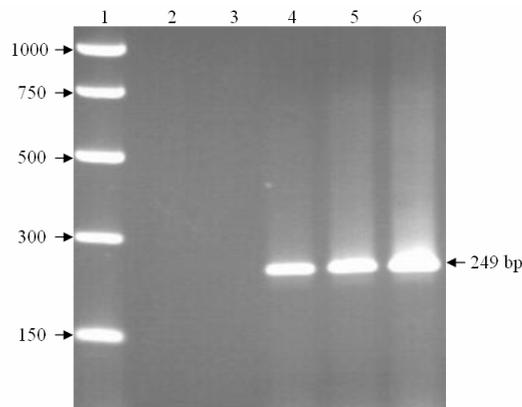


Figure 3.22: *CYP24A1* RT-PCR optimisation of template cDNA in MCF7. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: 100 ng, Lane 4: 150 ng, Lane 5: 200 ng, Lane 6: 250 ng.

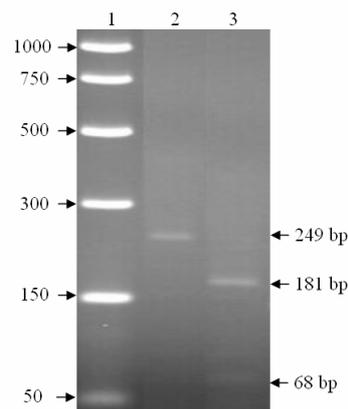


Figure 3.23: Restriction digestion of *CYP24A1* RT-PCR using *Bam*HI. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

3.3.3.6 CYP26 family RT-PCR

The expression of *CYP26A1* and *CYP26B1* mRNA was detected in both the MCF7 and MDA-MB-468 cells as 191 bp and 172 bp cDNA products respectively (Figures 3.24A and 3.24B). It was noted that a slightly higher level of *CYP26A1* mRNA expression was found in the MCF7 compared to the MDA-MB-468 cells, while a higher level of *CYP26B1* mRNA expression was observed in the MDA-MB-468 cells compared to the MCF7 cell line. To confirm *CYP26A1* mRNA expression, the PCR product was digested with *Alu*I and the correct digested fragment of 172 bp was observed (Figure 3.25A). The

presence of CYP26B1 mRNA was also confirmed by restriction digestion with *AluI* and observation of digested fragments of the correct size (104 bp and 68 bp); as shown in Figure 3.25B). Partial digest products, observed in Figures 3.25A and 3.25B, will be discussed in Section 3.4.

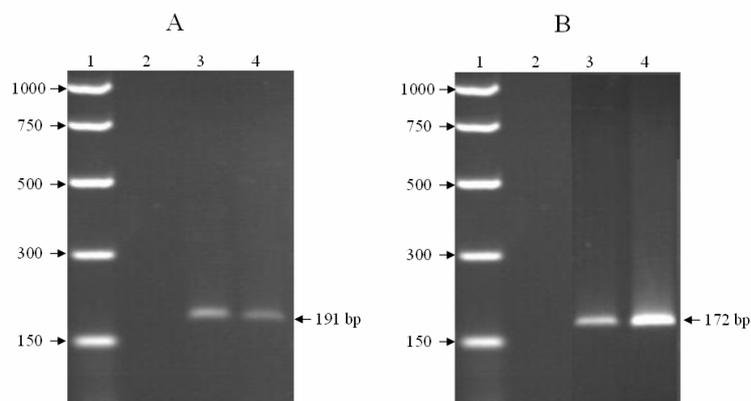


Figure 3.24: *CYP26A1* and *CYP26B1* RT-PCR. (A) *CYP26A1*. (B) *CYP26B1*. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF7, Lane 4: MDA-MB-468.

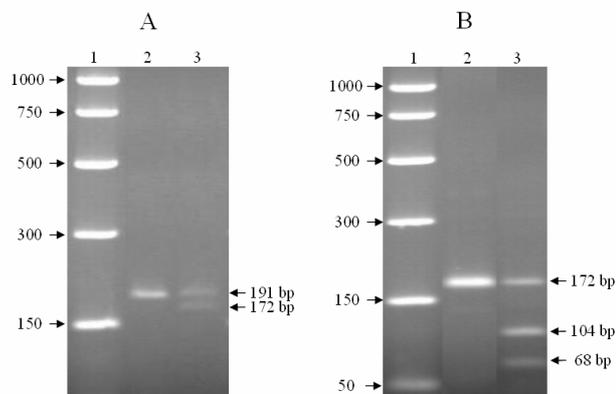


Figure 3.25: Restriction digestion of *CYP26A1* and *CYP26B1* RT-PCR using *AluI*. (A) *CYP26A1*. (B) *CYP26B1*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

3.3.4 Cytochrome P450-regulatory nuclear receptors RT-PCR

The presence of hypoxia-inducible factor-1 alpha (HIF-1 α), aryl hydrocarbon receptor (AhR), aryl hydrocarbon receptor nuclear translocator (ARNT) and aryl hydrocarbon receptor repressor (AhRR) mRNA was detected in both the MCF7 and MDA-MB-468 cells as 167 bp, 262 bp, 387 bp and 171 bp cDNA products respectively. A product of 250 bp was correctly determined for vitamin D receptor (VDR) in the MCF7 cells although no

corresponding product was observed in the MDA-MB-468 cells (Figure 3.26). All experiments were performed using 100 ng of template cDNA with 0.5 μ M of each primer set.

Stronger intensity of PCR product of HIF-1 α mRNA expression was clearly observed in the MCF7 compared to the MDA-MB-468 cells, whereas slightly higher levels of AhR and ARNT mRNA expression were found in the MDA-MB-468 compared to the MCF7 cells. Similar levels of AhRR mRNA expression were detected in both the MCF7 and MDA-MB-468 cells.

Restriction digestion of HIF-1 α mRNA by the enzyme *SspI* generated a fragment of 142 bp (Figure 3.27). To confirm the AhR mRNA expression, the PCR product was restricted with the enzyme *MspI* and the correct fragments of 163 bp and 99 bp were detected (Figure 3.28). The presence of ARNT and VDR mRNA was confirmed by restriction digestion with *PstI* which generated digested fragments of 269 bp and 118 bp for ARNT mRNA (Figure 3.29) and a fragment of 198 bp for VDR mRNA (Figure 3.31). To confirm AhRR mRNA expression, the PCR product was digested with *HaeIII* and the correct digested fragments of 92 bp and 79 bp were observed (Figure 3.30).

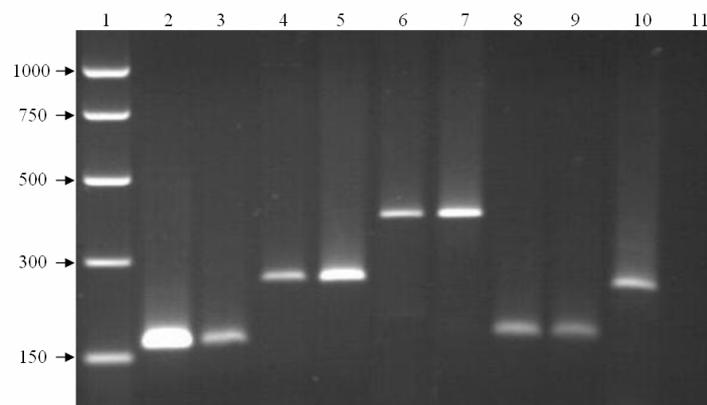


Figure 3.26: RT-PCR products of HIF-1 α , AhR, ARNT, AhRR and VDR. Lane 1: 1000 bp PCR markers, Lanes 2-3: HIF-1 α , Lanes 4-5: AhR, Lanes 6-7: ARNT, Lanes 8-9: AhRR, Lanes 10-11: VDR. Lanes number 2, 4, 6, 8, 10 represent MCF7 and lanes number 3, 5, 7, 9, 11 indicate MDA-MB-468.

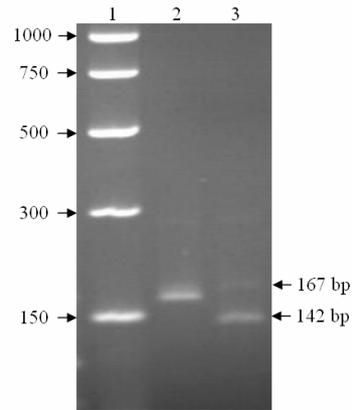


Figure 3.27: Restriction digestion of HIF-1 α RT-PCR using *SspI*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

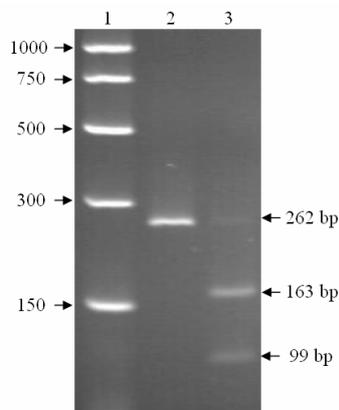


Figure 3.28: Restriction digestion of AhR RT-PCR using *MspI*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

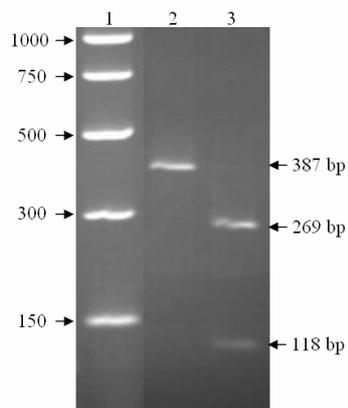


Figure 3.29: Restriction digestion of ARNT RT-PCR using *PstI*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

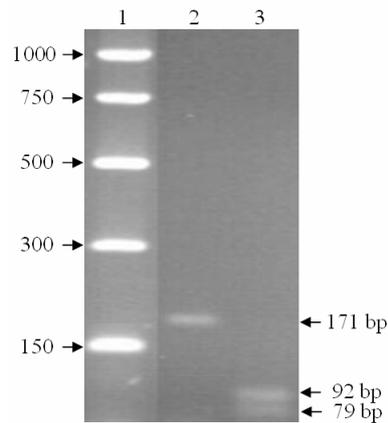


Figure 3.30: Restriction digestion of AhRR RT-PCR using *HaeIII*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

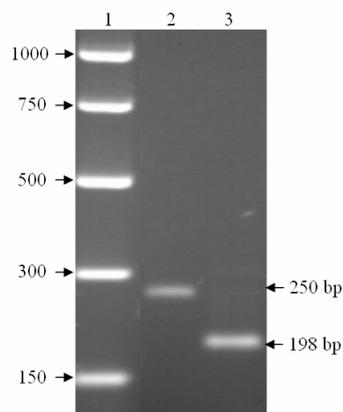


Figure 3.31: Restriction digestion of VDR RT-PCR using *PstI*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

3.3.4.1 Oestrogen receptor (ER) RT-PCR

A band of the correct size at 208 bp was observed for ER mRNA expression in both the MCF7 and MDA-MB-468 cells. However, an additional fainter band at 450 bp was also detected in this experiment (Figure 3.32). The DNA products of 208 bp and 450 bp were purified by the method described in Section 2.10, and the purified DNA products were digested with the restriction enzyme *RsaI*. The correct fragments of 111 bp and 97 bp were observed only in the purified 208 bp DNA fragment (Figure 3.33), not in the purified 450 bp DNA product.

The purified DNA products of 208 bp and 450 bp fragments were further analysed by DNA sequencing. The product of 208 bp fragment was sequenced and found to be a 92% match (69/75) to *Homo sapiens* ER mRNA (GenBank accession number: NM_000125) (Appendix D.4), whereas the product of the 450 bp fragment was sequenced and found to consist of unrelated ER sequences. This will be discussed in Section 3.4.

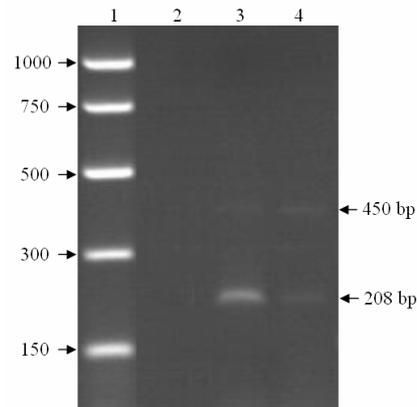


Figure 3.32: ER RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF7, Lane 4: MDA-MB-468.

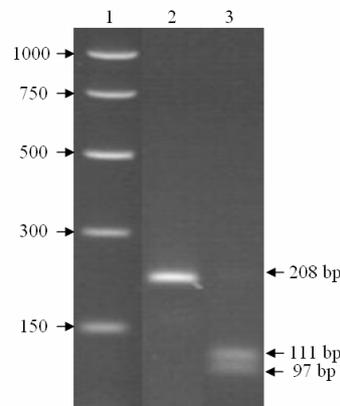


Figure 3.33: Restriction digestion of ER RT-PCR using *RsaI*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

3.3.4.2 Constitutive androstane receptor (CAR) RT-PCR

An initial experiment using the primer sequence designed for CAR by Primer3 (Table 2.6) failed to detect CAR mRNA in either the MCF7 or MDA-MB-468 cells. As mentioned in Section 1.7.3, CAR plays an important role in the transcriptional regulation of certain P450s (e.g. CYP2B6) and a study by Chang and co-workers (Chang et al. 2003)

demonstrated a positive correlation between CAR and CYP2B6 mRNA level in human liver tissues. In this study, the expression of CYP2B6 mRNA was detected and confirmed in the MDA-MB-468 cells (Figures 3.11 and 3.12); therefore, CAR mRNA was considered for investigation in this experiment using the published CAR primer set (Table 2.7). Results showed the expected PCR product (417 bp) for both MCF7 and MDA-MB-468 cells, though non-specific products were detected in the MCF7 cells (Figure 3.34).

Following the non-specificity of the published primer above, the nested CAR primer set (Table 2.6) was employed to enhance specificity of the first round PCR product under the conditions shown in Table 2.8. To obtain optimal conditions for this experiment, the volume of the first PCR product was varied (i.e. 1, 2, 3, 4 and 5 μ L) with 0.5 μ M of the nested CAR primer (Figure 3.35A), and the nested CAR primer concentration was varied (i.e. 0.1, 0.2, 0.3, 0.4 and 0.5 μ M) with 5 μ L of the first PCR product (Figure 3.35B). The optimal conditions were found to be 0.2 μ M of the nested primers with 5 μ L of the first PCR product and this was used for the remainder of this study. Expression of CAR mRNA was found in both the MCF7 and MDA-MB-468 cells which was confirmed by restriction digestion with *Pst*I and observation of the correct fragments of 170 bp and 62 bp (Figure 3.36).

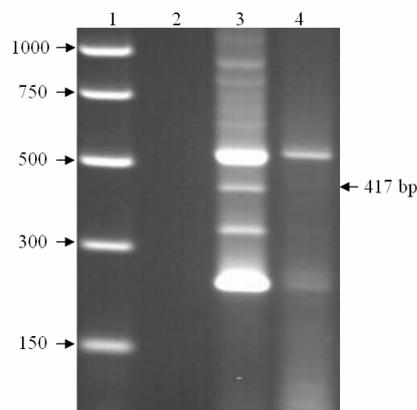


Figure 3.34: Nested CAR RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF7, Lane 4: MDA-MB-468.

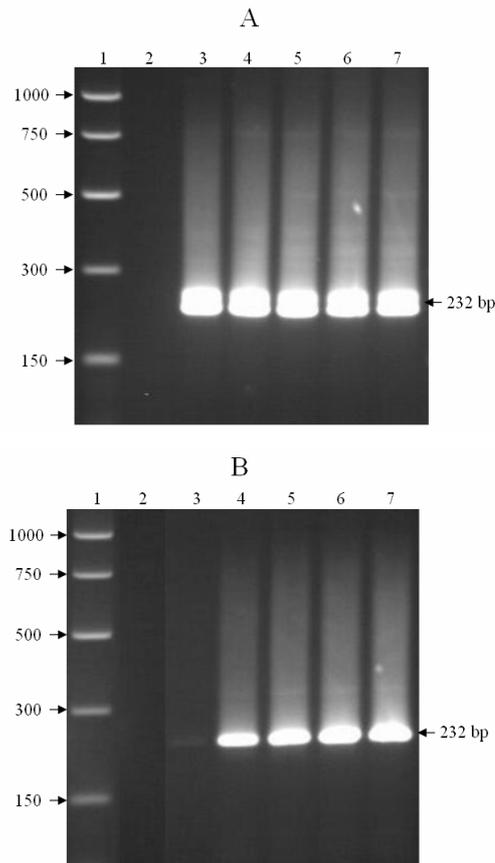


Figure 3.35: Nested CAR RT-PCR optimisation of the volume of the first PCR product and concentration of nested CAR primers in the MCF7. Lane 1: 1000 bp PCR markers, Lane 2: negative control (A) Varied the volume of the first PCR products. Lane 3: 1 μL , Lane 4: 2 μL , Lane 5: 3 μL , Lane 6: 4 μL , Lane 7: 5 μL . (B) Varied the concentration of nested CAR primers. Lane 3: 0.1 μM , Lane 4: 0.2 μM , Lane 5: 0.3 μM , Lane 6: 0.4 μM , Lane 7: 0.5 μM .

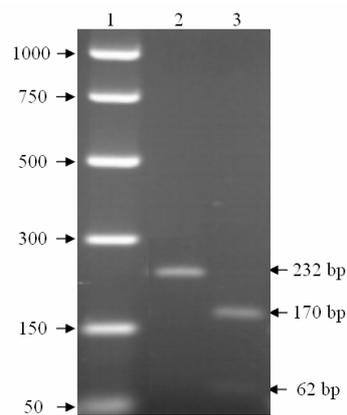


Figure 3.36: Restriction digestion of nested CAR RT-PCR using *Pst*I. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

3.3.4.3 Pregnane X receptor (PXR) RT-PCR

Initially, the primer sequence designed for PXR by Primer3 (Table 2.6) failed to demonstrate PXR mRNA expression in either the MCF7 or MDA-MB-468 cells. However, the expression of PXR mRNA in MCF7 cells has been demonstrated in previous studies (Miki et al. 2006, Sakai et al. 2006). In this research, the published PXR primer set (Table 2.7) was therefore used to determine the expression of PXR mRNA. No PCR product was detected after routine amplification so the optimisation of PCR analysis was performed. Amount of template cDNA was varied (i.e. 100, 150, 200 and 250 ng) with 0.5 μ M of PXR primer (Figure 3.37), and 250 ng of template cDNA was chosen for PXR amplification because the product appeared as an intense DNA band when compared to either 150 ng or 200 ng. The expression of PXR mRNA was found in only the MCF7 cells, not in the MDA-MB-468 cells. The presence of PXR mRNA in the MCF7 cells was confirmed by digestion with restriction enzyme *MspI*, which generated fragments of the correct size (142 bp and 90 bp; Figure 3.38).

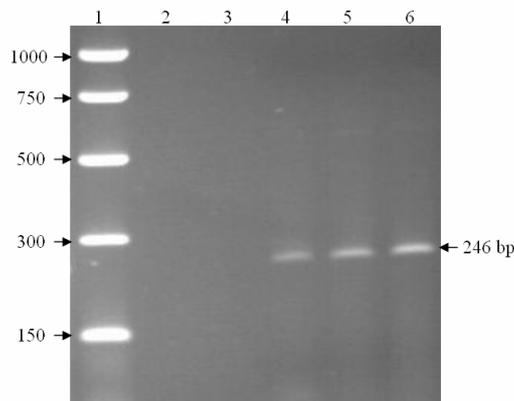


Figure 3.37: PXR RT-PCR optimisation of template cDNA in MCF7. Lane 1: 1000 bp PCR markers as indicated, Lane 2: negative control, Lane 3: 100 ng, Lane 4: 150 ng, Lane 5: 200 ng, Lane 6: 250 ng.

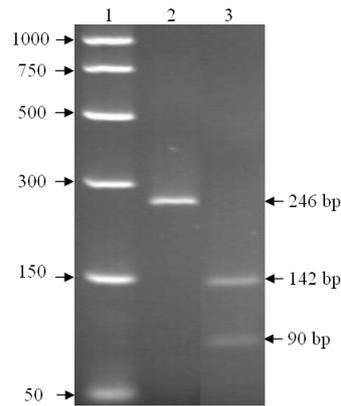


Figure 3.38: Restriction digestion of PXR RT-PCR using *MspI*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

3.4 Discussion

This chapter describes an RT-PCR protocol to optimise the detection of cytochrome P450 and P450-regulatory nuclear receptor mRNA. The advantages of using end-point PCR in this study are that it is a simple and rapid method for screening and detecting amplification products of high numbers of candidate genes in cell lines. This study assessed a number of variables affecting RT-PCR analysis including the methods of RNA isolation, cDNA verification, PCR and restriction digest reaction conditions, and DNA purification and sequencing.

Isolating total RNA of high purity was critical for validation of the PCR system as cellular contaminants such as genomic DNA (gDNA) can interfere with reaction efficiency and complicate interpretation of results. Initially the RNA purity in this study was low with an A_{260}/A_{280} ratio between 1.3 and 1.6 (instead of 1.8-2.1) and this seriously affected the specificity of the RT-PCR reactions. To overcome this problem several technical measures were implemented, in particular it was felt that there had been overloading of cells onto the RNeasy spin column and that the method of disrupting the cells was critical. The use of a cell number between 5×10^5 and 2×10^6 per purification, and an increased efficiency in the homogenisation process resulted in higher RNA purity, non-degraded RNA (Figure 3.2)

and less gDNA contamination in downstream applications. Upon successful validation, this methodology was utilised for the remainder of the study.

In this investigation, β_2 -microglobulin was used as an internal control to detect false negative results by comparing mRNA expression with the gene of interest in different cell lines. Low variation in β_2 -microglobulin mRNA between the MCF7 and MDA-MB-468 cells was observed (Figure 3.4), suggesting that this gene was an appropriate reference gene in this study. As mentioned in Section 3.1, the use of negative controls (no DNA template) is critical for conducting PCR as it can monitor false-positive results in the PCR assay. No product was detected in the negative controls in any of the experiments in this chapter, indicating that PCR reaction mixtures were free from DNA carryover contamination.

Specificity in primer sequences is one of the most important keys to success in the PCR assay. In this research, the candidate sequences designed by Primer3 programme were assessed via a direct BLAST search. Ten out of the nineteen cytochrome P450 enzymes (i.e. CYP1A1, CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP3A4, CYP3A5, CYP3A7 and CYP3A43), and two out of the eight P450-regulatory nuclear receptors (i.e. CAR and PXR) primers designed by Primer3 programme failed to demonstrate their mRNA expression in either the MCF7 or MDA-MB-468 cells, although sufficient validation following general guidelines used in this research was performed.

However, five out of the twelve undetectable genes (i.e. CYP1A1, CYP3A4, CYP3A5, CAR and PXR) were considered for further investigation using the published primers (Table 2.7) because evidence regarding the presence or the relevance of them has been demonstrated in breast cancer cells and they are involved in the metabolism of oestrogen and anticancer drugs. A single specific PCR product of CYP1A1 and PXR mRNA was

successfully detected after varying the concentration of cDNA template (Figures 3.6 and 3.37). The published CAR primer set was performed under routine amplification, but it generated non-specific PCR product (Figure 3.34). Therefore, nested PCR was used to improve specificity of the primer, a specific product was detected and its sequence identity confirmed by restriction digestion. The observation of non-specific bands in the first round PCR may be due to the fact published PCR conditions were not used in this experiment. In an attempt to generate high quality data, experiments were performed using the procedure described in Section 3.2.3 as standard. Unfortunately, the published primers of CYP3A4 and CYP3A5 fail to demonstrate mRNA expression in the cell lines used despite their validation.

It is well-known that CYP2D6 catalyses the N-demethylation and 4-hydroxylation of tamoxifen which have potent anti-oestrogen effects. Tamoxifen is widely used in the treatment of breast cancer (Schroth et al. 2007). Previous studies demonstrated that CYP2D6 mRNA was expressed in normal breast as well as in breast tumour samples (Huang et al. 1996, Hellmold et al. 1998, Iscan et al. 2001, Bièche et al. 2007). In addition, Hellmond and colleagues (Hellmold et al. 1998) demonstrated CYP2D6 protein expression in normal breast. These findings were in disagreement with those of Modugno and co-workers (Modugno et al. 2003), who investigated CYP2D6 mRNA expression in breast tumour and normal adjacent breast tissues using quantitative RT-PCR. The present study is the first to examine the expression of CYP2D6 mRNA in breast cancer MCF7 and MDA-MB-468 cell lines, but no CYP2D6 mRNA was observed. As mentioned above, the importance of CYP2D6 in the metabolism of tamoxifen has been emphasised in the treatment of breast cancer. For this reason, the current project sought to ascertain whether CYP2D6 is present or absent in the test cells using the published primers. Preliminary work with previously published CYP2D6 mRNA in normal breast and seven breast cancer cell lines will be discussed in the next chapter.

According to the general guidelines for the optimisation of PCR analysis in this study (Figure 3.1), the findings indicated that optimal cDNA template amount for the genes of interest was between 100 and 250 ng in a PCR reaction. The optimal primer concentration for each individual gene was generally 0.5 μ M because non-specific PCR products were seen at higher primer concentration of 1.0 μ M to 1.5 μ M. Regarding the thermocycling conditions, twenty-five to thirty-five cycles is generally standard for a PCR reaction. In the current investigation, thirty amplification cycles were selected and performed for all candidate genes, but not for β_2 -microglobulin. Twenty cycles were sufficient for β_2 -microglobulin amplification as the product generated a clear and sharp band (Figure 3.3).

This research aimed to detect the expression of a number of target mRNAs in a series of cell lines. Due to time constraints, a single repetition was performed in individual genes. However, in experiments where genes were undetectable after routine amplification, the procedure was repeated with varying amounts of cDNA template and/or primer and any detectable products were then confirmed by restriction digestion and/or DNA sequencing.

PCR products of detectable genes were confirmed by restriction digestion and observation of digested fragments of the correct size on gels. It should be noted that partial restriction digestion was observed for products of CYP2C18 (Figures 3.16), CYP26A1 (Figures 3.25A), CYP26B1 (Figures 3.25B), and AhR (Figures 3.28). These results may be due to excessive amounts of starting material being used, insufficient incubation time for reactions and/or low activity of restriction enzymes. This problem could be investigated by repeating experiments with lower DNA template concentrations, an increased incubation time and a new batch/fresh supply of restriction enzymes for further study.

In section 3.3.3.4, it was shown that MDA-MB-468 cells express CYP4Z1 mRNA (159 bp) with the presence of an additional band of ~250 bp. To ensure specificity of primers,

annealing temperature of PCR analysis was varied between 55°C and 63°C. However, two bands were still visualised on the gel, and this experiment showed that an annealing temperature of 58°C was optimal (Figure 3.20). Purified DNA of the two fragments was digested using restriction enzyme *RsaI* and a restricted fragment size of 112 bp was found in both purified fragments. This finding suggested that these fragments may contain similar DNA and they were therefore sequenced. The results of DNA sequencing were accessed directly by a BLAST search, and this predicted that both fragments were human CYP4Z1 mRNA (Appendix D). However, more than fifty percent of CYP4Z1 (~250bp) sequences were unreadable, possibly due to poor quality of the purified DNA samples or an unknown factor involved in the expression of CYP4Z1 mRNA. Prior to further characterisation of the CYP4Z1 mRNA (Chapter 5), the previously published primers which have been demonstrated a single PCR product were used to confirm the presence of CYP4Z1 mRNA in the cDNA samples employed in this study. Results will be shown and discussed in Chapter 4.

Several novel extrahepatic P450 enzymes may be of particular interest in breast cancer cells in terms of potential targets for cancer therapy as they are preferentially expressed in extrahepatic tissues, and overexpression of some cytochrome P450s have been identified in certain types of tumours. A preliminary investigation demonstrated the expression of CYP2F1, CYP2J2, CYP2R1, CYP2U1 and CYP4X1 in a panel of breast cancer cell lines and compared to a normal breast cell line performed by the Honours students within the School of Pharmacy who employed the same cDNA samples and established methods used in the current investigation. This preliminary work also investigated the expression of retinoic acid receptor (RAR) and retinoid X receptor (RXR), because their physiological roles have been shown to be involved in the development of various types of cancer (Germain et al. 2003, Altucci et al. 2007) and the regulation of some cytochrome P450 enzymes (Meyer 2007). These results will be shown in Chapter 4.

Table 3.1 summarises the presence of cytochrome P450 and cytochrome P450-regulatory nuclear receptor mRNA in the MCF7 and MDA-MB-468 cells after optimisation and confirmation of their expression. In summary, this chapter provides information on validation of PCR conditions derived from the model cell lines, MCF7 and MDA-MB-468. These conditions will be used for the remaining breast cancer cell lines and compared to a normal breast cell line in the next chapter.

Table 3.1: *The presence of cytochrome P450 and P450-regulatory nuclear receptor mRNA in the MCF7 and MDA-MB-468 cells*

P450	MCF7	MDA-MB-468	NR	MCF7	MDA-MB-468
CYP1A1	+	-	HIF-1α	+	+
CYP 1A2	NS	NS	AhR	+	+
CYP1B1	+	+	ARNT	+	+
CYP2A6	-	-	AhRR	+	+
CYP2B6	-	+	CAR	+	+
CYP2C8	+	-	PXR	+	-
CYP2C18	+	+	VDR	+	-
CYP2C9	-	-	ER	+	+
CYP2C19	-	-			
CYP2D6	-	-			
CYP2S1	+	+			
CYP3A4	-	-			
CYP3A5	-	-			
CYP3A7	-	-			
CYP3A43	-	-			
CYP4Z1	-	+			
CYP24A1	+	-			
CYP26A1	+	+			
CYP26B1	+	+			

Abbreviations: P450; cytochrome P450 enzyme, NR; P450-regulatory nuclear receptor, NS; non-specific product by RT-PCR analysis, +; PCR product detected at predicted size and sequence specificity, -; not detected by visual inspection.

Chapter 4

Cytochrome P450 expression in breast cancer cell lines

4.1 Introduction

Clinically, the majority of breast tumours express oestrogen receptor (ER) and it is well established that local production of oestrogen is particularly high in breast tumours (Suzuki et al. 2005). Oestrogen is involved in the development and progression of breast cancer (Yager and Davidson 2006). Women with ER-positive breast tumours are more likely to benefit from endocrine therapy than patients with ER-negative tumours. Approximately 60-65% of patients with ER-positive tumours respond to anti-oestrogen therapy (i.e. tamoxifen) whereas in women with ER-negative tumours, only low levels of response are achieved. Moreover, ER-negative women have a poorer survival and prognosis than patients who have node positive and ER-positive tumours (Putti et al. 2005).

However, the overall cytochrome P450 profile in breast cancer and the role of these enzymes in breast carcinogenesis is yet to be defined. A better understanding of the differential expression of these enzymes in different oestrogen receptor status, invasive phenotypes and racial groups could potentially be of enormous value in the development of novel cancer therapeutics (targeted at these enzymes) and the design of more effective drug regimens. Therefore, this chapter was designed to identify the cytochrome P450 and P450-regulatory nuclear receptor mRNA profile in a panel of breast cancer cell lines derived from different ethnic backgrounds (i.e. Caucasians and Afro-Caribbeans) and including different breast tumour characteristics (i.e. oestrogen receptor status and invasive phenotype). A normal breast cell line was also examined for comparative purposes.

4.2 Materials and Methods

4.2.1 Cell lines and cell culture

The eight cell lines used were obtained from different sources (Section 2.2.2) and a summary of their characteristics is presented in Table 1.1. All cells were maintained under the growth conditions described in Section 2.2.3. All experiments were conducted using cells cultured for no more than 15 passages after resuscitation of the first cell stock. All culture techniques were performed as explained previously (Sections 2.2.4 to 2.2.7).

4.2.2 RNA isolation and cDNA synthesis

Total RNA was isolated from all cell lines using the RNeasy Plus Mini kit from Qiagen as detailed in Section 2.3. Approximately 5×10^5 to 2×10^6 cells were extracted per purification as discussed in Section 3.3.1. The quantity and quality of total RNA in each preparation were determined by A_{260}/A_{280} ratios and inspection of the 28S and 18S rRNA bands in 1% (w/v) agarose gels. As described in Section 2.4, reverse transcription (RT) reactions were conducted in a final volume of 20 μ L (Table 2.1) using the reaction conditions summarised in Table 2.2.

4.2.3 Qualitative PCR amplification

PCR was performed under optimal conditions (Section 3.3) in order to profile P450 and P450-regulatory nuclear receptor mRNA expression in the test cell lines. The PCR products obtained from the housekeeping gene β_2 -microglobulin and the target genes from each cell line were visualised on 1.5% agarose gels containing ethidium bromide.

Following visualisation of bands in the PCR products, the band intensity of photographed gel (positive) images was subjected to densitometric scanning using UVI-BandMap software (UVItec, Cambridge, UK). Densitometry is the quantitative measurement of optical density which can be expressed as the number of dark spots in a given volume of intensity. The sensitivity (maximum spot size) was set at 5 mm for all samples to measure

the volume density of the amplified products. To demonstrate a relative expression of candidate genes' mRNA in each cell line tested, mRNA expression of each gene was normalised against β_2 -microglobulin mRNA expression. In the result section, some gel images were inverted from positive to negative images for better visualisation.

In this chapter, the PCR assay was used qualitatively i.e. it was used to determine whether mRNA was present or absent in all individuals from the test cell lines. To facilitate comparisons between the cell lines, the volume density of the β_2 -microglobulin RT-PCR and the relative intensities of target genes normalised against β_2 -microglobulin were determined.

4.2.4 Oligonucleotide primer design

Prior to further elucidation of the CYP4Z1 sequence the present investigation confirmed the expression of CYP4Z1 mRNA in the eight cDNA samples using the previously published primers (Table 2.7, Thomas et al. 2006) which had been designed to cross between exon 7 and exon 8 boundaries (Figure 5.2). Thomas and colleagues found that this primer set generated a single band of the CYP4Z1 amplification product.

During the course of the current study, the expression of CYP2D6, CYP2F1, CYP2J2, CYP2R1, CYP2U1, CYP4X1, RAR and RXR was investigated using cDNA samples as mentioned in Section 3.4. These were subjected to routine amplification (Tables 2.3 and 2.4) with the previously published primers (Table 2.7) and the products obtained were digested with restriction enzymes (Table 2.10) under the conditions described in Table 2.11 to confirm the mRNA expression of candidate genes.

4.3 Results

4.3.1 RNA isolation

Total RNA isolated from the cell lines had A_{260}/A_{280} ratios between 1.8 and 2.1. One microgram of total RNA isolated from the eight cell lines was loaded onto a 1% (w/v) agarose gel to confirm RNA quality (Figure 4.1).

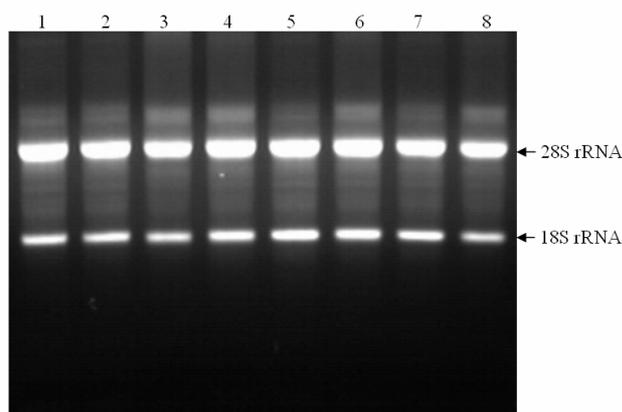


Figure 4.1: *Quality of total RNA isolated. Lane 1: MCF10A, Lane 2: MCF7, Lane 3: T47D, Lane 4: ZR-75-1, Lane 5: ZR-75-30, Lane 6: MDA-MB-231, Lane 7: MDA-MB-157, Lane 8: MDA-MB-468.*

4.3.2 β_2 -microglobulin RT-PCR

A band of 319 bp corresponding to the cDNA product size for β_2 -microglobulin was detected for all cell lines. There was no visible difference in the housekeeping gene β_2 -microglobulin mRNA expression between the breast cancer cells and the normal breast cell line used. No band was detected in the negative controls (no template cDNA, Figure 4.2). This data was used for each subsequent analysis in this chapter.

4.3.3 CYP1 family RT-PCR

CYP1A1 mRNA was detected in five out of the seven breast cancer cell lines as the correct band at 392 bp and it was expressed at a very low level in the normal breast MCF10A cell line. No CYP1A1 mRNA expression was detected in the ZR-75-30 and MDA-MB-468 cells (Figure 4.3).

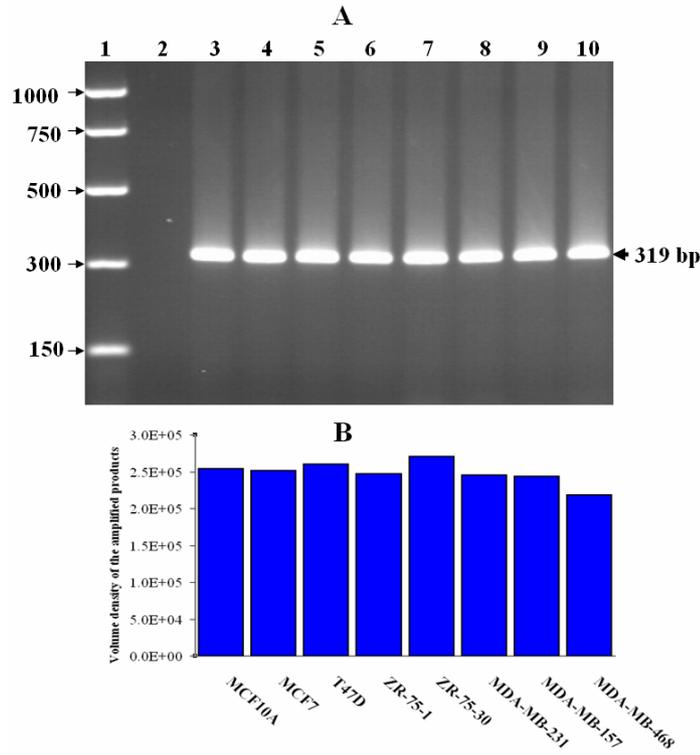


Figure 4.2: β_2 -microglobulin RT-PCR. (A) Visualisation of the β_2 -microglobulin amplified products. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468. (B) Volume density of the β_2 -microglobulin amplified products (pixels).

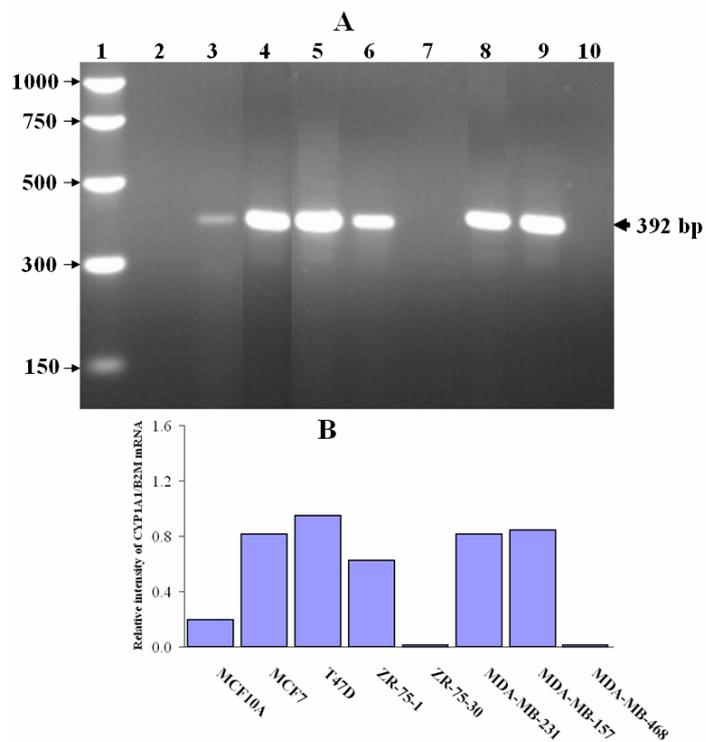


Figure 4.3: CYP1A1 RT-PCR. (A) Visualisation of the CYP1A1 amplified products. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468. (B) Relative intensities of CYP1A1 normalised against β_2 -microglobulin.

As mentioned in Section 3.3.3.1, the CYP1A2 primer sequence designed by Primer3 failed to detect the presence of CYP1A2 mRNA in either the MCF7 or MDA-MB-468 cells.

This primer set was also used for the remaining cell lines, but unfortunately the results still showed non-specific amplification in all cases.

A band of 397 bp was observed for CYP1B1 mRNA in both the normal breast (MCF10A) and breast cancer cell lines. MCF7, T47D, ZR-75-1, MDA-MB-231 and MDA-MB-157 demonstrated higher levels of expression than the ZR-75-30 cells. Only very weak expression was detected in the MDA-MB-468 cells (Figures 4.4).

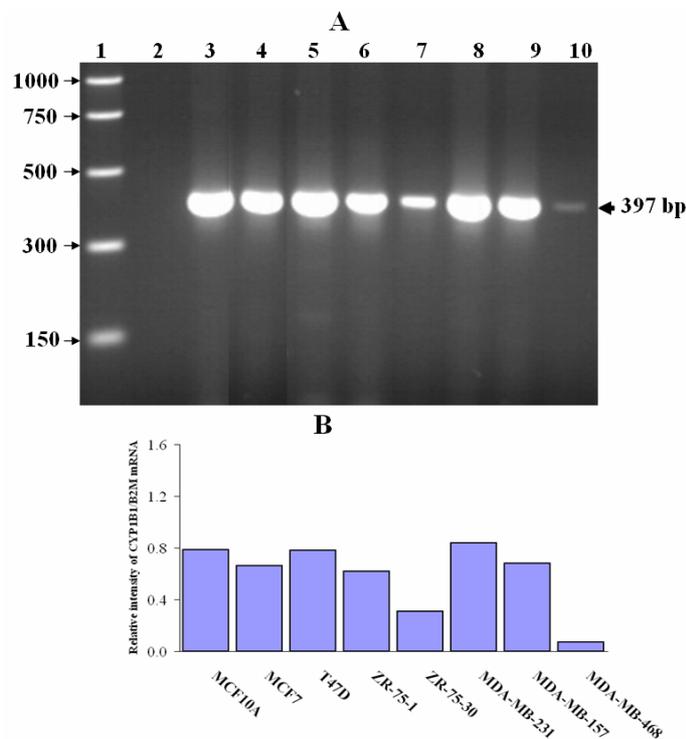


Figure 4.4: CYP1B1 RT-PCR. (A) Visualisation of CYP1B1 amplified products. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468. (B) Relative intensities of CYP1B1 normalised against β_2 -microglobulin.

4.3.4 CYP2 family RT-PCR

As mentioned in Section 3.3.3.2, both the MCF7 and MDA-MB-468 cell lines failed to show the presence of CYP2A6 mRNA during PCR validation. However, a band of 186 bp corresponding to the cDNA product size for CYP2A6 mRNA was detected in three of the cell lines. CYP2A6 mRNA was expressed in the ZR-75-1 and T47D cells at higher levels

than the MDA-MB-157 cells (Figures 4.5). The expression of CYP2A6 mRNA was confirmed by restriction digestion with *Hae*III, which generated digested fragments of the correct size (72 bp and 66 bp, Figure 4.6).

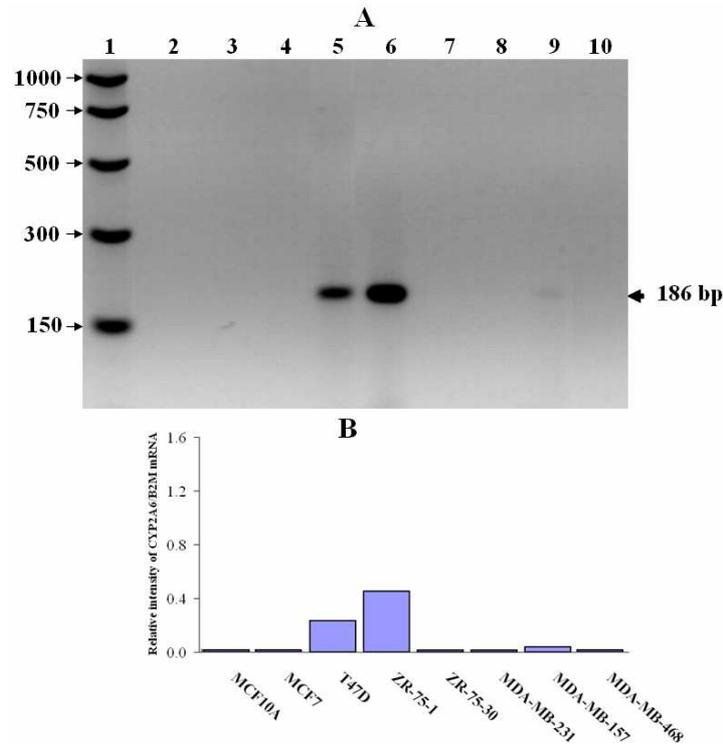


Figure 4.5: CYP2A6 RT-PCR. (A) Visualisation of CYP2A6 amplified products (negative image). Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468. (B) Relative intensities of CYP2A6 normalised against β_2 -microglobulin.

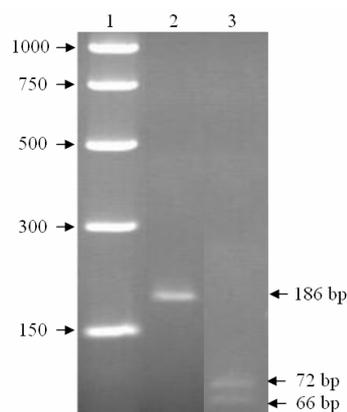


Figure 4.6: Restriction digestion of CYP2A6 RT-PCR with *Hae*III. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

A band of 194 bp corresponding to the cDNA product size for CYP2B6 mRNA was found in five out of the seven breast cancer cell lines but no product was visible in the normal breast cells. A high level of expression was observed in the T47D, ZR-75-1 and MDA-MB-157 cells, while very low levels were detected in the ZR-75-30 and MDA-MB-468 cells. CYP2B6 mRNA expression was not detected in the MCF7 and MDA-MB-231 cells (Figure 4.7).

CYP2C8 mRNA was detected in three out of the seven breast cancer cell lines as a cDNA product at 230 bp but no mRNA was observed in the normal breast cell line. A higher level of CYP2C8 expression was found in the MDA-MB-157 cells compared to the MCF7 and ZR-75-1 cells (Figure 4.8).

A band of 199 bp was observed for the presence of CYP2C18 mRNA in four out of seven breast cancer and the normal breast cell lines. It was expressed in the T47D cells at a high level, whereas very weak levels were detected in the normal breast cell line, and the breast cancer cell lines MCF7, MDA-MB-157 and MDA-MB-468 (Figure 4.9).

During PCR validation (Section 3.3.3.2) both the MCF7 and MDA-MB-468 cells failed to demonstrate expression of CYP2C9/19 and CYP2D6 mRNA. Similarly, no amplification product was detected in the cell lines investigated. However CYP2D6, an enzyme which plays a crucial role in the metabolism of antioestrogen tamoxifen and is associated with breast cancer was considered for PCR experiments using previously published sequences (Table 2.7, Thomas et al. 2006). Preliminary results are shown in Figures 4.27 and 4.28.

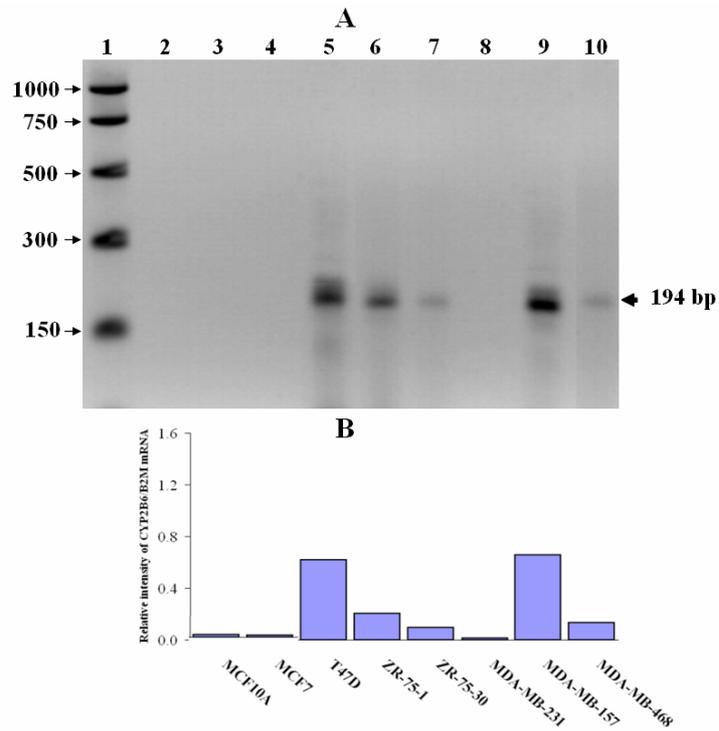


Figure 4.7: CYP2B6 RT-PCR. (A) Visualisation of CYP2B6 amplified products (negative image). Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468. (B) Relative intensities of CYP2B6 normalised against β_2 -microglobulin.

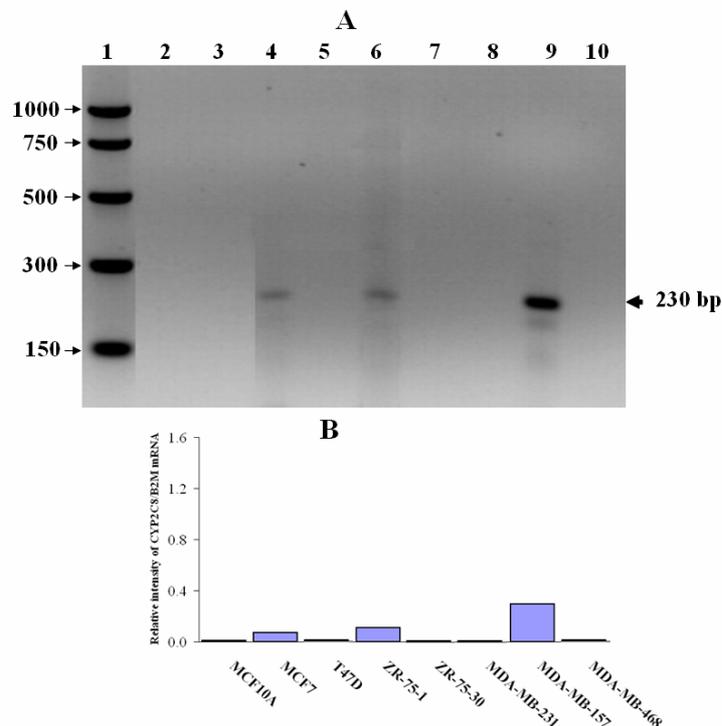


Figure 4.8: CYP2C8 RT-PCR. (A) Visualisation of CYP2C8 amplified products (negative image). Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468. (B) Relative intensities of CYP2C8 normalised against β_2 -microglobulin.

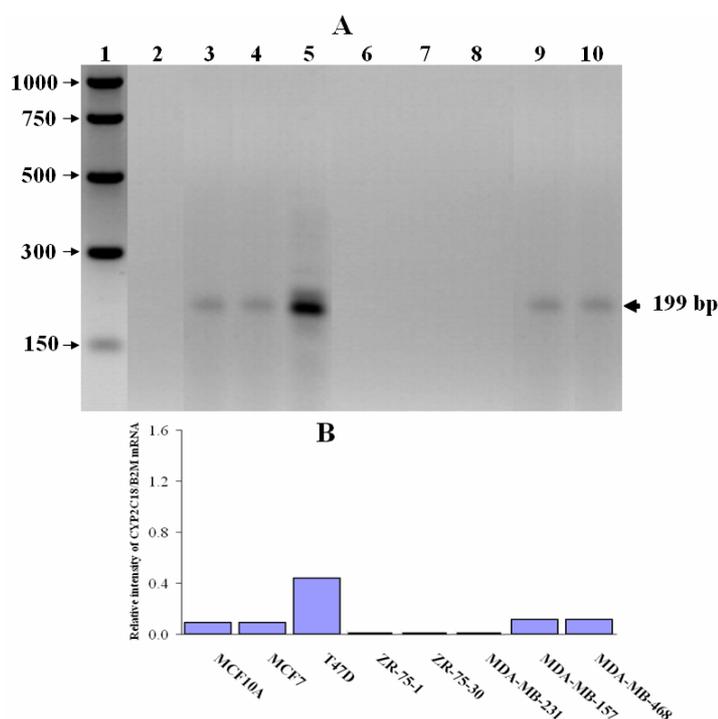


Figure 4.9: CYP2C18 RT-PCR. (A) Visualisation of CYP2C18 amplified products (negative image). Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468. (B) Relative intensities of CYP2C18 normalised against β_2 -microglobulin.

A band of 216 bp corresponding to the cDNA product size for CYP2S1 mRNA was highly expressed in all of the cell lines except MCF10A and ZR-75-30. The highest level of expression was found in the T47D cells. Moderate expression was detected in the MCF7, ZR-75-1, MDA-MB-231, MDA-MB-157 and MDA-MB-468 cells (Figure 4.10).

4.3.5 CYP3 family RT-PCR

In the CYP3A subfamily, the primer sequences designed for CYP3A4/5/7/43 by Primer3 (Table 2.6) failed to detect CYP3A mRNA expression in the MCF7 and MDA-MB-468 cells during PCR validation (Section 3.3.3.3). When profiling these genes against a series of cell lines, only a very weak level of CYP3A7 mRNA expression was detected in the normal breast cell line MCF10A as a cDNA product of 223 bp (Figure 4.11). The expression of CYP3A7 mRNA was confirmed by restriction digestion with *EcoRI* which generated fragments of the correct size (158 bp and 65 bp) (Figure 4.12). However, no

CYP3A4/5/43 mRNA expression was detected in either the normal breast or breast cancer cell lines under the conditions used in this study.

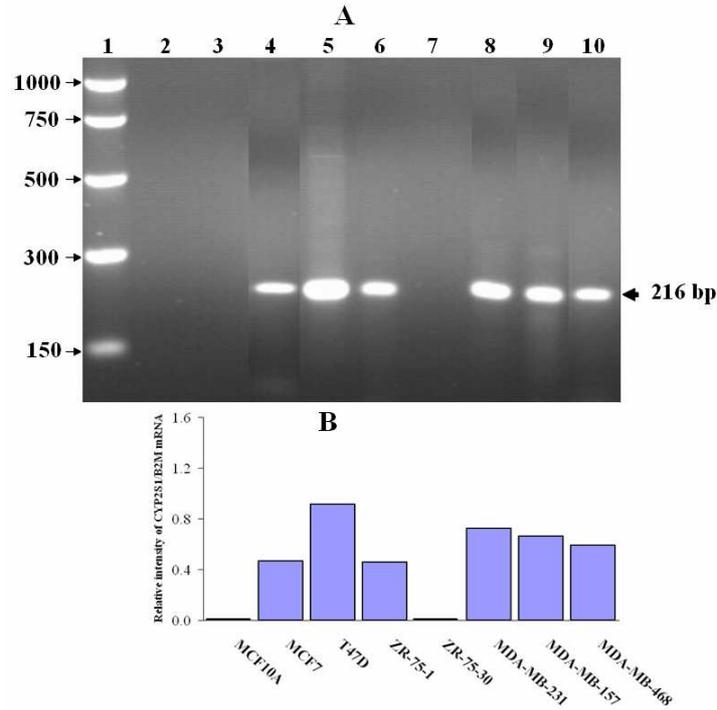


Figure 4.10: CYP2S1 RT-PCR. (A) Visualisation of CYP2S1 amplified products. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468. (B) Relative intensities of CYP2S1 normalised against β_2 -microglobulin.

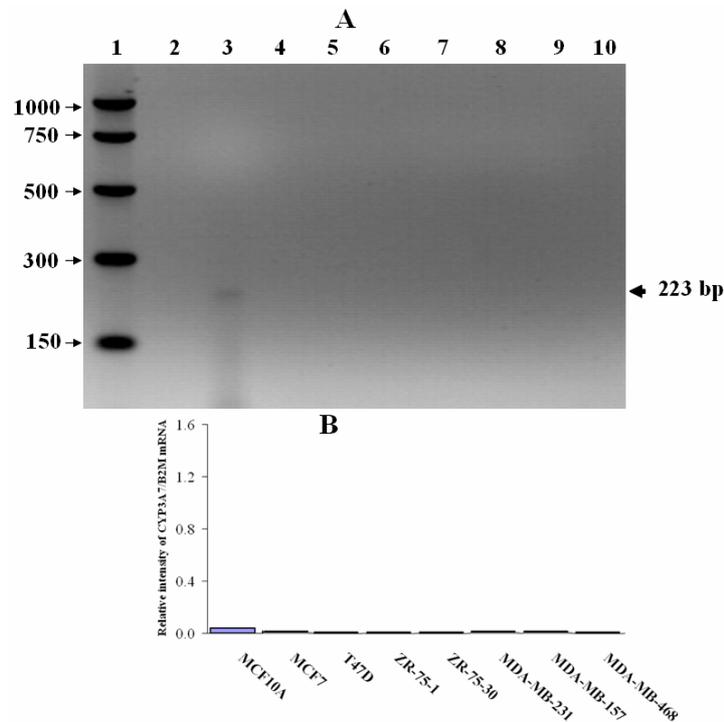


Figure 4.11: CYP3A7 RT-PCR. (A) Visualisation of CYP3A7 amplified products (negative image). Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468. (B) Relative intensities of CYP3A7 normalised against β_2 -microglobulin.

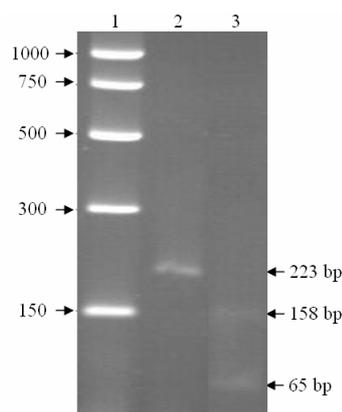


Figure 4.12: Restriction digestion of *CYP3A7* RT-PCR with *EcoRI*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

4.3.6 CYP4 family RT-PCR

A cDNA product at 159 bp indicated *CYP4Z1* mRNA was found in three out of the seven breast cancer cell lines and the normal breast cell line. *CYP4Z1* was expressed in the MDA-MB-468 and T47D cells at higher levels than in the MCF10A and ZR-75-1 cells. An additional band of ~250 bp was observed with the presence of *CYP4Z1* mRNA in all four cell lines (Figure 4.13).

A single band of 257 bp corresponding to the cDNA product size for the published *CYP4Z1* mRNA was detected in four of the seven breast cancer cell lines and the normal breast cell line (Figure 4.14). There was no additional band in this primer set. The *CYP4Z1* mRNA in the MDA-MB-468 cells seemed to be overexpressed compared with the MCF10A, T47D and ZR-75-1 cells. *CYP4Z1* mRNA was expressed in the ZR-75-30 cells at a very low level. Expression of *CYP4Z1* mRNA in the MDA-MB-468 cells was confirmed by restriction digest with *AluI*, and the correct fragments of 196 bp and 61 bp were observed (Figure 4.15).

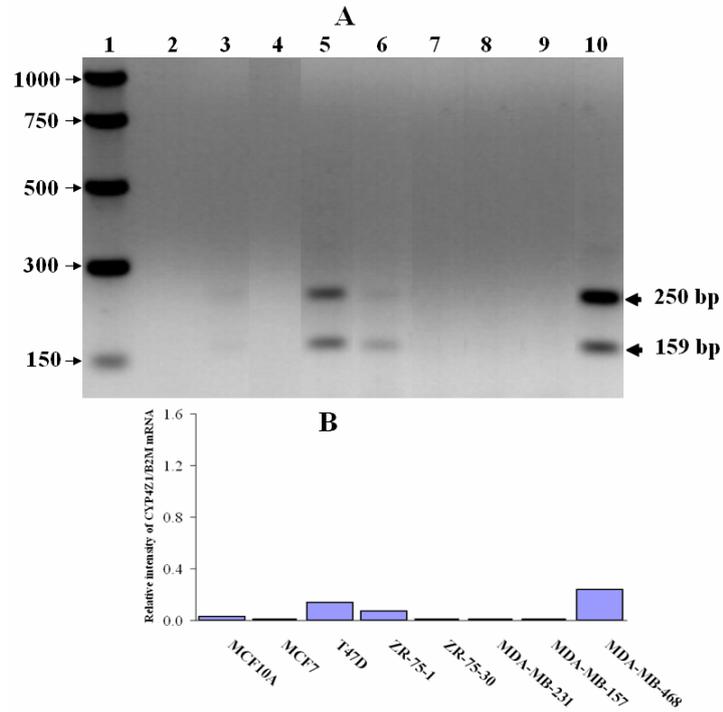


Figure 4.13: CYP4Z1 RT-PCR. (A) Visualisation of CYP4Z1 amplified products (negative image). Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468. (B) Relative intensities of CYP4Z1 (159 bp) normalised against β_2 -microglobulin.

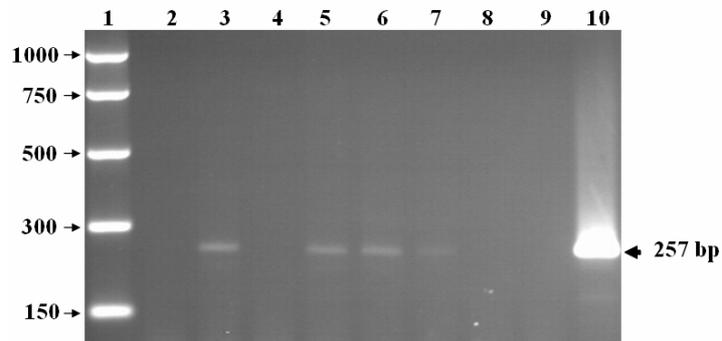


Figure 4.14: CYP4Z1 RT-PCR using the previously published primer set. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468.

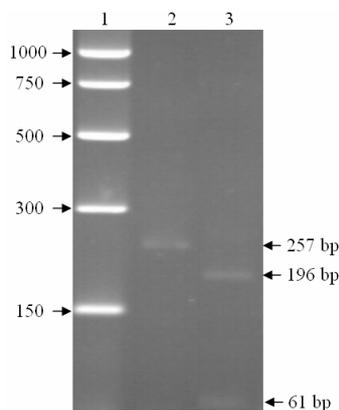


Figure 4.15: Restriction digestion of CYP4Z1 RT-PCR (using the published primers) with AluI. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

4.3.7 CYP24 family RT-PCR

A band at 249 bp was observed for CYP24A1 mRNA expression and it was detected in almost all the cell lines tested with the exception of ZR-75-30 and MDA-MB-468. Very low levels of CYP24A1 were observed in the MCF10A and MDA-MB-231 cells, whereas moderate expression was found in the MCF7, T47D, ZR-75-1 and MDA-MB-157 cells (Figure 4.16).

4.3.8 CYP26 family RT-PCR

CYP26A1 mRNA, at 191 bp, was detected in all cell lines except ZR-75-1 and the normal breast cell line. CYP26A1 was expressed in MDA-MB-231 and MDA-MB-468 at very low levels, while a higher level of expression was observed in the MDA-MB-157 cells (Figure 4.17).

A band indicating the presence of CYP26B1 mRNA at 172 bp was detected in the normal breast cell line and six out of the seven breast cancer cell lines. No CYP26B1 mRNA was expressed in the ZR-75-30 cells. Very weak levels of CYP26B1 mRNA were found in the MCF7, T47D and ZR-75-1 cells, whereas higher levels were detected in both the normal breast and the breast cancer cell lines (MDA-MB231, MDA-MB-157 and MDA-MB-468; Figure 4.18).

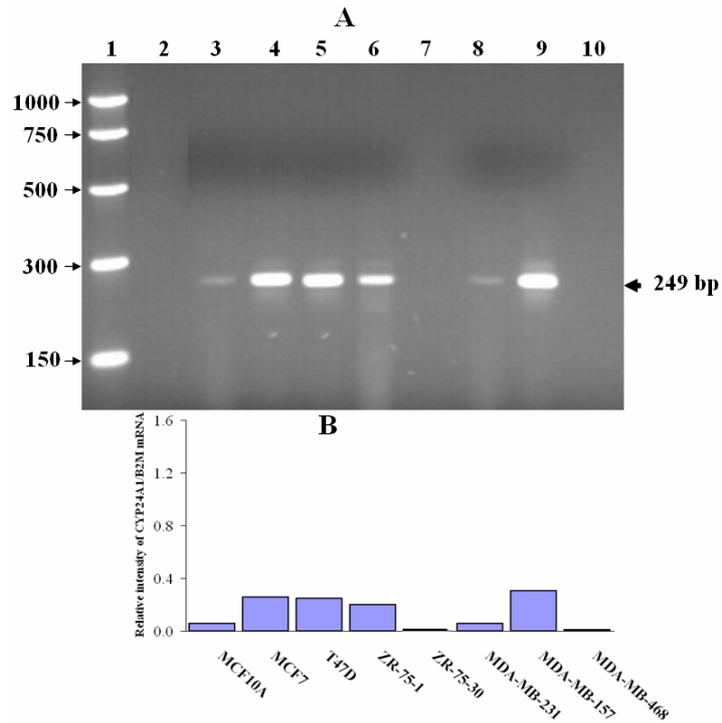


Figure 4.16: CYP24A1 RT-PCR. (A) Visualisation of CYP24A1 amplified products. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468. (B) Relative intensities of CYP24A1 normalised against β_2 -microglobulin.

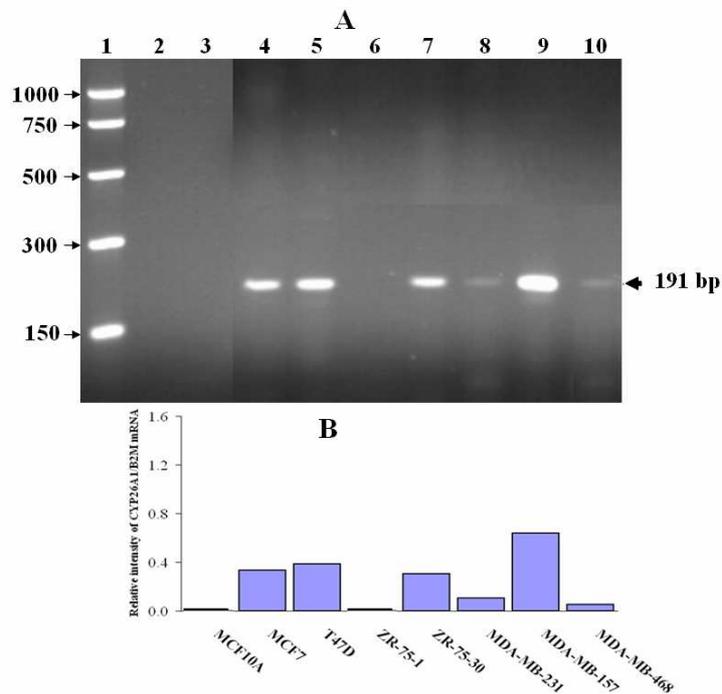


Figure 4.17: CYP26A1 RT-PCR. (A) Visualisation of CYP26A1 amplified products. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468. (B) Relative intensities of CYP26A1 normalised against β_2 -microglobulin.

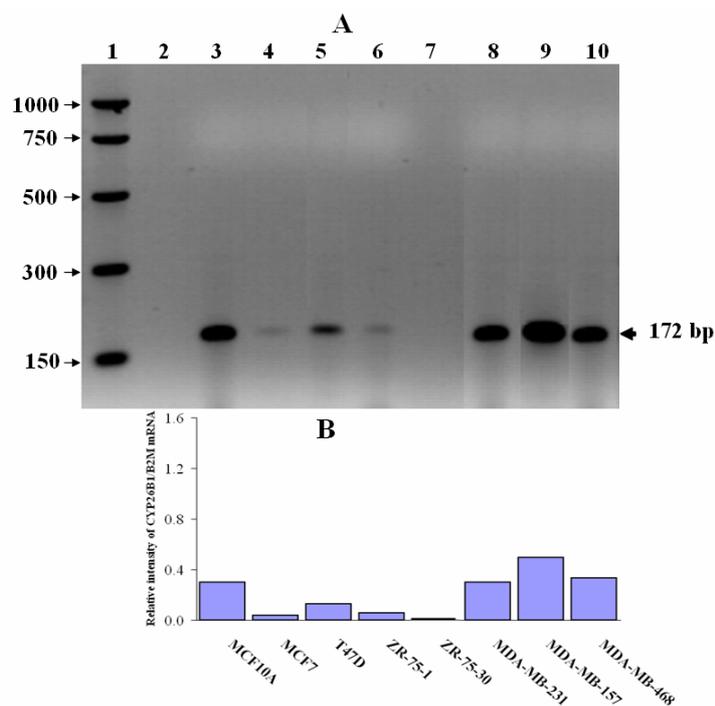


Figure 4.18: *CYP26B1* RT-PCR. (A) Visualisation of *CYP26B1* amplified products (negative image). Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468. (B) Relative intensities of *CYP26B1* normalised against β_2 -microglobulin.

4.3.9 Cytochrome P450-regulatory nuclear receptor RT-PCR

The expression of HIF-1 α , AhR and ARNT mRNA was detected in both the normal breast and breast cancer cell lines at the correct band size of 167 bp, 262 bp and 387 bp respectively (Figures 4.19, 4.20 and 4.21). HIF-1 α mRNA was expressed at high levels in the normal breast cells MCF10A, and the breast cancer cell lines T47D and MDA-MB-231. It was detected at lower levels in the ZR-75-1, ZR-75-30 and MDA-MB-468 cells. AhR and ARNT mRNA were expressed at similar levels in the cell lines used, and low levels of both AhR and ARNT mRNA expression were observed in the ZR-75-30 cells.

A band for the expression of AhRR mRNA at 171 bp was detected in six out of the seven breast cancer cell lines and in the normal breast cell line. Very weak levels of AhRR mRNA were found in the normal breast cells and ZR-75-30. AhRR mRNA expression was not detected in the ZR-75-1 cells (Figure 4.22).

A band of 250 bp corresponding to the cDNA product size for VDR mRNA was found in five out of the seven breast cancer cell lines and in the normal breast cell line but no product was detected in the ZR-75-30 and MDA-MB-468 cells (Figure 4.23).

ER mRNA was found in six out of the seven breast cancer cell lines as a correct band at 208 bp but it was not detected in the normal breast cell line or the breast cancer cell line MDA-MB-231. ER was expressed in the T47D, ZR-75-1, ZR-75-30 and MDA-MB-157 cells at higher levels than in the MCF7 and MDA-MB-468 cells. An additional fainter band at 450 bp was only observed in the MCF7 and MDA-MB-468 cells as mentioned in Section 3.3.4.1 (Figure4.24).

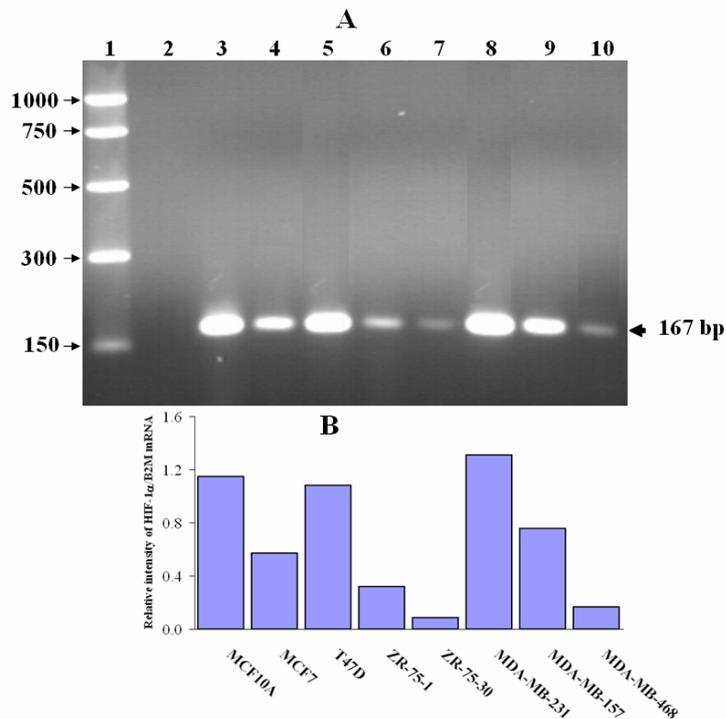


Figure 4.19: *HIF-1α* RT-PCR. (A) Visualisation of *HIF-1α* amplified products. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468. (B) Relative intensities of *HIF-1α* normalised against β_2 -microglobulin.

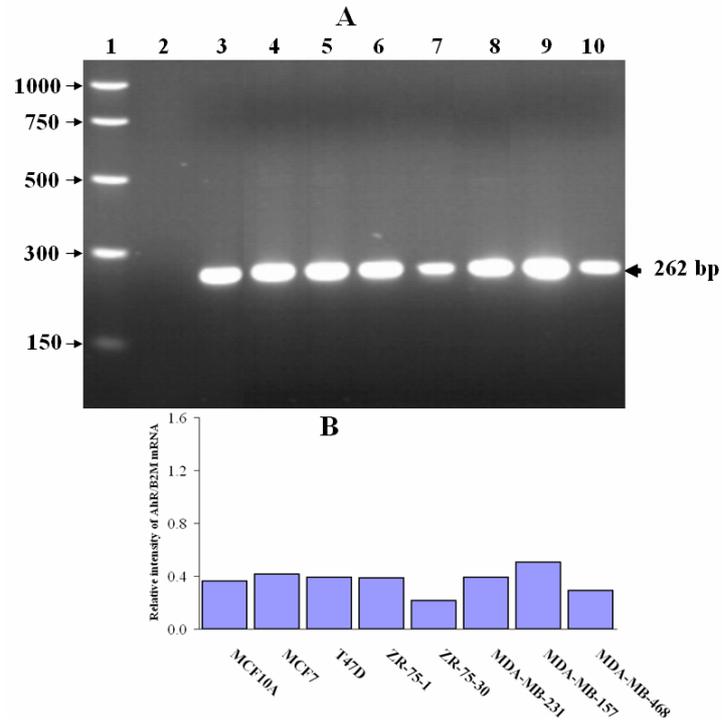


Figure 4.20: AhR RT-PCR. (A) Visualisation of AhR amplified products. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468. (B) Relative intensities of AhR normalised against β_2 -microglobulin.

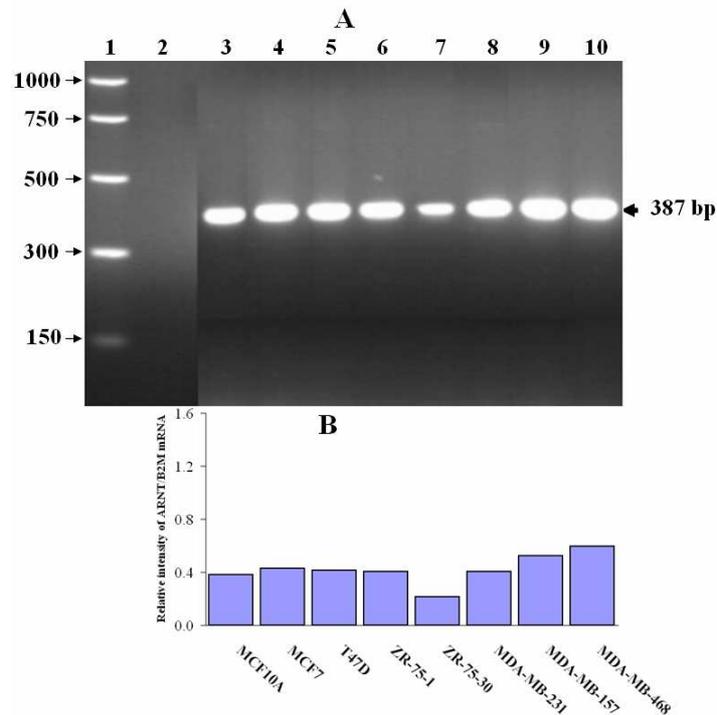


Figure 4.21: ARNT RT-PCR. (A) Visualisation of ARNT amplified products. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468. (B) Relative intensities of ARNT normalised against β_2 -microglobulin.

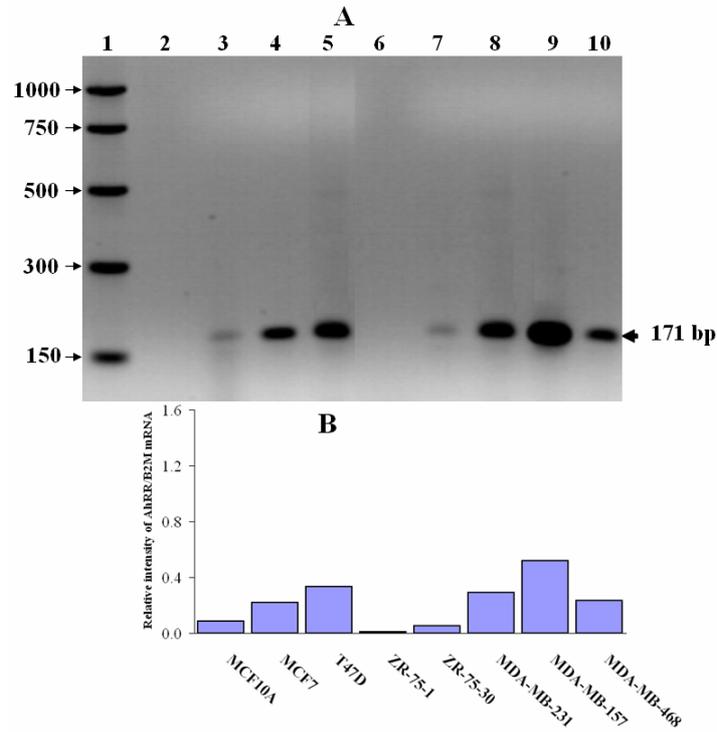


Figure 4.22: AhRR RT-PCR. (A) Visualisation of AhRR amplified products (negative image). Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468. (B) Relative intensities of AhRR normalised against β_2 -microglobulin.

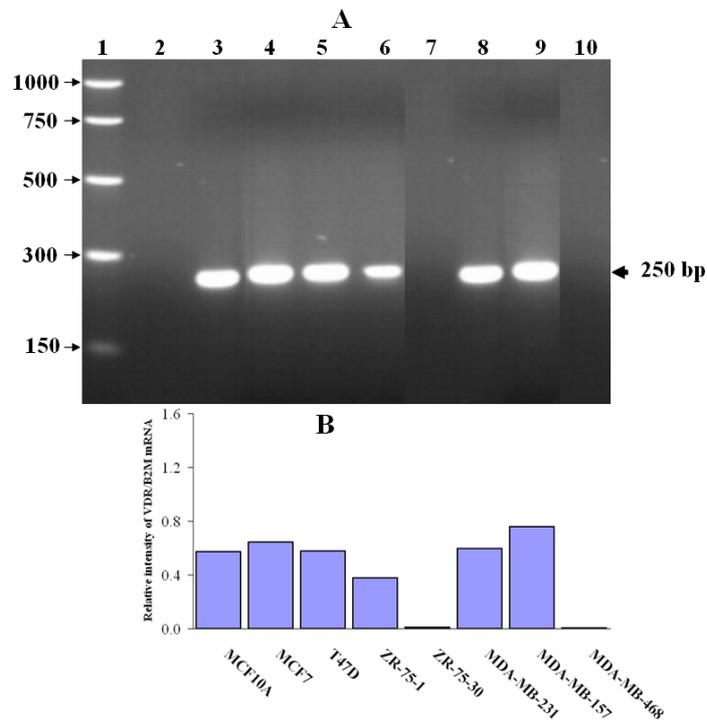


Figure 4.23: VDR RT-PCR. (A) Visualisation of VDR amplified products. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468. (B) Relative intensities of VDR normalised against β_2 -microglobulin.

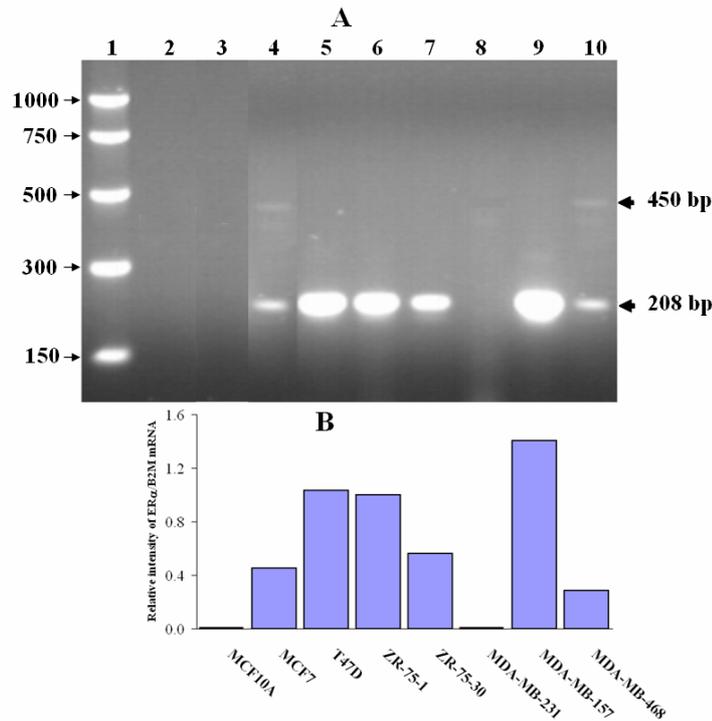


Figure 4.24: ER RT-PCR. (A) Visualisation of ER amplified products. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468. (B) Relative intensities of ER α normalised against β_2 -microglobulin.

A band of 232 bp was observed for the CAR mRNA using nested PCR in the breast cancer cell lines but it was not detected in the normal breast cell line. CAR mRNA (nested) was expressed at lower levels in the ZR-75-1 and MDA-MB-468 cells than in the MCF7, T47D, ZR-75-1, MDA-MB-231 and MDA-MB-157 cells. It was noticed that there were multiple bands of nested CAR RT-PCR product in the T47D, ZR-75-30, MDA-MB-231 and MDA-MB-468 cells (Figure 4.25).

A band of 246 bp was observed at the correct size for PXR mRNA in four out of the seven breast cancer cell lines but it was not expressed in the normal breast cell lines. PXR mRNA was expressed at similar levels in the ZR-75-1 and MDA-MB-157 cells and at very low levels in the MCF7 and T47D cells (Figure 4.26).

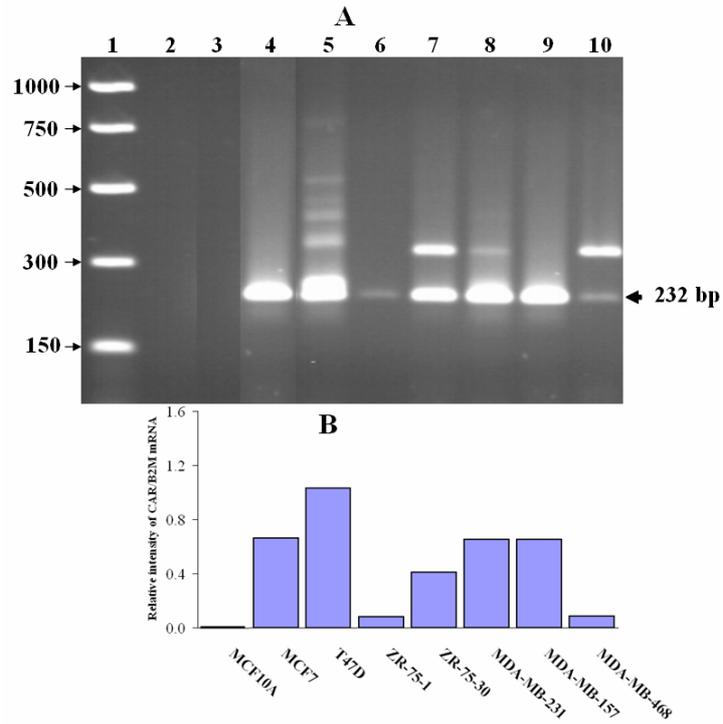


Figure 4.25: Nested CAR RT-PCR. (A) Visualisation of Nested CAR amplified products. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468. (B) Relative intensities of Nested CAR normalised against β_2 -microglobulin.

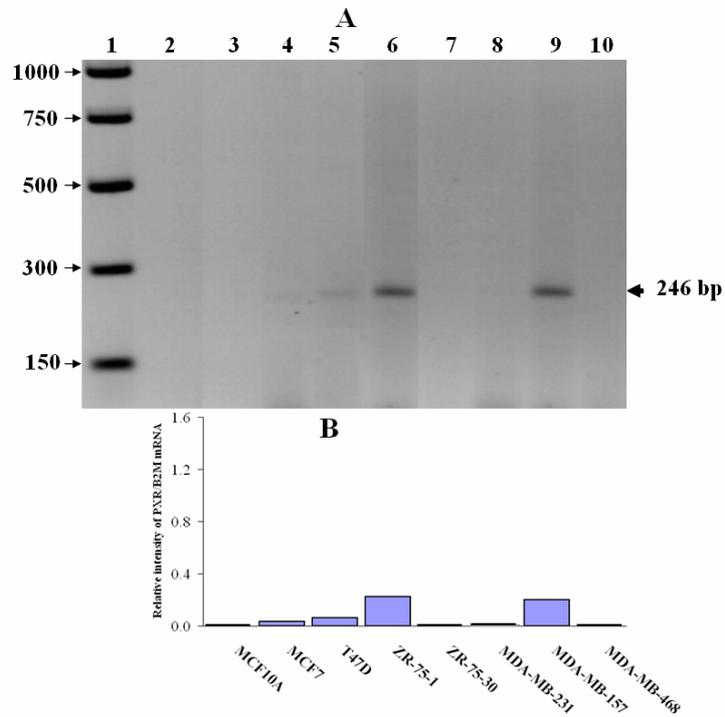


Figure 4.26: PXR RT-PCR. (A) Visualisation of PXR amplified products (negative image). Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468. (B) Relative intensities of PXR normalised against β_2 -microglobulin.

4.3.10 Preliminary work

A band of 261 bp indicated the cDNA product size for the published CYP2D6 mRNA was detected in both the normal breast and breast cancer cell lines (Figure 4.27). CYP2D6 mRNA was expressed at a lower level in the MDA-MB-231 cells than in the MCF7, T47D, ZR-75-1 and MDA-MB-157 cells. It should be noted that the presence of a cDNA product at 261 bp in MCF10A (Lane 2), MDA-MB-468 (Lane 6) and ZR-75-30 (Lane 9) shows up as visible smear in the background between ~250 bp and ~800 bp. However, the expression of CYP2D6 mRNA in the T47D cells was confirmed by restriction digestion with *RsaI*, which generated digested fragments of the correct size (142 bp and 119 bp, Figure 4.28).

The expression of CYP2F1 mRNA was detected in two out of the seven breast cancer cell lines (i.e. ZR-75-1 and MDA-MB-468) at the expected band size of 305 bp but it was not detected in the normal breast cell line (Figure 4.29). CYP2F1 was expressed in the MDA-MB-468 cells at a higher level than in the ZR-75-1 cells. An additional fainter band at ~900 bp was only observed in the ZR-75-1 cells. Expression of CYP2F1 mRNA in the MDA-MB-468 cells was confirmed by restriction digestion with *MspI* and observation of the correct fragments of 195 bp and 110 bp (Figure 4.30).

A single band of 714 bp corresponding to the cDNA product size for the CYP2J2 mRNA was found in both the normal breast and breast cancer cell lines (Figure 4.31). CYP2J2 mRNA was expressed in the MCF10A and MDA-MB-231 cells at very low levels while high levels of CYP2F1 mRNA were observed in almost breast cancer cell lines. To confirm CYP2J2 mRNA expression, the PCR product from MDA-MB-468 cells was restricted with the enzyme *AluI* and the correct fragments of 330 bp and 170 bp were detected (Figure 4.32).

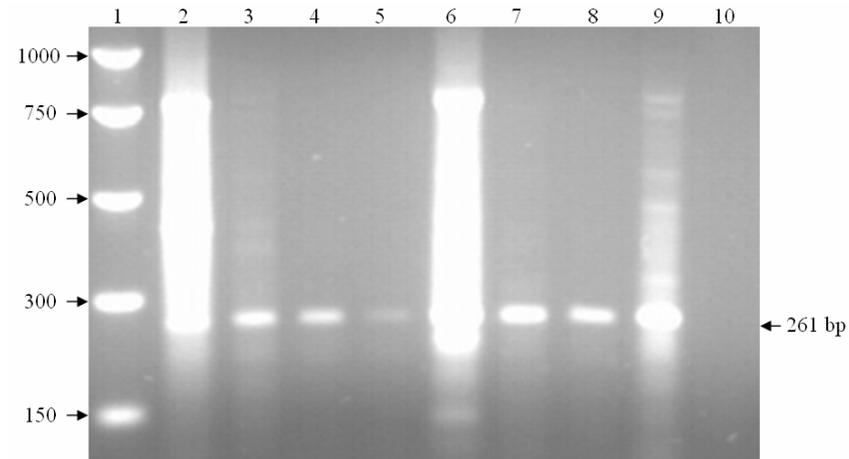


Figure 4.27: Visualisation of *CYP2D6* RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: MCF10A, Lane 3: MCF7, Lane 4: MDA-MB-157, Lane 5: MDA-MB-231, Lane 6: MDA-MB-468, Lane 7: T47D, Lane 8: ZR-75-1, Lane 9: ZR-75-30, Lane 10: negative control.

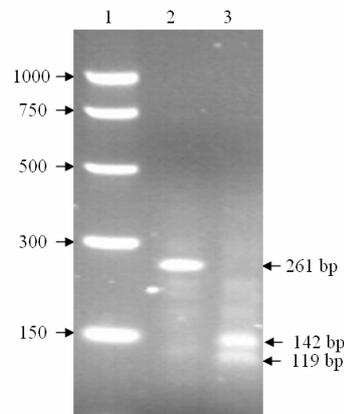


Figure 4.28: Restriction digestion of *CYP2D6* RT-PCR with *RsaI*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

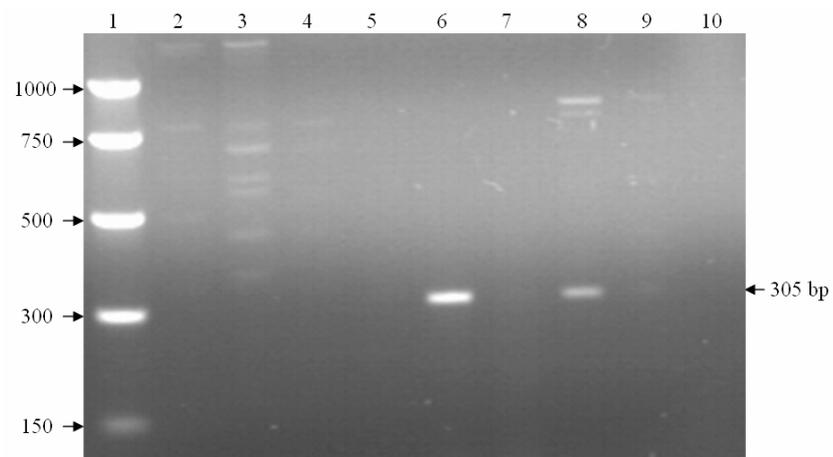


Figure 4.29: Visualisation of *CYP2F1* RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: MCF10A, Lane 3: MCF7, Lane 4: MDA-MB-157, Lane 5: MDA-MB-231, Lane 6: MDA-MB-468, Lane 7: T47D, Lane 8: ZR-75-1, Lane 9: ZR-75-30, Lane 10: negative control.

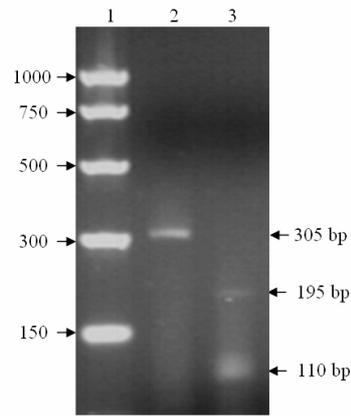


Figure 4.30: Restriction digestion of *CYP2F1* RT-PCR with *MspI*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

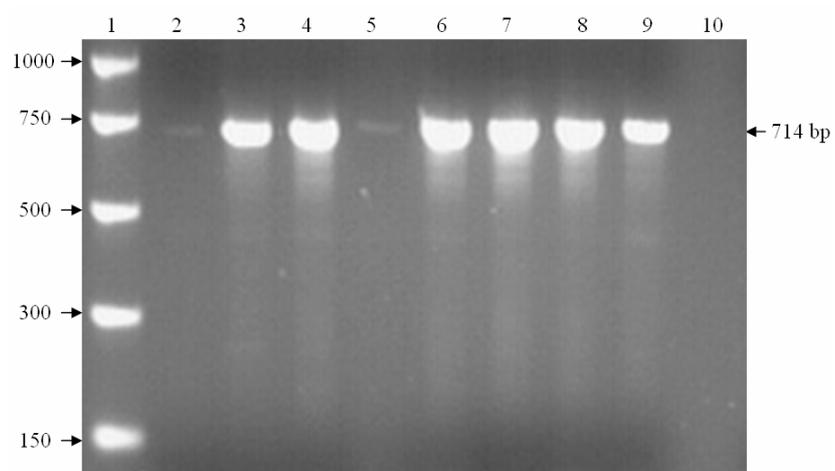


Figure 4.31: Visualisation of *CYP2J2* RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: MCF10A, Lane 3: MCF7, Lane 4: MDA-MB-157, Lane 5: MDA-MB-231, Lane 6: MDA-MB-468, Lane 7: T47D, Lane 8: ZR-75-1, Lane 9: ZR-75-30, Lane 10: negative control.

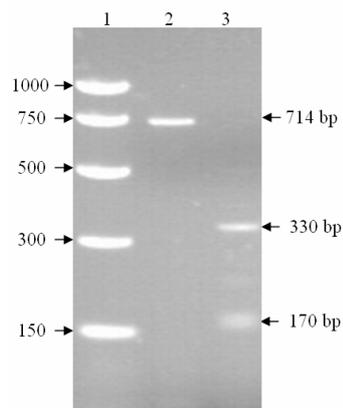


Figure 4.32: Restriction digestion of *CYP2J2* RT-PCR with *AluI*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

During the validation procedure of CYP2R1 RT-PCR analysis, a primer concentration of 0.2 μM was used for RT-PCR experiments as a higher concentration of primer (from 0.3 to 0.5 μM) generated non-specific amplification products. A band of 308 bp was observed for the CYP2R1 mRNA in both the normal breast and breast cancer cell lines (Figure 4.33). CYP2R1 mRNA was expressed at very low levels in the ZR-75-30 and MDA-MB-468 cells whereas higher levels of CYP2R1 mRNA were observed in the normal breast MCF10A cells and the breast cancer MCF7, T47D, MDA-MB-157, MDA-MB-231 and ZR-75-1 cells. Restriction digestion using *Hae*III confirmed the expression of CYP2R1 mRNA in the ZR-75-1 cells, and the correct fragment of 130 bp was observed but not a restricted band of 118 bp (Figure 4.34). It is possible that there was an overlapping band of the two fragments as they are close in size.

A cDNA product at 320 bp indicated CYP2U1 mRNA was found in both the normal breast and breast cancer cells (Figure 4.35). CYP2U1 was expressed in the breast cancer cell lines (except in the ZR-75-30 cells) at higher levels than in the normal breast MCF10A cells. Expression of CYP2U1 mRNA in the ZR-75-1 cells was confirmed by restriction digestion *Hae*III, and the correct fragments of 252 bp and 60 bp were detected (Figure 4.36).

CYP4X1 mRNA was detected in both the normal breast and breast cancer cell lines at the correct cDNA product size of 364 bp (Figure 4.37). Very weak levels of CYP4X1 mRNA were observed in the MCF7, MDA-MB-157 and MDA-MB-231 cells. High levels of expression were found in the normal breast MCF10A and the breast cancer MDA-MB-468 and T47D cells. Restriction digestion of CYP4X1 mRNA by the enzyme *Rsa*I generated fragments (278 bp and 86 bp) of the correct size (Figure 4.38).

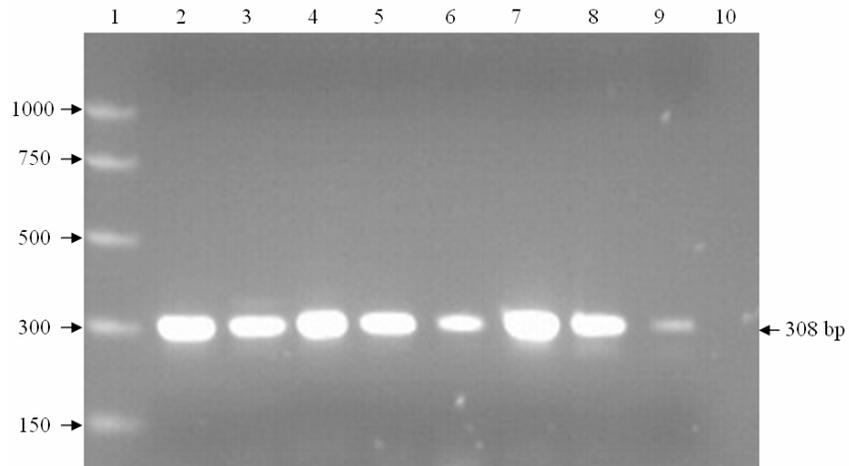


Figure 4.33: Visualisation of *CYP2R1* RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: MCF10A, Lane 3: MCF7, Lane 4: MDA-MB-157, Lane 5: MDA-MB-231, Lane 6: MDA-MB-468, Lane 7: T47D, Lane 8: ZR-75-1, Lane 9: ZR-75-30, Lane 10: negative control.

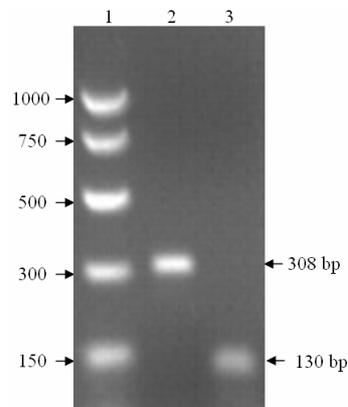


Figure 4.34: Restriction digestion of *CYP2R1* RT-PCR with *HaeIII*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

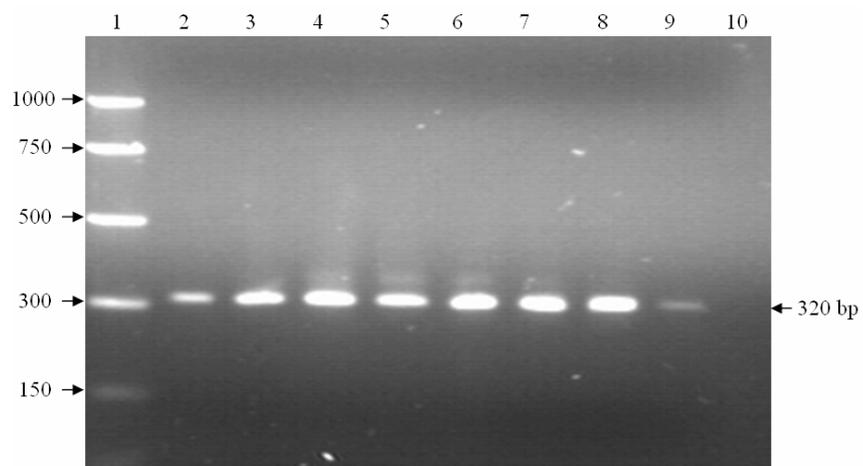


Figure 4.35: Visualisation of *CYP2U1* RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: MCF10A, Lane 3: MCF7, Lane 4: MDA-MB-157, Lane 5: MDA-MB-231, Lane 6: MDA-MB-468, Lane 7: T47D, Lane 8: ZR-75-1, Lane 9: ZR-75-30, Lane 10: negative control.

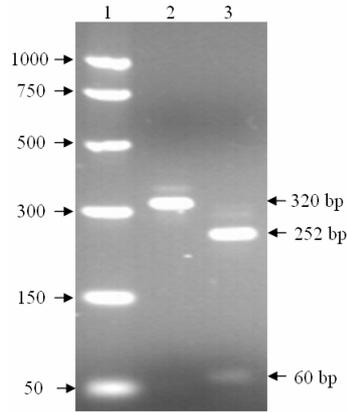


Figure 4.36: Restriction digestion of *CYP2U1* RT-PCR with *HaeIII*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

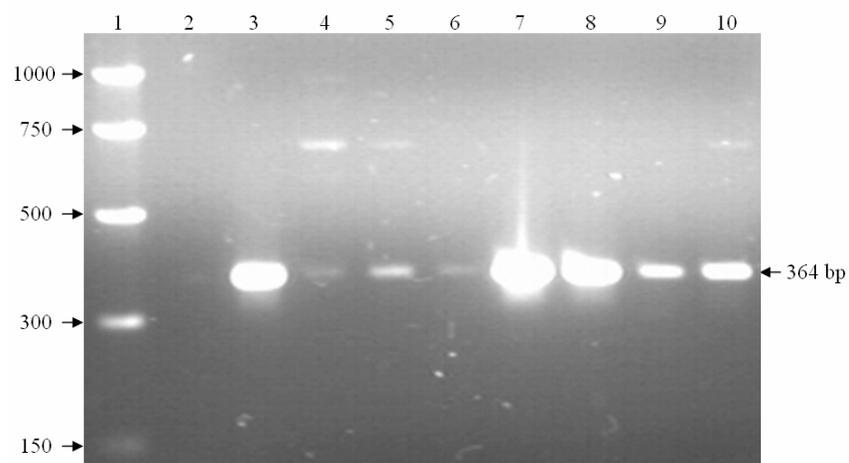


Figure 4.37: Visualisation of *CYP4X1* RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: MCF10A, Lane 3: MCF7, Lane 4: MDA-MB-157, Lane 5: MDA-MB-231, Lane 6: MDA-MB-468, Lane 7: T47D, Lane 8: ZR-75-1, Lane 9: ZR-75-30, Lane 10: negative control.

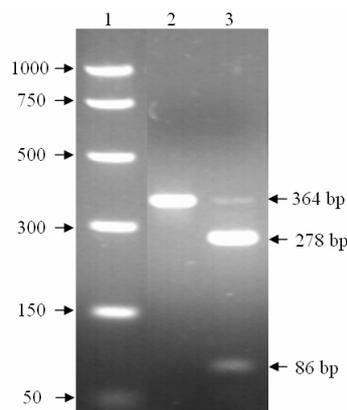


Figure 4.38: Restriction digestion of *CYP4X1* RT-PCR with *RsaI*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

RAR mRNA was detected in both the normal breast and breast cancer cell lines at the expected cDNA product size of 756 bp (Figure 4.39). Higher levels of RAR mRNA were observed in the T47D, ZR-75-1, MDA-MB-157 and MDA-MB-468 cells than in the MCF10A, MCF7, ZR-75-30 and MDA-MB-231 cells. To confirm the RAR mRNA expression, the PCR product was digested with restriction enzyme *HaeIII* and the correct fragments were found at 456 bp, 193 bp and 107 bp (Figure 4.40).

A band of 521 bp corresponding to the cDNA product size for RXR mRNA was found in both the normal breast and breast cancer cell lines. The highest expression was detected in the ZR-75-1 cells, and RXR mRNA was detected at weak to moderate levels in the MCF10A, T47D and MDA-MB-157 cells. Very low mRNA levels were detected in the MCF7, ZR-75-30 MDA-MB-231 and MDA-MB-468 cells (Figure 4.41). To confirm the RXR mRNA expression, the PCR product was digested with restriction enzyme *BamHI* and the correct fragments were found at 280 bp, 153 bp and 88 bp (Figure 4.42).

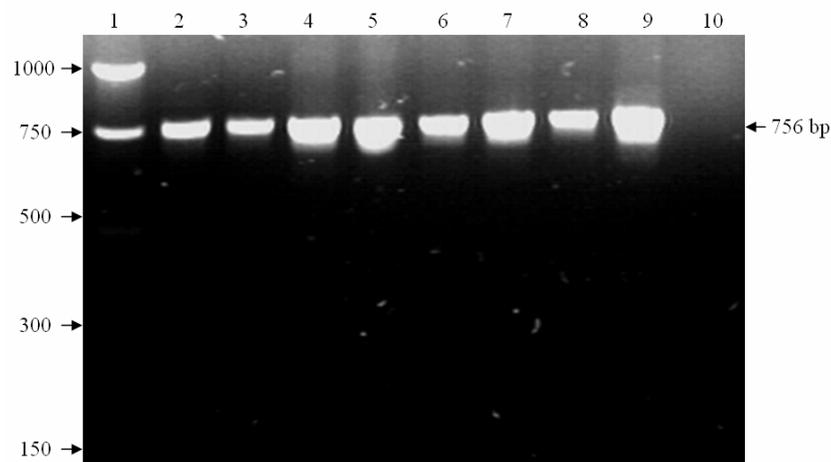


Figure 4.39: Visualisation of RAR RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: MCF10A, Lane 3: ZR-75-30, Lane 4: ZR-75-1, Lane 5: T47D, Lane 6: MCF7, Lane 7: MDA-MB-468, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: negative control.

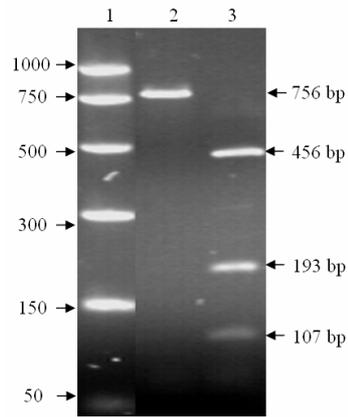


Figure 4.40: Restriction digestion of RAR RT-PCR with HaeIII. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

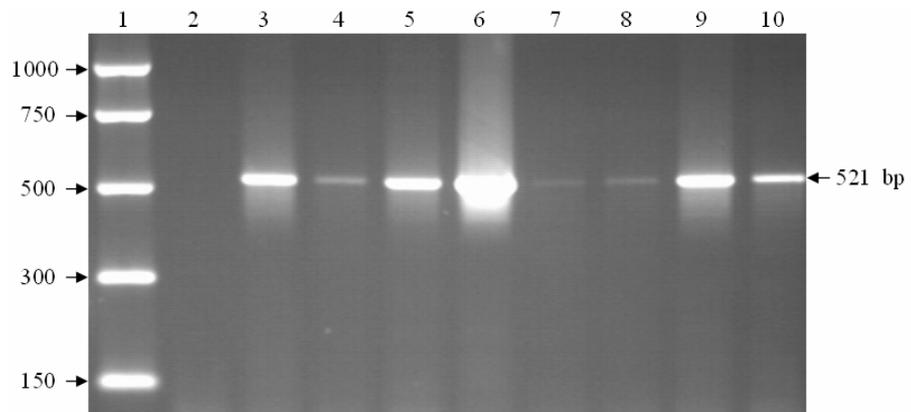


Figure 4.41: RXR RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468.

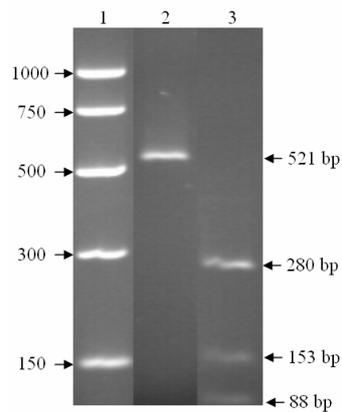


Figure 4.42: Restriction digestion of RXR RT-PCR using BamHI. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

4.4 Discussion

Results presented in this chapter provide a comprehensive profile of cytochrome P450 mRNA expression in a series of seven breast cancer cell lines as compared with the expression profile in a normal breast cell line using RT-PCR. Cytochrome P450-regulatory nuclear receptors were also investigated to explore the relationship between expression of cytochrome P450 mRNA and corresponding receptors. In research presented here, the qualitative comparative analysis of target gene expression between human normal breast and breast cancer cell lines was determined. The expression of CYP1A1, CYP1B1, CYP2C18, CYP2D6, CYP2R1, CYP2U1, CYP4X1, CYP4Z1 CYP24A1 and CYP26B1 mRNA was detected in both the normal breast and individual breast cancer cell lines. CYP3A7 mRNA was expressed only in the normal breast cell line, while CYP2A6, CYP2B6, CYP2C8, CYP2F1, CYP2J2, CYP2S1 and CYP26A1 mRNA were found only in individual breast cancer cell lines. The findings demonstrate differential mRNA expression of individual cytochrome P450s between normal breast and breast cancer cells, and some cell line-specific expression of individual cytochrome P450s. The findings showed seven out of the ten cytochrome P450 corresponding receptors (i.e. HIF-1 α , AhR, ARNT, AhRR, VDR, RAR and RXR) were expressed in both the normal breast and breast cancer cells while the expression of CAR, PXR and ER mRNA was detected only in individual breast cancer cell lines (Table 4.1).

The relationship between candidate mRNA profile and breast cancer cell lines from different ethnic groups (i.e. Caucasian and Afro-Caribbean) with oestrogen status (i.e. ER-positive and ER-negative) was also explored. CYP1B1, CYP2D6, CYP2J2, CYP2R1, CYP2U1, CYP4X1 and CYP26A1 mRNA were found in all cell lines. The expression of CYP1A1, CYP2S1, CYP24A1 and CYP26B1 mRNA was not detected in the ER-positive Afro-Caribbean breast cancer cell line (ZR-75-30) and no CYP2B6 expression was observed in the ER-negative Caucasian breast cancer cell line (MDA-MB-231). CYP2A6

(T47D, ZR-75-1 and MDA-MB-157), CYP2C8 (MCF7, ZR-75-1 and MDA-MB-157), CYP2C18 (MCF7, T47D, MDA-MB-468 and MDA-MB-157), CYP2F1 (ZR-75-1 and MDA-MB-468) and CYP4Z1 (T47D, ZR-75-1 and MDA-MB-468) mRNA were expressed in the ER-positive Caucasian and ER-negative Afro-Caribbean breast cancer cell lines. Although no differences in cytochrome P450 mRNA expression were observed between the different ethnic groups, these preliminary results suggest potential similarities in the ER-positive Caucasian and ER-negative Afro-Caribbean breast cancer cells which warrant further investigation.

In addition to comparing cytochrome P450 expression with ethnicity (i.e. Caucasian and Afro-Caribbean) and ER status, the relationship between ethnic difference and invasive phenotype was also examined. CYP1B1, CYP2D6, CYP2J2, CYP2S1, CYP2R1, CYP2U1, CYP4X1 mRNA were found in all cell lines. CYP1A1, CYP2A6, CYP2C8 and CYP24A1 mRNA were not detected in the non-invasive Afro-Caribbean breast cancer cell lines. CYP26A1 mRNA was below the levels of detectability in only the normal breast MCF10A and ZR-75-1 ER-positive breast cancer cell lines. CYP26B1 mRNA was below limits of detectability only in the ER-positive ZR-75-30 breast cancer cell line. No cytochrome P450 mRNA was specific for the invasive phenotype, but the expression of CYP4Z1 mRNA appears to be specific for the non-invasive phenotype. CYP2F1 was observed in only two out of the eight cell lines both of the non invasive phenotype. No CYP2B6 or CYP2C18 expression was observed in the invasive Caucasian breast cancer cells. Although of a preliminary nature these results suggest an inverse relationship of cytochrome P450 expression (i.e. CYP2A6 and CYP2C8 mRNA) between ethnic group and invasive phenotype, particularly the non-invasive Caucasian and invasive Afro-Caribbean breast cancer cell lines. These preliminary findings warrant further investigation potentially in relation to patient samples.

Table 4.1: The presence of cytochrome P450 and cytochrome P450-regulatory nuclear receptor mRNA in breast cancer cell lines compared to the normal breast cell line.

ER status	ER-positive					ER-negative		
Ethnicity	Cau	Cau	Cau	Cau	Afro	Cau	Afro	Afro
Invasive phenotype	NBC	Non-inv	Non-inv	Non-inv	Non-inv	Inv	Inv	Non-inv
Cell lines	MC10A	MCF7	T47D	ZR-75-1	ZR-75-30	MDA-MB-231	MDA-MB-157	MDA-MB-468
Cytochrome P450 mRNA								
CYP1A1*	+	+	+	+	-	+	+	-
CYP 1A2	NS	NS	NS	NS	NS	NS	NS	NS
CYP1B1	+	+	+	+	+	+	+	+
CYP2A6	-	-	+	+	-	-	+	-
CYP2B6	-	-	+	+	+	-	+	+
CYP2C8	-	+	-	+	-	-	+	-
CYP2C18	+	+	+	-	-	-	+	+
CYP2C9	-	-	-	-	-	-	-	-
CYP2C19	-	-	-	-	-	-	-	-
CYP2D6*	+	+	+	+	+	+	+	+
CYP2F1*	-	-	-	+	-	-	-	+
CYP2J2*	+	+	+	+	+	+	+	+
CYP2R1*	+	+	+	+	+	+	+	+
CYP2S1	-	+	+	+	-	+	+	+
CYP2U1*	+	+	+	+	+	+	+	+
CYP3A4*	-	-	-	-	-	-	-	-
CYP3A5*	-	-	-	-	-	-	-	-
CYP3A43	-	-	-	-	-	-	-	-
CYP3A7	+	-	-	-	-	-	-	-
CYP4X1*	+	+	+	+	+	+	+	+
CYP4Z1	+	-	+	+	-	-	-	+
CYP24A1	+	+	+	+	-	+	+	-
CYP26A1	-	+	+	-	+	+	+	+
CYP26B1	+	+	+	+	-	+	+	+
Cytochrome P450-regulatory nuclear receptor mRNA								
HIF-1 α	+	+	+	+	+	+	+	+
AhR	+	+	+	+	+	+	+	+
ARNT	+	+	+	+	+	+	+	+
AhRR	+	+	+	-	+	+	+	+
CAR*	-	+	+	+	+	+	+	+
PXR*	-	+	+	+	-	-	+	-
VDR	+	+	+	+	-	+	+	-
ER	-	+	+	+	+	-	+	+
RAR*	+	+	+	+	+	+	+	+
RXR*	+	+	+	+	+	+	+	+

Note: Blue and yellow labelled indicate the detected and undetected mRNA in all cell lines respectively.

Abbreviations: ER; oestrogen receptor, Cau; Caucasian, Afro; Afro-Caribbean, NS; non-specific product, *; published primers, NBC; normal breast cell line, Inv; invasive phenotype

The CYP1 family comprises three enzymes, CYP1A1, CYP1A2 and CYP1B1, and they are involved in the metabolism of oestrogen as well as procarcinogens (e.g. PAHs). Research conducted by several groups, with both tissues and cell lines, suggests an association between the roles of these enzymes in oestrogen metabolism and breast carcinogenesis (Singh et al. 2005, Tsuchiya et al. 2005, Cavalieri et al. 2006, Fernandez et al. 2006, Yager and Davidson 2006).

The expression of CYP1A1 mRNA has been detected in normal breast tissue (Huang et al. 1996, Hellmold et al. 1998, Iscan et al. 2001, Modugno et al. 2003) and breast tumours (Huang et al. 1996, Iscan et al. 2001, Modugno et al. 2003). Hellmold and co-workers showed CYP1A1 protein to be present in (5/15) normal breast (Hellmold et al. 1998). El-Rayes and colleagues reported that CYP1A1 protein was significantly higher ($P < 0.05$) in normal adjacent breast tissues than in breast tumours by immunoblotting (El-Rayes et al. 2003). However, these findings are in disagreement with those of Albin and colleagues who did not detect CYP1A1 protein in breast tumours and normal adjacent breast tissues using the same method (Albin et al. 1993).

In the present study, CYP1A1 mRNA was found in the normal breast MCF10A cells which is in agreement with previous studies (Spink et al. 1998, Thomas et al. 2006). The expression of CYP1A1 mRNA was detected in all breast cancer cell lines except for the ER-positive Afro-Caribbean ZR-75-30 and ER-negative Afro-Caribbean MDA-MB-468 breast cancer cells. The finding that CYP1A1 mRNA is present in the MCF7, ZR-75-1, T47D and MDA-MB-231 cells correlates well with results from a study by Spink and colleagues who investigated CYP1A1 mRNA using Northern hybridisation (Spink et al. 1998). The present investigation also found CYP1A1 mRNA in the MDA-MB-157 cells, though this was not detected in the previous study (Spink et al. 1998). The lack of CYP1A1 expression in the MDA-MB-468 cells is in agreement with an earlier

investigation (Wang et al. 1997). However, the expression of CYP1A1 mRNA in the ZR-75-30 cells has not been previously reported. In addition, McFadyen and co-workers reported that the MCF7 cells express CYP1A1 mRNA but did not express the protein (McFadyen et al. 2003). These observations suggest that CYP1A1 protein expression in these cell lines warrants further investigation to clarify the functional significance of the CYP1A1 expression.

The association between the CYP1A1 polymorphisms and cancer susceptibility, lung cancer in particular, have been demonstrated to be dependent upon ethnicity of population. However, investigation into a possible relationship between the CYP1A1 alleles and breast cancer risk has generated inconsistent results and this remains unclear (Masson et al. 2005). Preliminary findings from the present study suggest that CYP1A1 is expressed in the majority of cell lines apart from Afro-Caribbean cell lines of the non-invasive phenotype.

Several breast cancer research groups have demonstrated the expression of CYP1B1 mRNA in normal breast (Huang et al. 1996, Hellmold et al. 1998, Iscan et al. 2001) and breast tumour tissues (Huang et al. 1996, Iscan et al. 2001). A higher level of CYP1B1 mRNA was found in breast tumours than in normal breast tissue (Modugno et al. 2003). In addition, previous studies demonstrated that CYP1B1 protein was overexpressed in breast tumour samples and undetectable in normal breast tissue (Murray et al. 1997, McFadyen et al. 1999). On this basis, tumour-specific CYP1B1 expression appears to be a potential target for the development of new anticancer treatments and a CYP1B1 inhibitor and CYP1B1 vaccine are currently in clinical trials (Section 1.8). In this research, CYP1B1 mRNA was detected in the normal breast MCF10A cells and the seven breast cancer cell lines. No difference in CYP1B1 mRNA expression was observed between the different ER status, ethnic groups or invasive phenotype. However, it is essential to confirm

CYP1B1 protein expression in these cell lines before it can be verified as a target for the development of new anticancer drugs.

The CYP1A1 and CYP1B1 genes are regulated by the aryl hydrocarbon receptor (AhR) and induced by AhR agonists such as TCDD and benzo(a)pyrene (Iwanari et al. 2002). The AhR belongs to the basic helix-loop-helix/Per-ARNT-Sim (bHLH-PAS) family which consists of the AhR dimerisation partners such as AhR nuclear translocator (ARNT), hypoxia inducible factor-1 alpha (HIF-1 α) (Semenza 2000) and AhR repressor (AhRR) (Tsuchiya et al. 2003). These findings suggest that the AhR-mediated pathway may interact with other nuclear receptor signalling pathways which are associated with the expression of CYP1A1 and CYP1B1.

It is well known that CYP1A1 and CYP1B1 are involved in oestrogen metabolism and Angus and colleagues demonstrated that oestradiol can also induce the transcriptional expression of CYP1A1 and CYP1B1 (Angus et al. 1999). The induction of CYP1B1 by oestradiol has been demonstrated in breast cancer oestrogen-dependent (i.e. MCF7) and oestrogen-independent (i.e. MDA-MB-435) cells. An increased CYP1B1 mRNA level was found in the MCF7 cells but not in the MDA-MB-435 cells, indicating that the induction of CYP1B1 by oestradiol may be transcriptionally regulated by oestrogen receptor as previously demonstrated (Tsuchiya et al. 2004, Tsuchiya et al. 2005). In addition, Beischlag and colleagues (Beischlag and Perdew 2005) demonstrated that AhR agonist TCDD can increase the expression of CYP1A1 and that it activates inhibitory crosstalk between ER and the AhR-ARNT complex.

In the current project, expression of AhR, ARNT and HIF-1 α mRNA was found in all the test cells, which is similar to CYP1B1 mRNA but not CYP1A1 mRNA expression. Allan and co-workers found that decreased CYP1A1 expression under hypoxic conditions.

These findings may have been caused by competition for dimerisation with ARNT, leading to inhibition of target genes of AhR (i.e. CYP1A1), as dimerisation of AhR/ARNT and HIF-1 α /ARNT is required for transcription initiation in their target genes including CYP1A1 and CYP1B1 (Allen et al. 2005). These cell lines could be useful models to clarify their relationship in breast cancer cells.

Previous studies have failed to detect CYP1A2 mRNA expression in normal human breast or breast tumour tissues (Hellmold et al. 1998, Modugno et al. 2003). However, a recent study using immunohistochemistry detected CYP1A2 protein expression in 51 out of the 393 breast tumours tested (Haas et al. 2006). Although multiple bands were observed in the present study, no specific product at the correct size was identified for CYP1A2 mRNA in either the normal breast or breast cancer cell lines. The lack of CYP1A2 mRNA expression in the normal breast MCF10A cell line is in agreement with the recent study by Thomas and colleagues (Thomas et al. 2006). The lack of CYP1A2 mRNA expression is surprising as CYP1A2 is involved in the metabolism of oestradiol (Tsuchiya et al. 2005, Yager and Davidson 2006) and the catalysis of a wide range of procarcinogens e.g. heterocyclic amines, nitrosamine, aflatoxin B1 and arylamine (Kim and Guengerich 2005). It should be noted that CYP1A2 mainly catalyses flutamide (a nonsteroidal antiandrogenic anticancer drug) which is commonly used for prostate cancer treatment (Goda et al. 2006).

CYP2A6 mRNA expression was detected in (10/15) normal breast tissues (Hellmold et al. 1998) whereas Iscan and co-workers reported no mRNA expression of CYP2A6 in normal breast tissues or breast tumour tissues (Iscan et al. 2001). Using real-time quantitative RT-PCR analysis, Bieche and colleagues (Bièche et al. 2004) demonstrated that the CYP2A6 mRNA level in ER-positive breast tumours is higher than in either ER-negative breast tumours or normal breast tissue. As was observed in present investigation, the expression level of CYP2A6 was higher in the breast cancer ER-positive Caucasian ZR-75-1 and

T47D than in the ER-negative Afro-Caribbean MDA-MB-157. However, it is important to note that CYP2A6 expression was not observed in the invasive Caucasian MDA-MB-231 or the non-invasive Afro-Caribbean MDA-MB-468 breast cancer cells. No CYP2A6 mRNA was found in the normal breast MCF10A cells either, which correlates well with results from a study by Thomas and colleagues (Thomas et al. 2006).

The importance of CYP2A6 in the metabolism of anticancer drugs (i.e. letrozole) and procarcinogens (i.e. nitrosamine and food mutagens) has been emphasised, and it has been reported that this cytochrome P450 enzyme may play a role in the development of breast cancer (von Weymarn et al. 2006). CYP2A6 metabolises a third-generation aromatase inhibitor, letrozole, which is superior to tamoxifen for first-line treatment in breast cancer (Simpson et al. 2004). Therefore, the findings in the present study suggest that expression of CYP2A6 mRNA in the opposite oestrogen receptor status/invasive phenotype of Caucasian and Afro-Caribbean breast cancer cell lines could be of benefit in the design of more effective drug regimens in cancer patients.

The expression of CYP2B6 mRNA was found in (12/14) breast tumours and (13/14) normal breast tissues (Iskan et al. 2001), and Hellmond and co-workers detected CYP2B6 mRNA expression in (9/15) normal breast tissues (Hellmold et al. 1998). In the present study, CYP2B6 mRNA was observed in five out of the seven breast cancer cell lines but not in the normal breast MCF10A cells. The lack of CYP2B6 mRNA expression in the MCF7 and MCF10A cells is in agreement with the studies of Soulez and Parker (Soulez and Parker 2001), and Thomas and colleagues (Thomas et al. 2006) respectively. The presence of CYP2B6 mRNA in the ZR-75-1 cells is in agreement with the research of Soulez and Parker (Soulez and Parker 2001). The absence of CYP2B6 mRNA in the MDA-MB-231 cells, classified as invasive and ER-negative Caucasian breast cancer cells,

is important to note as CYP2B6 converts the prodrug cyclophosphamide to an active phosphoramidate mustard and acrolein (Zanger et al. 2007).

Targeting drugs at CYP2B6 is an attractive concept for cancer research as this would lead to local activation of the prodrug and higher concentrations of the active metabolites at the tumour site. MetXia is a recombinant retroviral vector encoding human CYP2B6 which is designed to activate cyclophosphamide. MetXia is currently in Phase I trial and being investigated in advanced breast cancer patients (Braybrooke et al. 2005).

The CYP2B6 gene is regulated by CAR (Honkakoski and Negishi 2000) and a positive correlation between CYP2B6 and CAR mRNA expression has been demonstrated in human liver (Chang et al. 2003). A study by Lamba and colleagues showed that ethnic and gender differences influence the expression of CYP2B6 and CAR (Lamba et al. 2003).

The expression level of CYP2B6 mRNA was significantly higher in females than in males ($p < 0.001$) and CYP2B6 activity in Hispanic females higher than in Caucasian ($p < 0.022$) or African-American ($p < 0.038$) females. These findings can be related to female hormones including oestrogen. However, Min and colleagues (Min et al. 2002) demonstrated inhibitory crosstalk between ER and CAR in human hepatoma HepG2 cell line and that CAR inhibits ER-mediated transactivation of endogenous and exogenous oestrogen. Recent research by the same group found that ER-mediated transactivation can be regulated by CAR which is dependent on the oestrogen response elements (EREs) and oestrogen receptor status of target cells (Min 2006).

In research presented here, a direct correlation between CYP2B6 and CAR mRNA was observed in the ER-positive Caucasian T47D and the ER-negative Afro-Caribbean MDA-MB-157 and MDA-MB-468 breast cancer cells. Further investigation of the expression of CYP2B6 protein in these cell lines is warranted prior to determination in clinical samples.

The expected product size of CAR is 232 bp following nested PCR, but multiple bands are seen in lanes 5, 7, 8 and 10 (Figure 4.25). The presence of non-specific products may be due to too much template (first round PCR product) and/or insufficient primer. This problem can be resolved by diluting the concentration of template. However, during the validation of CAR in this research, the PCR product was confirmed by restriction digestion and the expected products were clearly seen (Figure 3.36).

There are four members of the CYP2C subfamily CYP2C8, CYP2C9, CYP2C18 and CYP2C19. They are highly homologous genes and are clinically relevant in anticancer drug treatments. In cancer chemotherapy, CYP2C8 catalyses the metabolism of paclitaxel (Taniguchi et al. 2005) whereas CYP2C9 and CYP2C19 catalyse the bioactivation of cyclophosphamide and ifosfamide (Goldstein 2001, Schmidt et al. 2004) as well as the metabolism of tamoxifen. CYP2C18 is involved in the activation of ifosfamide (Zhu-Ge and Yu 2004). Modugno and colleagues reported that the expression of CYP2C mRNA was not detected in normal breast tissues or breast tumours (Modugno et al. 2003). In contrast, previous studies showed that CYP2C mRNA expression was found in normal breast as well as breast tumour tissues (Huang et al. 1996, Iscan et al. 2001). In addition, CYP2C protein was expressed in normal breast tissues (Hellmold et al. 1998). These findings disagreed with those of Albin and colleagues who did not detect CYP2C in normal breast or breast tumours (Albin et al. 1993). All four CYP2C isoforms were investigated in the current study. Unfortunately, no CYP2C9 or CYP2C19 mRNA was detected in the eight cell lines, while the expression of CYP2C8 and CYP2C18 mRNA was detected in the ER-positive Caucasian and ER-negative Afro-Caribbean breast cancer cell lines. These preliminary findings suggest potential ethnic differences in the relationship between the expression of CYP2C8/18 mRNA and ER status should be taken into consideration in the development of novel cancer therapeutics. CYP2C18 mRNA

expression was also observed in the normal breast MCF10A cells, a result which differs from the finding of Thomas and colleagues (Thomas et al. 2006).

CYP2D6 catalyses the N-demethylation and 4-hydroxylation of tamoxifen which have potent antioestrogen effects. Tamoxifen is widely used in the treatment of breast cancer (Coller et al. 2002). Previous studies demonstrated that CYP2D6 mRNA was expressed in normal breast as well as breast tumour samples (Huang et al. 1996, Hellmold et al. 1998, Iscan et al. 2001, Bièche et al. 2007). In addition, Hellmond and colleagues demonstrated CYP2D6 protein expression in normal breast (Hellmold et al. 1998). These findings are in disagreement with those of Modugno and co-workers, who did not detect CYP2D6 mRNA expression in breast tumour and normal adjacent breast tissue using quantitative RT-PCR (Modugno et al. 2003). The present study is the first to examine the expression of CYP2D6 mRNA in breast cancer cell lines. An initial experiment using the primer sequence designed for CYP2D6 by Primer3 (Table 2.6) failed to detect CYP2D6 mRNA in the test cell lines. However, the previously published CYP2D6 primer set (Table 2.7) was employed to confirm the expression of CYP2D6 mRNA and this primer set successfully demonstrates a cDNA product of CYP2D6 mRNA in the eight cell lines.

In the preliminary work, CYP2D6 mRNA was detected in all four ER-positive breast cancer MCF7, T47D, ZR-75-1 and ZR-75-30 cells, suggesting that a local tamoxifen metabolism mediated by CYP2D6 in these cells may be of particular interest in the development of hormonal therapy for breast cancer. It is known that approximately 35% of ER-positive breast cancer patients do not respond to tamoxifen therapy and some patients who do respond to tamoxifen eventually become resistant to this treatment (Osborne 1998, Jin et al. 2005).

In addition, preliminary results showed that the presence of CYP2D6 mRNA is detected with the appearance of smearing bands in the two Afro-Caribbean breast cancer cells lines MDA-MB-468 and ZR-75-30 (Figure 4.27, lanes 2 and 9). This is likely to be related to the presence of a splice variant of this gene as currently more than eighty alleles have been identified for the CYP2D6 gene. Variant alleles of the CYP2D6 gene may lead to increased, decreased or absent functionality of the CYP2D6 gene (Klein et al. 2007). The activity of CYP2D6 is extremely variable, and inter-ethnic variations in drug metabolisms are well recognised. Poor metabolisers (PMs) lacking CYP2D6 activity have been found in 7% of Caucasians and 1-3% of other ethnic groups, whereas ultra-rapid metabolisers (UMs) displaying unusually high activity of CYP2D6 have been observed in 29% of people from North Africa and the Middle East (de Leon et al. 2006a). Unfortunately, information on the specific country of origin of the breast cancer cells used in the current study is not available. However, these findings suggest that the breast cancer cell line models may be of particular relevance in the development of cancer therapy for Afro-Caribbean breast cancer patients.

Unlike the MDA-MB-468 and ZR-75-30 cells, CYP2D6 mRNA was observed with smearing in the normal breast MCF10A cells derived from a Caucasian woman. This data is in agreement with the original study (Thomas et al. 2006) using the same primers but Thomas and co-workers detected a fainter expression. This difference may be due to the use of an annealing temperature and concentration of MgCl₂. The current project used a lower annealing temperature (55°C) and 1.5 mM MgCl₂ whereas Thomas and co-workers employed an annealing temperature at 60°C and 5 mM MgCl₂.

CYP2F1 is predominantly expressed in human lung, with low level or no expression being detected in the liver or other extrahepatic tissues (Carr et al. 2003, Tournel et al. 2007a).

CYP2F1 is involved in the activation of some pneumotoxicants including naphthalene and

3-methylindole (Karthan and Yost 2008). The current project is the first to examine expression of CYP2F1 mRNA in a series of breast cancer cell lines. No CYP2F1 mRNA was observed in the normal breast MCF10A cells whereas the original study demonstrated weak expression of CYP2F1 in sub-confluent but not confluent MCF10A cells (Thomas et al. 2006). This finding is in disagreement with preliminary work from the present investigation (see discussion in following section; CYP2S1).

CYP2F1 was detected only in the ER-positive Caucasian ZR-75-1 cells and the ER-negative Afro-Caribbean MDA-MB-468 cells. Recently, Tournel and colleagues (Tournel et al. 2007b) demonstrated inter-ethnic variations in CYP2F1 genetic polymorphism, and results from their studies suggest that a higher frequency of poor metabolisers occur in Afro-Caribbean populations (i.e. Gabonese and Senegalese) compared to French Caucasians and Tunisians. Although this association was derived from healthy volunteers, it may be of interest to assess inter-ethnic variation in cancer patients including those with breast cancer.

CYP2J2 is expressed in various normal tissues (i.e. liver, small intestine, colon and kidney) but higher levels occur in the coronary endothelial cells and cardiac myocytes (Wu et al. 1996). CYP2J2 is involved in the metabolism of arachidonic acids to form epoxyeicosatrienoic acids (EETs) which are which are potent endogenous vasodilators and inhibitors of vascular inflammation (Node et al. 1999, Scarborough et al. 1999). The physiological function of CYP2J2 in xenobiotic metabolism remains unknown.

Preliminary work showed high expression of CYP2J2 mRNA in all breast cancer cell lines but at a very low level in the invasive Caucasian breast cancer MDA-MB-231 cells. Weak expression of CYP2J2 mRNA was detected in the normal breast MCF10A cells which is in agreement with the original investigation (Thomas et al. 2006) using this primer set.

CYP2J2 mRNA in breast cancer cell lines has not been previously reported, but Jiang and co-workers detected higher levels of CYP2J2 mRNA and protein in breast cancer than in their corresponding normal tissues (Jiang et al. 2005). These findings suggest that CYP2J2 mRNA is specifically expressed in breast cancer cells and may be involved in breast carcinogenesis. It is important to further investigate the function of CYP2J2 in these cell lines prior to using them as models in breast cancer research.

Many research groups reported the expression of CYP2S1 mRNA and protein in several extrahepatic tissues (Choudhary et al. 2005, Karlgren et al. 2005, Saarikoski et al. 2005, Rivera et al. 2007), but it has not been previously reported in normal breast tissues or breast tumour samples. One previous study has demonstrated CYP2S1 mRNA in sub-confluent normal breast MCF10A cells but not in confluent cultures (Thomas et al. 2006), and this finding is in disagreement with the present study. The MCF10A cells used in this project were grown under the same conditions as the previous study (Thomas et al. 2006), and approximately 70-80% confluent MCF10A culture was employed to determine CYP2S1 mRNA. The observed differences in CYP2S1 mRNA expression in MCF10A between the two research groups could be attributable to differences in PCR conditions (i.e. RNA isolation method, primer sequences and MgCl₂ concentration) and primer sequences used. The present study is the first to report expression of CYP2S1 mRNA in a series of breast cancer cell lines, and it was detected in all cancer cell lines except for the ER-positive Afro-Caribbean ZR-75-30 cells.

CYP2S1 has several characteristics similar to members of the CYP1 family (i.e. CYP1A1 and CYP1B1). A recent study has reported that induction of CYP2S1 mRNA by dioxin and hypoxia is mediated by AhR/ARNT and HIF-1 α /ARNT complex pathway respectively (Rivera et al. 2007). In addition, CYP2S1 is known to be induced by retinoic acid (Smith et al. 2003), naphthalene (Karlgren et al. 2005) and PAHs (Rivera et al. 2002). There is no

information available on crosstalk between these receptors and CYP2S1 expression in breast cancer cells. However, the current study demonstrated the presence of AhR, ARNT, HIF-1 α mRNA in all the test cell lines and this could facilitate a better understanding of the role of CYP2S1 in cancer development and cancer treatment, following confirmation of CYP2S1 protein expression in these cell lines.

Previous studies demonstrated that CYP2S1 protein was associated with poor prognosis of colorectal tumours (Kumarakulasingham et al. 2005), and an increased expression of CYP2S1 protein was found in metastasis disease compared with primary ovarian cancer (Downie et al. 2005). In the present study, CYP2S1 mRNA was detected in the ER-positive/negative and the non-invasive/invasive phenotype.

When detecting CYP2S1 by RT-PCR, a faint band was observed above 216 bp in lane 5 (Figure 4.10). The presence of this non-specific amplification product may have been caused by excessive template cDNA. Importantly though, the expected product size of 216 bp representing CYP2S1 mRNA was seen as a clear band, and this was confirmed by restriction digest (Figure 3.18) and DNA sequencing (Figure D.1) during the validation step in this study.

Another novel extrahepatic cytochrome P450, CYP2U1, is highly expressed in human thymus and brain (Karlgen et al. 2004). Chuang and colleagues demonstrated that CYP2U1 plays a role in the metabolism of arachidonic acid and other long chain fatty acids (Chuang et al. 2004). CYP2U1 converts arachidonic acid into 19- and 20-hydroxyeicosatetraenoic acids (HETEs). The 20-HETE is potent vasoconstrictor in the cerebral circulation, and this indicates that CYP2U1 may control the brain blood flow (Karlgen et al. 2005). In addition, an association between the expression of CYP2U1 and tumour tissues has been shown by two studies and the findings suggest the same trend.

Currently, the role of CYP2U1 in the metabolism of xenobiotic compounds remains unknown. CYP2U1 protein in ovarian and colorectal tumours was higher than in normal ovary (Downie et al. 2005) and colon tissues (Kumarakulasingham et al. 2005) respectively. This may indicate that CYP2U1 plays a role in the carcinogenic process in certain types of tumour.

To date, CYP2U1 expression in breast cancer cells has not been demonstrated.

Preliminary results from the current study showed that CYP2U1 mRNA was detected in both the normal breast and breast cancer cell lines. CYP2U1 mRNA in the MCF10A cells confirmed the findings of a previous study (Thomas et al. 2006). With the exception of the ZR-75-30 cells, this enzyme was expressed at a lower level in MCF10A than in breast cancer cell lines. Preliminary results suggest that CYP2U1 mRNA in the breast cancer cells relative to the normal breast cells shows a similar trend in ovarian and colorectal cancer as mentioned above. The breast cancer cell line models could potentially be exploited in the development of novel cancer therapeutics and CYP2U1 could be used as a prognostic biomarker for breast cancer. This warrants further investigation.

The human CYP3A subfamily comprises four enzymes, CYP3A4, CYP3A5, CYP3A7 and CYP3A43. CYP3A4 plays an important role in the metabolism of steroid hormones (i.e. oestrogen and glucocorticoid) (Guengerich 1999) as well as xenobiotic compounds (i.e. paclitaxel, cyclophosphamide and doxorubicin) (McFadyen et al. 2004, Michael and Doherty 2005). The CYP3A genes are induced by glucocorticoids, phenobarbital and vitamin D (Pascussi et al. 2003). They are regulated by pregnane X receptor (PXR) which binds DNA as heterodimer with retinoid X receptor (RXR) and activates gene transcription. However, Pascussi and colleagues report that CYP2B6, CYP2C8 and CYP2C9 are also regulated by PXR. These observations suggest that the interaction

among PXR, CAR and VDR may occur after heterodimerisation with RXR as they share DNA response elements, partners and target genes (Pascussi et al. 2003).

Many research groups have investigated the expression of CYP3A in breast tumour tissues, but their findings seem to be inconsistent. For example, Iscan and colleagues found no CYP3A4 or CYP3A5 or CYP3A7 mRNA in normal breast or breast tumours (Iscan et al. 2001), whereas Huang and co-workers reported that CYP3A4 or CYP3A5 mRNA was higher in non-tumour breast tissues than in breast tumour samples (Huang et al. 1996). Hellmond and colleagues demonstrated the presence of CYP3A mRNA in normal breast too but they did not detect CYP3A protein in these tissues (Hellmold et al. 1998). These finding correlate well with those of Albin and co-workers who detected no CYP3A4 protein in breast tumours or normal adjacent breast tissues (Albin et al. 1993). Modugno and colleagues demonstrated CYP3A4 mRNA in normal breast as well as in breast tumour tissues, but they did not detect CYP3A5 mRNA in normal breast or breast tumour tissues (Modugno et al. 2003). The CYP3A43 gene is the most recently discovered in the CYP3A subfamily (Domanski et al. 2001) and the expression of CYP3A43 in breast cells has not been previously reported.

The present study failed to detect any members of the CYP3A subfamily in the panel of breast cancer cell lines. The lack of CYP3A mRNA expression is surprising as the CYP3A enzymes are involved in the metabolism of oestradiol and the activation of procarcinogens (e.g. aflatoxin B1, PAHs and NNK), CYP3A4 in particular (Guengerich 1999). However, a weak expression of CYP3A7 mRNA was found in the normal breast MCF10A cells, a finding which is in accordance with the results of Thomas and co-workers who investigated CYP3A mRNA in the MCF10A cells (Thomas et al. 2006).

CYP3A7 is a human foetal liver P450 isoform which plays a role in the hydroxylation of retinoic acid and the 16 α -hydroxylation of steroids, suggesting it may be relevant to human normal development and hormone dependent cancer (Daly 2006). However, a previous study showed that a high level of mRNA expression was detected in well differentiated hepatocellular carcinoma (Tsunedomi et al. 2005) and its protein expression was found to be associated with primary ovarian cancer when compared to normal ovary tissues (Downie et al. 2005).

Transcriptional expression of the CYP3A genes is regulated by PXR. The induction of CYP3A4 by PXR has been shown to play a crucial role in endometrial cancer. PXR ligands induce PXR expression, resulting in altered regulation of PXR target genes including CYP3A4 (Masuyama et al. 2003, Masuyama et al. 2005). In addition, a recent report claimed that down-regulation of PXR in the presence of the anticancer drug paclitaxel increased the inhibition of cell growth and apoptosis, leading to a higher sensitivity to anticancer drugs and the overcoming of drug resistance in the treatment of endometrial cancer (Masuyama et al. 2007). Although the present study did not detect CYP3A mRNA in the breast cancer cell lines used, it should be noted that PXR mRNA was found in the MCF7, T47D, ZR-75-1 and MDA-MB-157 cells. These findings suggest that the regulation of CYP3A genes in breast cancer cells may be involved in other signalling pathways rather than the PXR signalling pathway.

CYP24A1 (1,25-(OH)₂D₃-24-hydroxylase) is involved in the inactivation of vitamin D₃ and is regulated by vitamin D receptor (VDR). Recently, overexpression of CYP24A1 mRNA has been demonstrated in a variety of tumour tissues such as breast, prostate and lung (Cross et al. 2003). In addition, CYP24A1 mRNA has been shown to be associated with poor prognosis of oesophageal cancer by Mimori and co-workers (Mimori et al. 2004). These findings suggested that CYP24A1 expression plays a role in the carcinogenic

process and that CYP24A1 is a candidate oncogene (Anderson et al. 2006). Consequently, an inhibitor of CYP24A1 (EB1089) which has been demonstrated to have an antiproliferative effect on breast cancer has been developed and is currently in clinical trials (Sundaram et al. 2006).

Using real-time quantitative RT-PCR analysis, Fisher and co-workers detected the expression of CYP24A1 wild-type and splice variants in the normal breast MCF10A and breast cancer MCF7 cells; though an association between splice variants and CYP24A1 activity was not clearly established (Fisher et al. 2007). The detection of CYP24A1 mRNA in the MCF10A and MCF7 cells correlates well with results from the present study. CYP24A1 mRNA was detected in all the test cell lines except for the ER-positive ZR-75-30 and ER-negative MDA-MB-468 Afro-Caribbean breast cancer cell lines. It is important to note that ER status differences in CYP24A1 expression may be of particular relevance to the use of an inhibitor of CYP24A1 in breast cancer treatment. Regarding qualitative comparison, a correlation between CYP24A1 and VDR mRNA was observed in this research. The absence of CYP24A1 mRNA (Figure 4.16) agrees with the lack of VDR mRNA (Figure 4.23) in the ZR-75-30 and MDA-MB-468 cells.

In addition to metabolism of vitamin D₃ by cytochrome P450 enzymes, the conversion of the vitamin D₃ precursor into the active metabolite 1,25-(OH)₂D₃ is mediated by CYP2R1 and CYP27A1 which are predominantly expressed in human liver, and by CYP27B1 which is mainly expressed in the kidney (Cheng et al. 2003). Vitamin D₃ is believed to exhibit potent anticancer properties as it inhibits proliferation and stimulates apoptosis of cells. For this reason, enzymes involved in the activation and inactivation of vitamin D₃ are of particular relevance to cancer therapy. To date no information on the physiological role of CYP2R1 in xenobiotic metabolism has been reported. CYP2R1 mRNA is highly

expressed in human liver and testis but lower levels are found in other tissues (Karlgrén et al. 2005).

The preliminary work presented in this chapter is the first evidence of CYP2R1 mRNA expression in a panel of breast cancer cell lines compared with the normal breast cells. CYP2R1 mRNA in the normal breast MCF10A cells was also observed in this work and is in agreement with the original study (Thomas et al. 2006) using these primers. CYP2R1 mRNA was highly expressed in almost all breast cancer cell lines but lower levels were observed in the Afro-Caribbean breast cancer MDA-MB-468 and ZR-75-30 cells.

Regarding the breast cancer cells derived from Caucasian patients, CYP2R1 mRNA seems to be expressed at similar levels both in ER-positive and ER-negative phenotype, suggesting that CYP2R1 may be of interest as a potential target for chemotherapy in either an oestrogen-independent or an oestrogen-dependent breast tumour once protein expression is confirmed in these cell lines.

Members of the CYP26 family (i.e. CYP26A1 and CYP26B1) specifically catalyse the 4-hydroxylation of all *trans*-retinoic acid (ATRA) (Sonneveld et al. 1998). CYP26A1 is overexpressed in various tumours including breast tumours (Mira-Y-Lopez et al. 2000) and these findings have led to the use of a CYP26A1 inhibitor (R116010) in oestrogen-independent mouse breast tumours (Njar 2002). Previous studies demonstrated CYP26A1 mRNA in ER-positive T47D (Sonneveld et al. 1998) and MCF7 (van Heusden et al. 1998, Mira-Y-Lopez et al. 2000) and these findings are in agreement with the present study. The current study also detected CYP26A1 in all the cancer cell lines except the ZR-75-1 cells, suggesting that CYP26A1 may be specifically expressed in breast cancer cells and that the test cell lines could be used as models to investigate novel anticancer drugs.

Earlier studies have shown that CYP26B1 is predominantly expressed in human adult brain, particularly the cerebellum and cephalic tissues (White et al. 2000, Trofimova-Griffin and Juchau 2002). However, there is no information on CYP26B1 mRNA in normal breast or breast tumour tissues. The current investigation is the first to screen a panel of breast cancer cell lines and a normal breast cell line for the presence of CYP26B1 mRNA. The presence of CYP26B1 mRNA was detected in the normal breast MCF10A cells and all breast cancer cell lines except the ZR-75-30 cells. It is important to note that CYP26B1 mRNA expression was elevated in the normal breast and ER-negative breast cancer cells compared to the ER-positive breast cancer cell lines, suggesting that oestrogen may affect the expression of CYP26B1 mRNA.

The CYP26 genes are regulated by nuclear receptor retinoic acid receptor (RAR) and retinoid X receptor (RXR) which bind to RA response elements and regulate transcription of target genes (Nezzar et al. 2007). RXR is a common heterodimeric partner for several nuclear receptors (i.e. CAR, PXR, PPAR α and VDR) and crosstalk between these receptors leads to unexpected expression of cytochrome P450 enzymes (Section 1.7.9). In the present study, the expression of RAR and RXR mRNA was detected in both the normal breast and breast cancer cell lines; however, no correlation between RAR and RXR mRNA was observed regarding ER status, invasive phenotype or ethnicity. These findings suggest that any applications of the inhibition of CYP24 or CYP26 in cancer therapy must consider a significant function of their corresponding nuclear receptors and possible crosstalk between nuclear receptors in the same superfamily.

Transcriptional expression of some cytochrome P450 genes is regulated by their corresponding nuclear receptors (Section 1.7). However, the existence of intracellular crosstalk between these receptors appears to have important consequences on cytochrome P450 gene regulation as the receptors share heterodimeric partner, ligands, DNA response

elements and target genes. A study of coexpression of cytochrome P450s and their corresponding nuclear receptors in breast cancer cells may lead to a better understanding of how the event alters the cancer biology.

CYP4X1 mRNA is widely expressed in several human tissues (i.e. breast, ovary, uterus, liver and kidney, trachea and aorta). Savas and colleagues demonstrated that peroxisome proliferator activated receptor-alpha (PPAR α) is involved in the activation of human CYP4X1, as investigated through used of human hepatoma HepG2 cells that stably express a mouse PPAR α mutant. A physiological role of CYP4X1 is currently unknown but the consistently high levels of CYP4X1 in the aorta and trachea may suggest that CYP4X1 plays a role in the generation of arachidonic acid metabolites, compounds with vasoactive properties (Savas et al. 2005). Preliminary results showed that CYP4X1 mRNA was expressed in both the normal breast and breast cancer cell lines. The detection of CYP4X1 mRNA in the normal breast MCF10A cells is in agreement with a previous study (Thomas et al. 2006) using these primers, but it is more likely to be overexpressed compared with those of Thomas and co-workers. The expression of CYP4X1 mRNA has not been previously investigated in breast cancer cells. CYP4X1 mRNA was expressed in the seven breast cancer cell lines but at very high levels in the ER-positive Caucasian breast cancer T47D and the ER-negative Afro-Caribbean breast cancer MDA-MB-468 cells. This finding warrants further investigation.

Using real-time quantitative RT-PCR, Savas and co-workers reported that CYP4Z1 mRNA was higher in normal breast tissue than other human normal tissues (Savas et al. 2005). The study of Rieger et al. (2004) demonstrated that CYP4Z1 mRNA was overexpressed in breast tumours compared with the corresponding normal breast tissue. In addition, a previous study demonstrated CYP4Z1 in the normal breast MCF10A cells and this is in agreement with results from the present study (Thomas et al. 2006). In breast cancer cell

lines, CYP4Z1 mRNA was detected in the MCF7 (Rieger et al. 2004, Savas et al. 2005) and T47D cells (Savas et al. 2005). The detection of CYP4Z1 mRNA in the T47D cells is in agreement with results from the present study, though this is not the case for the MCF7 cells. This discrepancy may be attributable to the different conditions used for cell culture and the sensitivity of the RT-PCR method. However, results from studies examining the expression of CYP4Z1 protein in breast cancer cells generally appear to be inconsistent. Rieger and colleagues detected CYP4Z1 protein in MCF7 cells (Rieger et al. 2004) but Savas and co-workers reported no CYP4Z1 protein expression in MCF7 (Savas et al. 2005).

The present study also demonstrated CYP4Z1 mRNA in the ZR-75-1 and MDA-MB-468 cells and this has not been previously reported. These findings suggest that CYP4Z1 mRNA is more likely to be specifically expressed in the non-invasive phenotype of breast cancer cells. A higher level of CYP4Z1 expression was observed in the ER-negative Afro-Caribbean MDA-MB-468 breast cancer cell line than in the ER-positive Caucasian T47D and ZR-75-1 breast cancer cells and this warrants further investigation.

Prior to further elucidation of the sequence, the present investigation confirmed the expression of CYP4Z1 mRNA in all the cDNA samples, using previously published primers (Thomas et al. 2006) that had been designed to cross between exon 9 and exon 12 boundaries (Figures 4.14 and 4.15). This primer set successfully demonstrates a single product of CYP4Z1 mRNA in the test cells. The presence of CYP4Z1 mRNA in the normal breast MCF10A cells is in agreement with the original study (Thomas et al. 2006) using this primer set. The presence of CYP4Z1 mRNA in the MCF10A, T47D, ZR-75-1 and MDA-MB-468 cells using primers designed by Primer3 is also in agreement with the original study (Figure 4.13). In addition, CYP4Z1 mRNA was observed with a faint band in the ZR-75-30 cells (Figure 4.14).

In summary, Chapter 4 has examined the profile of cytochrome P450 mRNA expression in a panel of breast cancer cell lines compared to that of a normal breast cell line. One of the target genes in this study, CYP4Z1, is a novel isoform of the CYP4 family and has been associated with metastatic disease and poor prognosis of specific types of tumours (Downie et al. 2005). Overexpression of CYP4Z1 has also been found in breast cancer (Rieger et al. 2004), though the mechanism of gene regulation and substrate-specificity are still unclear. The presence of two bands at 159 bp and ~250 bp in the CYP4Z1 RT-PCR warrants further investigation and this will be carried out in Chapter 5.

Chapter 5

Characterisation of CYP4Z1 mRNA in Breast Cancer Cells

5.1 Introduction

The previous two chapters of this study (Sections 3.3.3.4 and 4.3.6) detected CYP4Z1 amplification products by RT-PCR. Results with these primers showed a band of ~250 bp in addition to a band of the correct product size (159 bp). A similar pattern of two bands was found in four of the eight cell lines (i.e. MCF10A, T47D, ZR-75-1 and MDA-MB-468). Purified DNA of both fragments was further investigated by restriction digestion and DNA sequencing. It is important to note that the results of the restricted fragment sizes (Figure 3.21) and the BLAST analysis of DNA sequences did not distinguish between the fragment of 159 bp and ~250 bp. Further elucidation of the sequence is required to identify the source of the additional band. The reproducibility of the presence of this PCR product ruled out a non-specific product, and raises the possibility that alternate splicing of the gene is producing variations in the size of the mRNA.

Alternative splicing is one of the regulatory mechanisms that contribute to variation in the incorporation of coding regions (exons) into messenger RNA, leading to the production of protein. It is well-known that alternative splicing is of great importance to genetics because a splicing error can disrupt the open reading frame of mRNA, and this will generate different mature RNA encoding distinct protein products from a single gene.

There are four common types of gene splicing event (i.e. exon skipping, intron retention, alternative 5' site and alternative 3' site; Figure 5.1) and these can occur in the genes after the transcriptional process (Ferreira et al. 2007). In human cells, alternative splicing has been estimated in 40-60% of all genes, and more than 70% of alternative splicing generates significant functional diversity (Modrek and Lee 2002, Stamm et al. 2005).

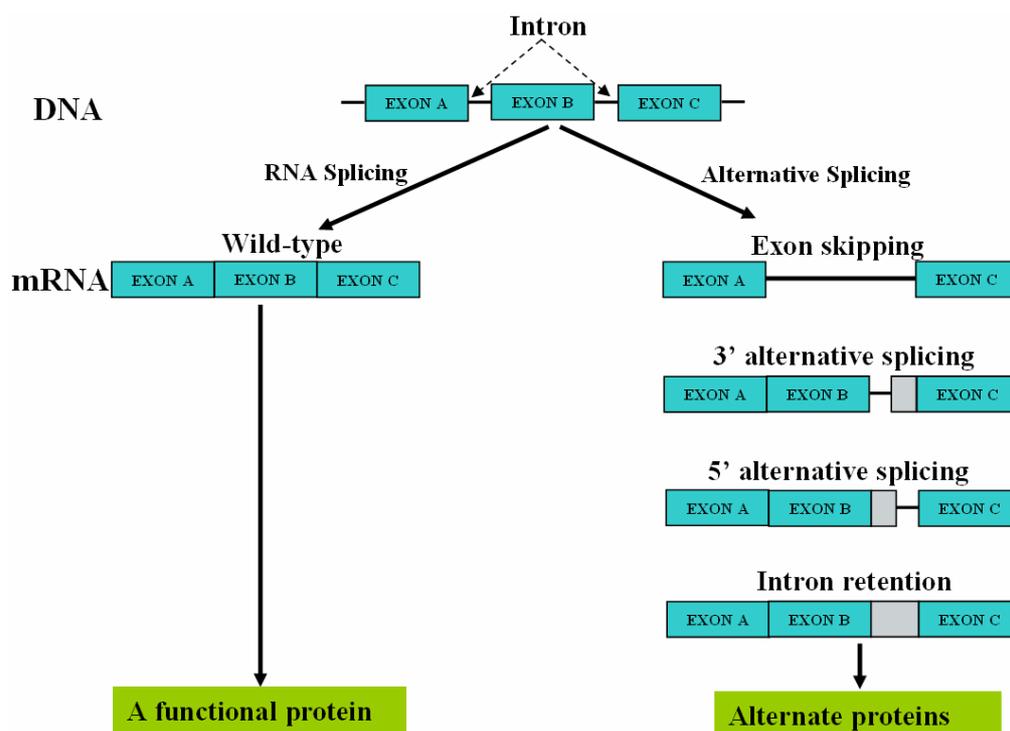


Figure 5.1: Patterns of alternative splicing. Constitutive and alternative exons are indicated by blue and grey boxes respectively and introns are shown by straight lines (Adapted from Ferreira et al. 2007).

Splicing errors in human genes appear to be associated with genetic polymorphisms which result in the alteration of signalling/enzymatic activities in several key genes involved in drug metabolism including cytochrome P450 enzymes (Stamm et al. 2005). CYP4F3 plays an important role in the metabolism of leukotriene B4 and arachidonic acid. Christmas and colleagues (Christmas et al. 2001) reported that alternative splicing events of CYP4F3 influence substrate-specificity, and tissue-specific alternative splicing of pre-mRNA has also been demonstrated. There are two isoforms of alternative splice variants of human CYP4F3. CYP4F3A was predominantly expressed in neutrophils, bone marrow and peripheral blood leukocytes while CYP4F3B was preferentially expressed in liver and kidney. With regard to catalytic activity, higher activities for arachidonic acid or leukotriene B4 were found in CYP4F3B than in CYP4F3A, indicating that alternative splice variants of CYP4F3 generate functional diversity of enzymes.

An alternative splicing of the CYP1A1 gene that results in deletion of 87 bp of exon 6 (exon 6 del CYP1A1) was detected in human brain but not in liver. This splice variant does not metabolise polycyclic aromatic hydrocarbon to genotoxic metabolites that causes formation of DNA adducts in the human brain, and therefore differs from wild-type CYP1A1. These observations suggest that splice variants play an important role in the metabolism of procarcinogens (Kommaddi et al. 2007). This effect may contribute to other CYP1A1 substrates including anticancer drugs.

Evidence of a link between alternative splice variants of some cytochrome P450 genes and a variety of cancers has emerged. Leung and co-workers detected overexpression of CYP1A1 protein and its splice variant (CYP1A1v) in ovarian cancer cells and found they can metabolise procarcinogens such as 17 β -oestradiol and benzo(a)pyrene. This suggests that the splice variant of CYP1A1 may be involved in both the initiation and progression of ovarian cancer (Leung et al. 2005). An alternative splicing of the CYP27B1 gene has been shown to be associated with breast cancer cells (Cordes et al. 2007) and endometrial cancer cells (Becker et al. 2007). These findings suggest that splice variants may be involved in the initiation and progression of specific types of tumours.

In the CYP4Z1 gene, no information on its alternative splicing has previously been reported although Rieger and colleagues found a truncated version of CYP4Z1 a pseudogene CYP4Z2P with 96% identity to CYP4Z1. Currently, there are two studies that have demonstrated a specific amplification product of CYP4Z1 mRNA using qualitative RT-PCR. Figure 5.2 highlights the structure of the CYP4Z1 gene and shows the positions of the primers used in this study and previous studies using a physical mapping of the CYP4Z1 genome (GenBank accession number: NC_000001; Human Genome Sequencing Consortium International 2004). Thomas and co-workers (Thomas et al. 2006) employed specific primers against CYP4Z1 mRNA, designed to cross between exon 9 and exon 12

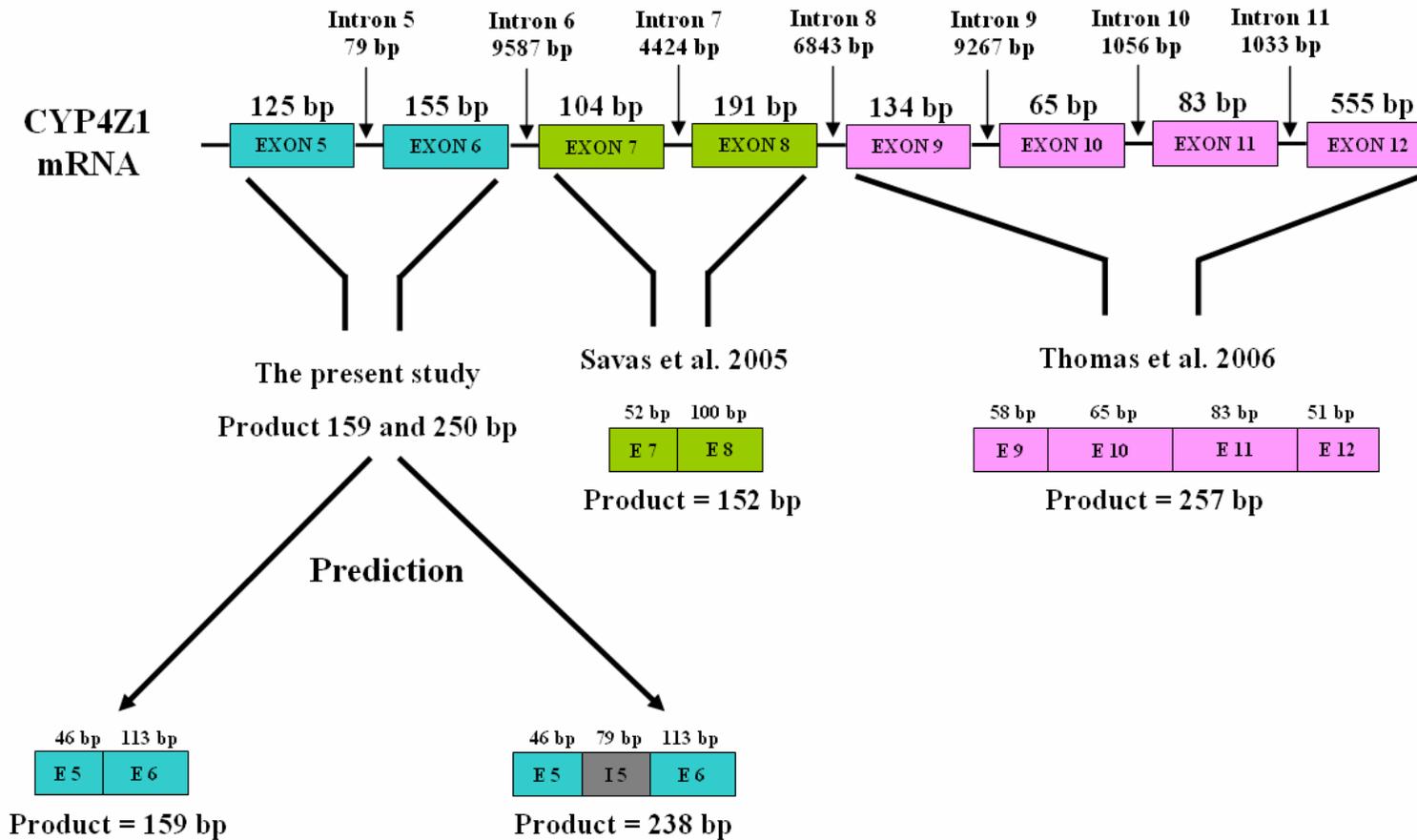


Figure 5.2: Schematic view of the structure of the CYP4Z1 gene showing the positions of the primers in this study and previous studies.
 Abbreviations: E; exon, I; intron

boundaries, whereas Savas and colleagues (Savas et al. 2005) used primers located crossing the exon 7 to exon 8 boundaries of the CYP4Z1 gene. These two separate studies detected CYP4Z1 mRNA in the normal breast MCF10A cells (Thomas et al. 2006), human normal tissues (i.e. breast, liver and kidney) and the breast cancer cell lines (i.e. MCF7 and T47D) (Savas et al. 2005). Both found a single band of the CYP4Z1 amplification product.

By contrast, the present study designed the CYP4Z1 primer set F1&R1 (Figure 5.3), crossing from exon 5 to exon 6 boundaries and detected the presence of two bands (159 bp and ~250 bp). These observations would suggest an alternative splicing event particularly intron retention in CYP4Z1. Prior to elucidation of the sequence, the previous chapter (Section 4.3.6) confirmed the expression of CYP4Z1 mRNA in all the cDNA samples using the previously published CYP4Z1 primer (Thomas et al. 2006). The primer set successfully demonstrates a single PCR product of CYP4Z1 mRNA in the test cells. The presence of CYP4Z1 mRNA in the normal breast MCF10A cells is in agreement with the original study (Thomas et al. 2006) using this primer set. The aims of this chapter were to identify the mRNA sequences (exon 5-6) and further elucidate the two bands at 159 bp and ~250 bp.

5.2 Materials and Methods

5.2.1 Qualitative and nested RT-PCR of CYP4Z1 mRNA

To further elucidate the sequence between exon 5 and exon 6 boundaries, the purified DNA of 159 bp and ~250 bp fragments (i.e. T47D, ZR-75-1 and MDA-MB-468), cDNA samples (i.e. MCF10A, T47D, ZR-75-1 and MDA-MB-468) and the first PCR products were used as template DNA for PCR reactions. It is important to note that the MCF10A cells were excluded from the experiments which required the use of purified DNA

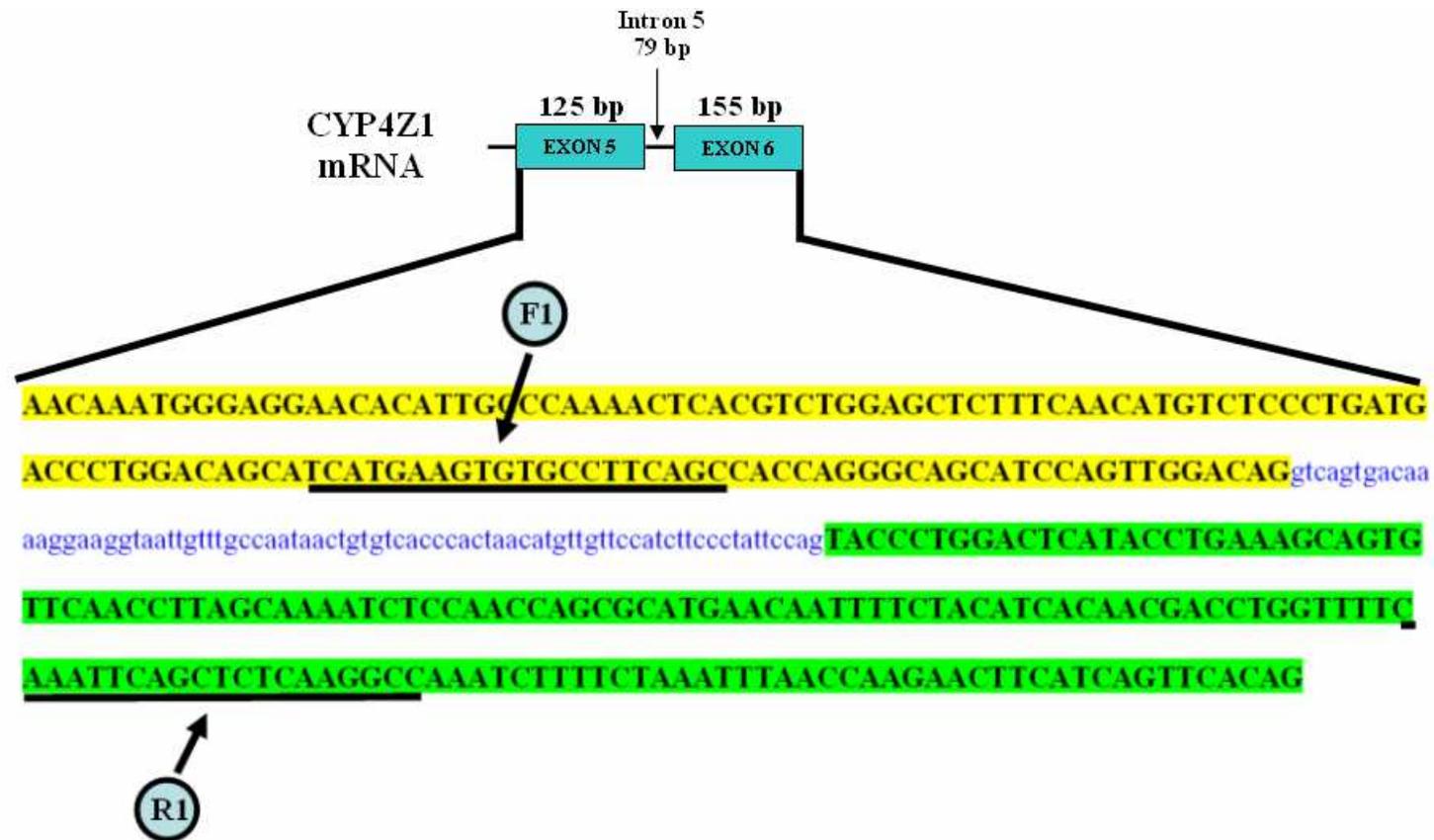


Figure 5.3: The sequence of exon 5-intron 5-exon 6 boundaries of the CYP4Z1 gene showing the position of the primer set F1&R1 (Table 2.6). Exon sequences are shown in upper cases and intron sequences in lower case letters. The sequences in exon 5 and exon 6 are indicated in yellow and green respectively. Abbreviations: F1; forward primer 1, R1; reverse primer 1

fragments as templates because CYP4Z1 mRNA in this cell line was expressed at a very low level and it was very difficult to purify a DNA sample. Templates were subjected to the PCR protocols with four different pairs of primers (Table 5.1). The first protocol employed a set of primers F1&r2 that are located in the exon 5 (46 bp) and the intron-retained (79 bp), and used the purified DNA (Figures 5.4 and 5.5) and cDNA samples as template (Figure 5.7). To amplify the intron 6-retained, the second PCR protocol was a nested PCR, using a pair of primers Ff2&r2 covering the exon 5 (10 bp) and the intron-retained (79 bp) and the first PCR product of the first protocol as template (Figure 5.6). The third protocol used primers F3&R3 that are located in the beginning of exon 5 (118 bp) and the middle of exon 6 (123 bp), and employed the cDNA samples as template (Figure 5.8). The final protocol was a nested PCR using primers F3&r2 that cover the beginning of exon 5 (118 bp) and the intron-retained (79 bp), and employing the first PCR product of the third PCR protocol as template DNA (Figure 5.9). The specificity of the primer sequences used was confirmed by BLAST analysis. The first PCR and nested PCR were performed under the conditions previously described (Tables 2.3, 2.4 and 2.8).

5.2.2 Purified DNA and DNA sequencing

PCR product was purified using the high pure PCR product purification kit, and the purified DNA samples were sent to the Rowett Research Institute for sequence analysis using automated dideoxy DNA sequencing (Section 2.10, Appendix D; Figure D.3). The acceptable A_{260}/A_{280} ratio of DNA extracted sample was between 1.8 and 2.1.

Table 5.1: Primer sequences for CYP4Z1 used to elucidate the sequence between exon 5 and exon 6.

Protocol	Name	Primer	Position	Sequence (5' to 3')	Length	T _m (°C)	T _a (°C)	Product size (bp)
1	CYP4Z1 (F1&r2)	Forward (F1)	Exon 5	TCATGAAGTGTGCCTTCAGC	20	64.1	55	125
		Reverse (r2)	Intron 5	ctggaataggaagatggaa	20	60.4		
2	CYP4Z1 (F/f2&r2)	Forward (F/f2)	Exon 5/Intron 5	AGTTGGACAGgtcagtgaca	20	60.8	55	89
		Reverse (r2)	Intron 5	ctggaataggaagatggaa	20	60.4		
3	CYP4Z1 (F3&R3)	Forward (F3)	Exon 5	GGGAGGAACACATTGCCCA	19	68.3	60	241
		Reverse (R3)	Exon 6	GAAAAGATTTGGCCTTGAGAGC	22	64.9		
4	CYP4Z1 (F3&r2)	Forward (F3)	Exon 5	GGGAGGAACACATTGCCCA	19	68.3	55	197
		Reverse (r2)	Intron 5	ctggaataggaagatggaa	20	60.4		

Note: Exon sequences are shown in capital letters and intron sequences in lower case letters

5.3 Results

5.3.1 Identification of CYP4Z1 splice variants in the cell lines

Initial experiments were performed using the primer set CYP4Z1 F1&R1, and the results showed fragments of 159 bp and ~250 bp in the amplification product of CYP4Z1 RT-PCR (Figures 3.20 and 4.13). Restriction digestion and DNA sequencing confirmed both of these products as CYP4Z1. Prediction of PCR products from whole genomic sequences (Figure 5.2) was a PCR product of 159 bp generated from an intron free mRNA (exon 5 (46 bp)/exon 6 (113 bp)) and a product of ~250 bp obtained from a retained intron mRNA (exon 5 (46 bp)-intron 5 (79 bp)-exon 6 (113 bp)).

The first PCR protocol (primer set CYP4Z1 F1&r2) was designed to investigate the exon 5 and a retained intron using the purified 159 bp and ~250 bp DNA fragments. First of all, purified 159 bp and ~250 bp DNA fragments from the T47D cells were amplified at different annealing temperatures (i.e. 55, 58 and 60°C) under the same PCR conditions. The purpose of this was to investigate the effect of annealing temperature on the specificity of primers. After varied temperature annealing, a single product of retained intron (125 bp) was detected in both purified fragments, and 55°C was selected as the optimum annealing temperature for CYP4Z1 F1&r2 amplification as DNA bands appeared sharp and clear (Figure 5.4). This annealing temperature would be employed for experiments with MDA-MB-468 and ZR-75-1 cells. Similarly, the expected size of 125 bp was found in the purified DNA of 159 bp and ~250 bp from the MDA-MB-468 and ZR-75-1 cells (Figure 5.5). However, the 125 bp product was expected only for the purified DNA of ~250 bp to confirm intron retention event in the CYP4Z1 gene and not for the purified DNA of 159 bp. These results were different to those expected (Figure 5.5A).

To further identify the complete intron 5 sequences, the second PCR protocol was performed using the first PCR products (125 bp) obtained from T47D, MDA-MB-468 and

ZR-75-1 cells as templates, and the primer set CYP4Z1 F/f2&r2 used as nested primers. A product size of 89 bp was expected for nested PCR obtained from the purified DNA of ~250 bp fragment. The results showed the presence of the expected product size (89 bp) but the product size of primary template (125 bp) was also detected in all PCR products. It should be noted that a fragment of ~250 bp was detected in the purified 159 bp DNA fragment derived from the ZR-75-1 cells (Figure 5.6). These observations suggest that there may have been too much template in the PCR reaction.

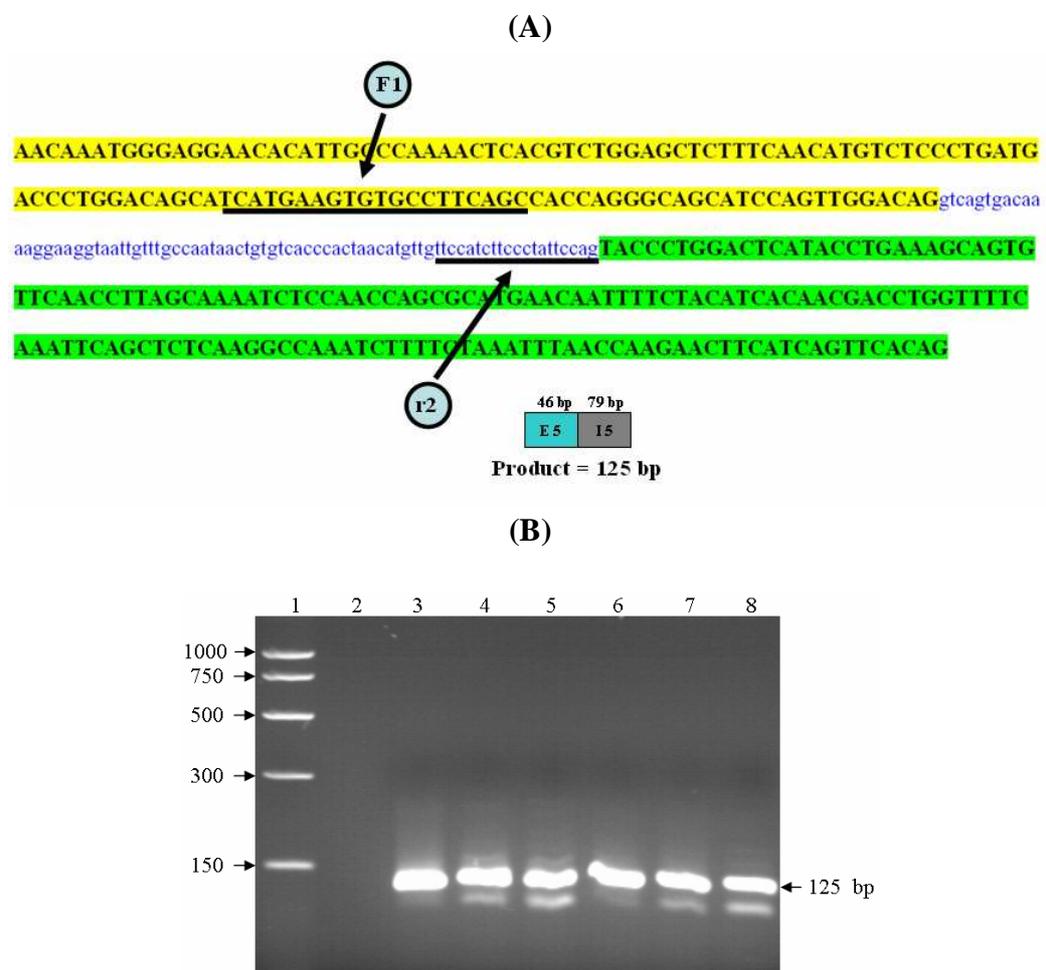


Figure 5.4: The first protocol (a pair of primers F1&r2) used purified DNA as templates. (A) The position of the primers F1&r2 in the CYP4Z1 gene and the expected product size. (B) Illustration of the effect of annealing temperature on the primer F1&r2 RT-PCR product in the T47D cells. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lanes 3-5: purified DNA of 159 bp, Lanes 6-8: purified DNA of 250 bp, Lanes 3&6: 55°C, Lanes 4&7: 58°C, Lanes 5&8: 60°C

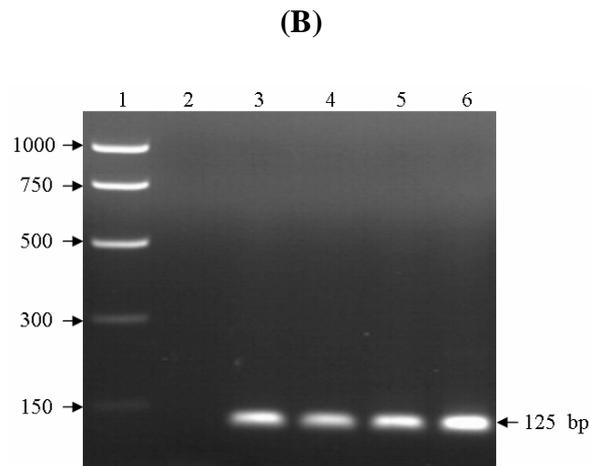
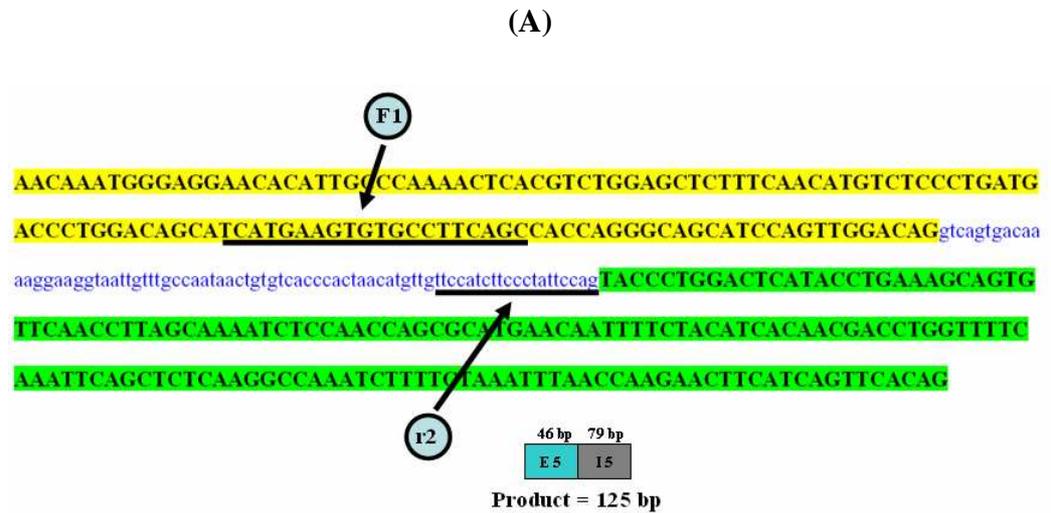


Figure 5.5: The first protocol (a pair of primers F1&r2) used purified DNA as templates. (A) The position of the primers F1&r2 in the CYP4Z1 gene and the expected product size. (B) CYP4Z1 F1&r2 RT-PCR in the ZR-75-1 and MDA-MB-468 cells. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lanes 3-4: ZR-75-1, Lanes 5-6: MDA-MB-468, Lanes 3&5: purified DNA of 159 bp, Lanes 4&6: purified DNA of 250 bp.

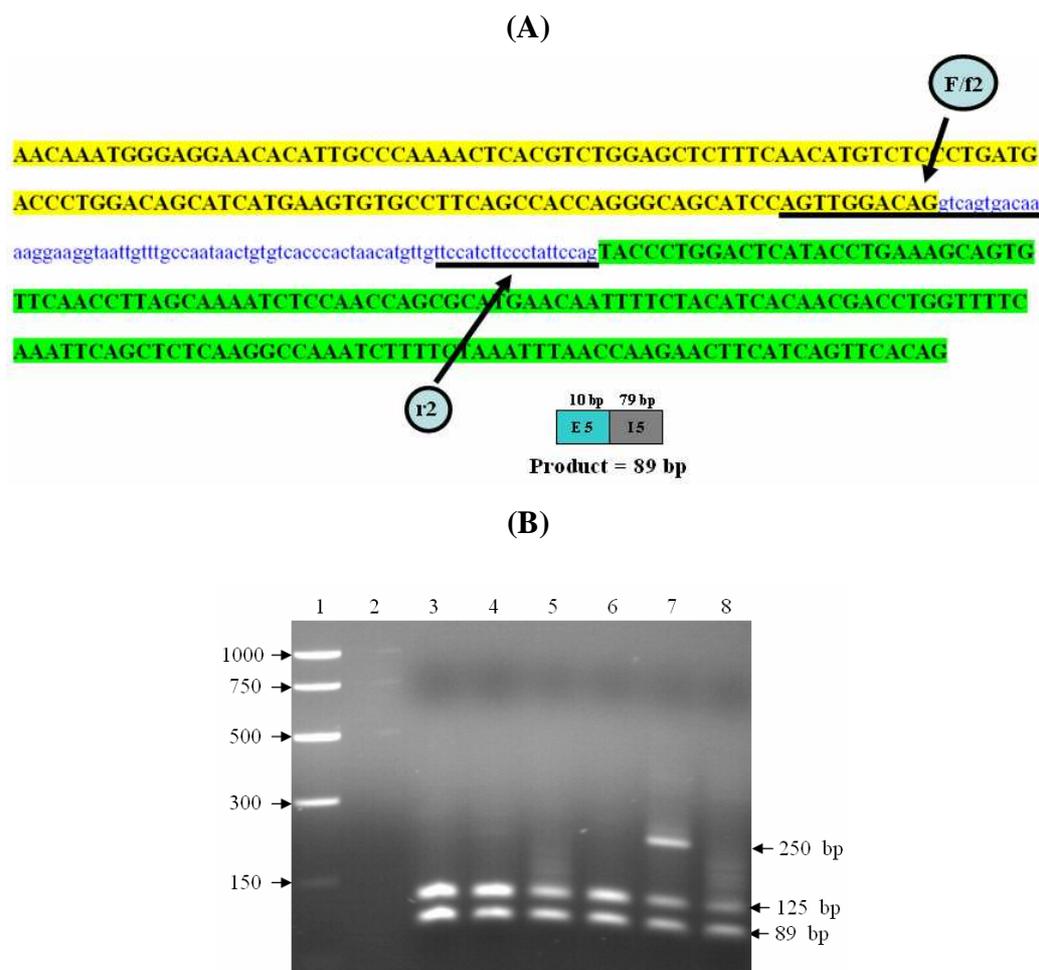


Figure 5.6: The second protocol (a pair of primers F/f2&r2) used the first round PCR product of the first protocol as templates. (A) The position of the primers F/f2&r2 in the CYP4Z1 gene and the expected product size. (B) Nested PCR CYP4Z1 F1&r2 RT-PCR using the primer set CYP4Z1 F/f2&r2. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lanes 3&6: MDA-MB-468, Lanes 4&7: T47D, Lanes 5&8: ZR-75-1, Lanes 3-5: purified DNA of 159 bp, Lanes 6-8: purified DNA of 250 bp.

In addition, the original cDNA samples isolated from the T47D, MDA-MB-468 and ZR-75-1 cells were also used with the first PCR protocol (primer set CYP4Z1 F1&r2). The expected product size of this protocol was 125 bp and this was detected, but there were also faint DNA fragments present at 159 bp, ~250 bp and ~450 bp in all cDNA used (Figure 5.7). The occurrence of non-specific amplification products may be due to low specificity of primers, although the BLAST analysis showed that the primers were specific for the CYP4Z1 gene.

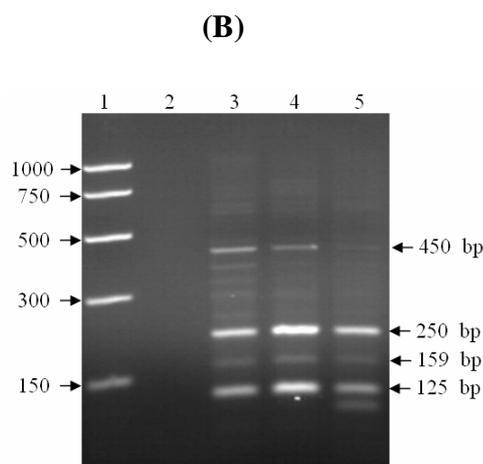
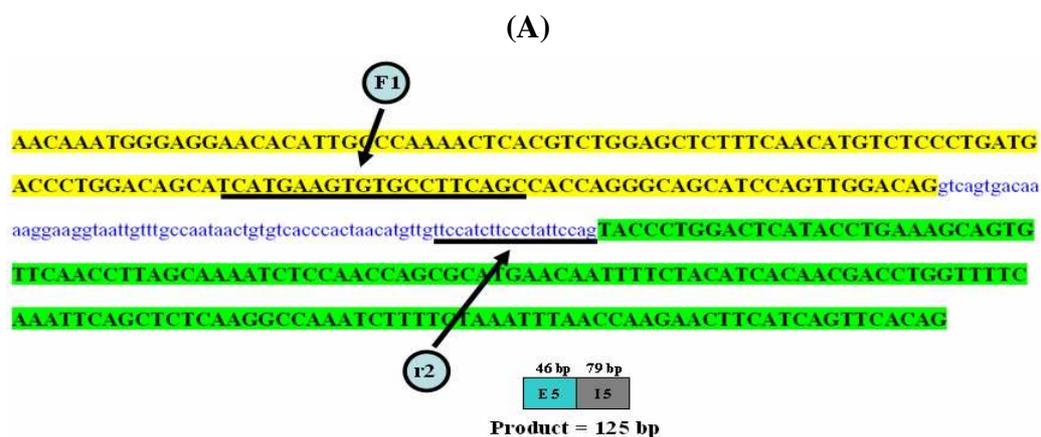


Figure 5.7: The first protocol (a pair of primers F1&r2) used cDNA samples as templates. (A) The position of the primers F1&r2 in the CYP4Z1 gene and the expected product size. (B) CYP4Z1 F1&r2 RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: T47D, Lane 4: ZR-75-1, Lane 5: MDA-MB-468.

The third PCR protocol (a primer pair F3&R3) was designed to further identify the sequence between exon 5 and exon 6 boundaries which were at different positions to the original primers F1&R1. This primer set was amplified with the cDNA isolated from MCF10A, T47D, MDA-MB-468 and ZR-75-1 cells. The expected product sizes for intron free mRNA [exon 5 (118 bp)/exon 6 (123 bp)] and retained intron mRNA [exon 5 (118 bp)-intron 5 (79 bp)-exon 6 (123 bp)] were 241 bp and 320 bp fragments. However, the presence of 241 bp and 320 bp fragments was detected and an additional band of 520 bp band was also found in all cDNA samples (Figure 5.8). It should be noted that a band of 241 bp detected in the MCF10A cells was expressed at a very low level when compared to

those of the T47D, MDA-MB-468 and ZR-75-1 cells. Although the primer set failed to detect a single band of PCR product, it behaved in the same pattern as primer set F1&R1.

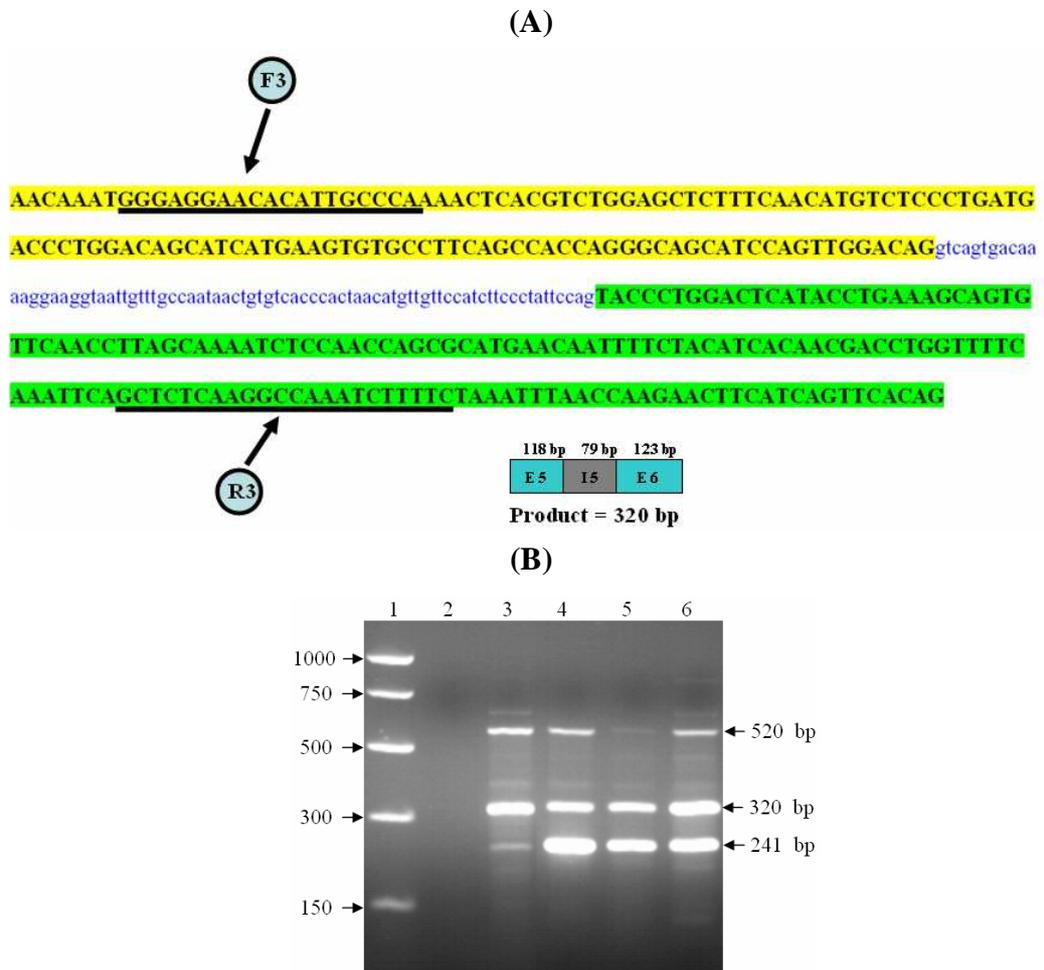


Figure 5.8: The third protocol (a pair of primers F3&R3) used cDNA samples as templates. (A) The position of the primers F3&R3 in the CYP4Z1 gene and the expected product size. (B) CYP4Z1 F3&R3 RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MDA-MB-468, Lane 5: T47D, Lane 6: ZR-75-1.

To further elucidate the retained intron sequence, the fourth PCR protocol was performed using the primary PCR products obtained from the third protocol as templates and primers F3&r2. The product size of 197 bp was expected for nested PCR but a band of 197 bp and the first PCR products used as templates (241 bp and 320 bp) were detected as well as a faint additional band of 520 bp (Figure 5.9).

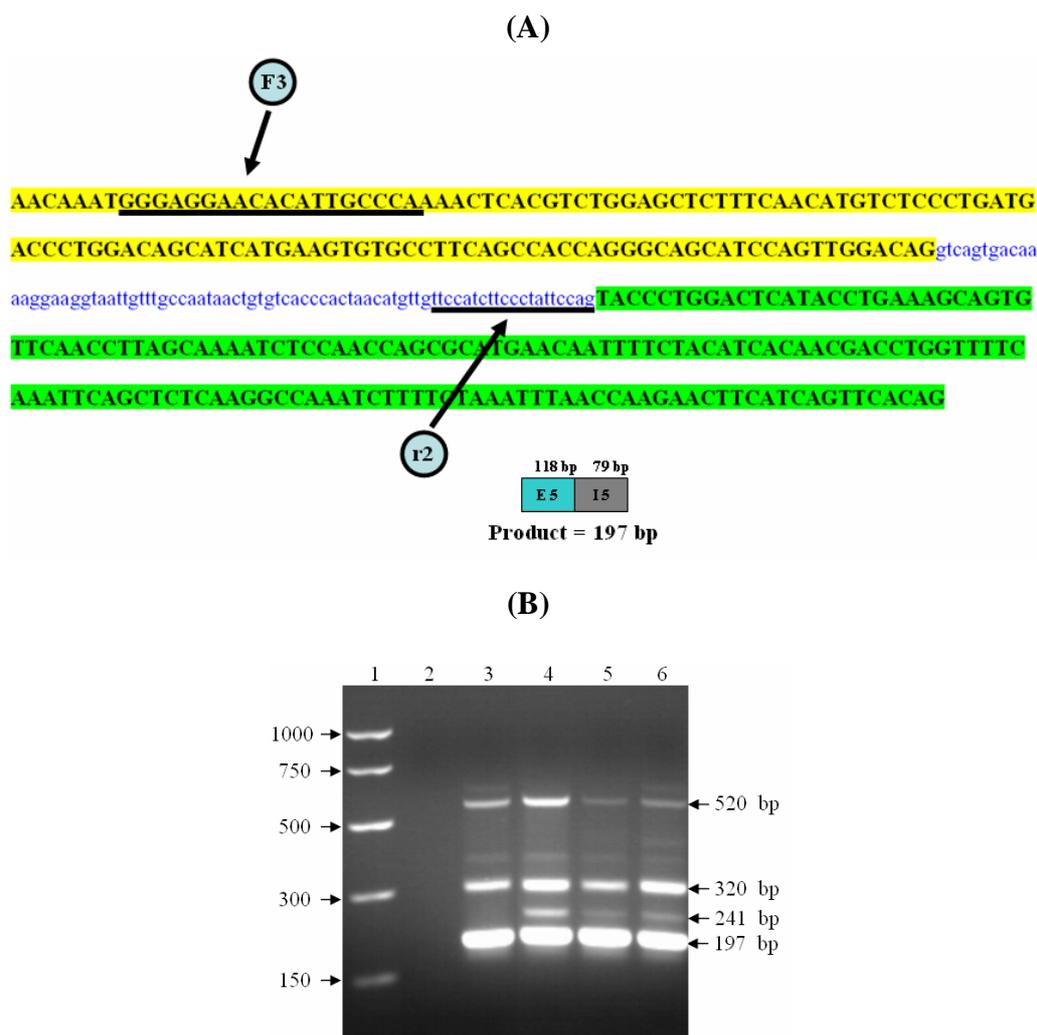


Figure 5.9: The fourth protocol (a pair of primers F3&r2) used the first round PCR product of the third protocol as templates. (A) The position of the primers F3&r2 in the CYP4Z1 gene and the expected product size. (B) Nested PCR CYP4Z1 F3&r3 RT-PCR using the primer set CYP4Z1 F3&r2. Nested PCR CYP4Z1 F3&r3 RT-PCR using the primer set CYP4Z1 F3&r2 using different templates. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MDA-MB-468, Lane 5: T47D, Lane 6: ZR-75-1.

5.4 Discussion

The investigation in this chapter provides the first evidence of alternative splicing pre-mRNA by intron retention in the CYP4Z1 gene. As mentioned in Section 5.1, alternative splicing events in cytochrome P450 enzymes, for example the CYP4F3 (Christmas et al. 2001) and CYP1A1 (Kommaddi et al. 2007) genes, are associated with some aspects of enzymatic activity such as substrate-specificity, catalytic activity and functional protein diversity. Tissue-specific alternative splice variants have also been shown. In addition,

alternative splicing events in cytochrome P450s such as CYP1A1 (Leung et al. 2005) and CYP27B1 (Becker et al. 2007, Cordes et al. 2007) have been demonstrated to play a key role in the carcinogenic process, suggesting that this could be a potential target for the development of cancer therapeutics. Alternative splicing in the CYP4Z1 gene has not been previously documented. The current study is the first to present the sequence between exon 5 and exon 6 boundaries and this does show potential alternative splicing intron retention in the normal breast MCF10A cells and the breast cancer T47D, MDA-MB-468 and ZR-75-1 cells.

The present study initially employed specific primers against CYP4Z1 mRNA designed to cross between the exon 5 and exon 6 boundaries that generated the fragments of 159 bp and ~250 bp in the amplification product of CYP4Z1 RT-PCR (Figures 3.20 and 4.13). These observations were required to further elucidate the sequence because restriction digestion and DNA sequencing confirmed that both of these products were indicated as *Homo sapiens* CYP4Z1 mRNA (GenBank accession number: NM_178134). In addition, the result obtained from digestion of purified ~250 bp DNA fragment with *RsaI* i.e. 112 bp and 120 bp fragments (Figure 3.21B) was unexpected. The recognition site for *RsaI* is GT//AC. In the physical mapping of CYP4Z1 genome (Figure 5.3), G and g are located at the end of exon 5 and intron 5 respectively, and TAC is shown at the beginning of exon 6, which might explain the two restricted products [i.e. 126 bp (exon 5 (46 bp) + intron 5 (79 bp) + exon 6 (1 bp)) + exon 6 (112 bp) = 238 bp fragment]. These findings suggested that the retained intron may be located at the junction of intron 5 and exon 6.

In PCR reactions, the four different primer sets covering the exon 5 to exon 6 boundaries were used to further elucidate the sequences. The primer sets F1&r2 (the first protocol) and F3&r2 (the fourth protocol) were designed to detect the sequence in exon 5 and intron 5 whereas primer pairs F/f2&r2 (the second protocol) cover only the retained intron region.

The primers F3&R3 (the third protocol) are located in the beginning of exon 5 and the middle of exon 6.

The primer set in the first protocol (F1&r2) was initially amplified with the purified DNA of 159 bp and ~250 bp fragments. A single band of 125 bp was detected in purified DNA of both fragments from the T47D, MDA-MB468 and ZR-75-1 cells (Figures 5.6 and 5.7). Originally, the 125 bp product was expected only for the purified fragment of ~250 bp as it could prove retained intron mRNA, but not in the purified fragment of 159 bp. This may be due to unknown sequences in this splice variant, perhaps duplicated coding sequence in exon 5. For clarification, further experiments were performed using the primer set in the second protocol (F/f2&r2) to detect the nucleotide sequences of intron 5. The presence of the expected product size of 89 bp was detected as well as the first PCR product (125 bp) (Figure 5.6), suggesting that there may have been too much template DNA or non-specificity of primers. The pair of primers F1&r2 were also amplified with the original cDNA, but non-specific products were detected (Figure 5.7). Varying annealing temperatures to confirm specificity of primers could be used to address this problem.

The third PCR protocol with the primer set F3&R3 was used to amplify cDNA samples. Non-specific amplification of PCR products was detected (Figure 5.8) though nested PCR using the primer set F3&r2 was performed to increase primer specificity and detect retained intron (Figure 5.7). It is essential to note that the primers used failed to detect a single band of expected product size, but the presence of predicted retained intron products was found in all experiments (Figures 5.4-5.9).

Currently, there are two available databases to search full genomes of the CYP4Z1 genes; the NCBI (<http://www.ncbi.nlm.nih.gov/sites/entrez>) and Genatlas (<http://www.genatlas.org>). The NCBI database (NC_000001) which was used to identify the intron 5 sequences

in the CYP4Z1 gene during this research was markedly different in sequences from those of the GenAtlas database (Figure 5.10). At a glance in the GenAtlas database, there are two regions of the CYP4Z1 gene which demonstrate duplicate repeat sequences, one between exon 5 and intron 5 (101 nucleotides, Figure 5.11A) and the other between intron 5 and intron 5/exon 6 (180 nucleotides, Figure 5.11B). This may help to explain the non-specific products obtained in this chapter because the duplicated sequences also cover the primer sequence used in this research. The forward primer F1 is found in exon 5 and intron 5 whereas the reverse primer r2 is located in two positions in intron 5. The forward primer F/f2 is found in the junction at exon 5/intron 5 and intron 5, and eight nucleotides of the reverse primer R1 were found in intron 5 (Figure 5.12). Obviously, the sequences in exon 5-intron 5-exon 6 in the CYP4Z1 gene appear to be complicated. Further analysis including advanced molecular analysis of genomic/proteomic data is required to achieve a better understanding of the alternative splicing event in the CYP4Z1 gene.

Introns with a high GC content are associated with lower excision rates, increased susceptibility to transcription factor binding and higher transcriptional activity (Goodall and Filipowicz 1991, Galante et al. 2004). The GC content within intron 5 of the CYP4Z1 genome (the NCBI database) is approximately 42% (33 sequences of GC in a total 79 sequences). Moreover, average lengths of retained and non-retained introns are ~115 bp and 3.3 kb respectively, and several studies have confirmed that retained introns are usually shorter than non-retained introns (Stamm et al. 2000, Galante et al. 2004, Chen et al. 2006). Intron 5 of the CYP4Z1 gene has only 79 nucleotide sequences (Figure 5.3). Therefore, the specific region of this gene is more likely to have retained intron.

Data presented in this chapter provides the first evidence of CYP4Z1 mRNA expression in normal breast and breast cancer cell lines, and also of a retained intron 5. Future work could identify the sequences of intron 5 by DNA sequencing and elucidate the distribution

of intron retention events on CYP4Z1 mRNA by cloning PCR products of interest in the cell lines used. The biological significance of this event warrants further investigation. This chapter provides information on basal mRNA expression of the CYP4Z1 gene. The next chapter will provide information on the regulation of CYP4Z1 mRNA by investigating the potential nuclear receptors that regulate CYP4Z1 expression in induced cells and determining if the proposed alternative spliced forms are differentially expressed.

The NCBI database

AACAAATGGGAGGAACACATTGCCAAAACCTCACGTCTGGAGCTCTTTCAACATGTCTCCCTGATG
 ACCCTGGACAGCATCATGAAGTGTGCCTTCAGCCACCAGGGCAGCATCCAGTTGGACAGgtcagtgacaa
 aaggaggtaattgfttgccaataactgtgtcaccactaacatgtgttccatcttccctattccagTACCCTGGACTCATACTGAAAAGCAGTG
 TTCAACCTTAGCAAAATCTCCAACCAGCGCATGAACAATTTCTACATCACAACGACCTGGTTTTG
 AAATTCAGCTCTCAAGGCCAAATCTTTCTAAATTTAACCAAGAACTTCATCAGTTCACAG

The Genatlas database

AACAAATGGGAGGAACACATTGCCAAAACCTCACGTCTGGAGCTCTTTCAACATGTCTCCCTGATG
 ACCCTGGACAGCATCATGAAGTGTGCCTTCAGCCACCAGGGCAGCATCCAGTTGGACAGgtcagtgacaa
 aaggaggtaattgfttgccaataactgtgtcaccactaacatgtgttccatcttccctattccagtagctaccctggactcataacctgaagcagtggtcaaccttagcaaaatctcc
 aaccagcgcatgaacaattttctacatcacaacgacctggtttcaaatcacaanaactcagctctggagctcttcaacatgtctccctgatgacctggacagcatcatgaagt
 gtgccttcagccaccagggcagcatccagttggacaggtcagtgacaaaaggaggtaattgfttgccaataactgtgtcaccactaacatgtgttccatcttccctattcca
 gTACCCTGGACTCATACTGAAAAGCAGTGTTCAACCTTAGCAAAATCTCCAACCAGCGCATGAACA
 ATTTCTACATCACAACGACCTGGTTTTCAAATTCAGCTCTCAAGGCCAAATCTTTCTAAATTTAA
 CCAAGAACTTCATCAGTTCACAG

Figure 5.10: Sequence comparison of the CYP4Z1 gene from the NCBI and Genatlas database. Exon sequences are shown in upper cases and intron sequences in lower case letters. The sequences in exon 5 and exon 6 are indicated in yellow and green respectively.

AACAAATGGGAGGAAACACATTGCCCAAAACTCACGTCTGGAGCTCTTTCAACATGTCTCCCTGATG
F1 F/f2
ACCCTGGACAGCATCATGAAGTGTGCCTTCAGCCACCAGGGCAGCATCCAGTTGGACAGgtcagtgacaa
r2
aaggaggfaaftgfttgccaataactgtgcaccactaacatgftgttccatctccctattccagtacctggactcatactgaaagcagtgftcaaccttagcaaaatctcc
R1 (8 nt) F1
aaccagegcatgaacaatfttctacatcacaacgacctggtttcaaatcaaaaactcacgtctggagctctttcaacatgtctccctgatgacctggacagcatcatgaagt
F/f2 r2
gtgcctcagccaccaggcagcatccagttggacaggtcagtgacaaaaggaggtaattgfttgccaataactgtgcaccactaacatgftgtccatctccctattcca

TACCCTGGACTCATACCTGAAAGCAGTGTTC AACCTTAGCAAAATCTCCAACCAGCGCATGAACA
R1
ATTTTCTACATCACAACGACCTGGTTTTCAAATTCAGCTCTCAAGGCCAAAATCTTTTCTAAATTTAA

CCAAGA ACTTCATCAGTTCACAG

Figure 5.12: Repeated DNA sequences in the CYP4Z1 gene using the GenAtlas database associated with primer sequences used. Exon sequences are shown in upper cases and intron sequences in lower case letters. The sequences in exon 5 and exon 6 are indicated in yellow and green respectively.

Chapter 6

Regulation of CYP4Z1 expression in Breast Cancer Cell Lines

6.1 Introduction

In Chapter 5, evidence was found of alternative pre-mRNA splicing of the CYP4Z1 gene in the normal breast MCF10A cells and the breast cancer T47D, MDA-MB-468 and ZR-75-1 cells. Although experiments have not been successful in fully characterising the alternative splicing by intron retention, the expected product size of the retained intron 5 (79 bp) was present in all the test cells. Usually, the molecular basis of eukaryotic transcription is controlled by a complex regulatory system as gene expression can be regulated at a variety of steps in the pathway from DNA to RNA and RNA to protein. Transcription is the process by which a monocistronic mRNA is transcribed from DNA and it is controlled by the level of RNA polymerase. The resulting pre-mRNA is capped at the 5' end and the 3' end is polyadenylated. Introns are removed by splicing before the mature mRNA is released from the nucleus to be translated by ribosomes in the cytoplasm into cellular protein (Figure 6.1) (Turner 2005).

Most members of cytochrome P450 family 4 catalyse the ω -hydroxylation of fatty acids, steroid hormones (i.e progesterone, testosterone and growth hormones) and arachidonic acids (i.e. leukotrienes and prostanoid) and these enzymes appear to be implicated in vascular tone and blood pressure regulation (Hsu et al. 2007). Currently, the cytochrome P450 subfamilies 4A and 4F are the most widely characterised and they are mainly expressed in human liver and kidney. Information on expression, function and genetic variation of these genes has been well established in animal models (i.e. rat, mouse and rabbit) (Hsu et al. 2007). The expression of CYP4A genes is induced by peroxisome proliferators (i.e. hypolipidemic drug fibrates and Wy14643 or pirinixic acid). The

induction of CYP4A expression by peroxisome proliferators is mediated by the peroxisome proliferator activated receptor alpha (PPAR α) (Waxman 1999, Okita and Okita 2001), which requires heterodimerisation with the retinoid X receptor (RXR) for transcriptional regulation of CYP4A (Waxman 1999, Okita and Okita 2001). PPAR α is believed to play an important role in the regulation of lipid homeostasis as well as lipid metabolism. However, crosstalk between PPAR α and other nuclear receptors such as retinoic acid receptor (RAR), vitamin D receptor (VDR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR) may occur as they share a dimeric partner (RXR) and substrates (i.e. steroid hormones) (Honkakoski and Negishi 2000).

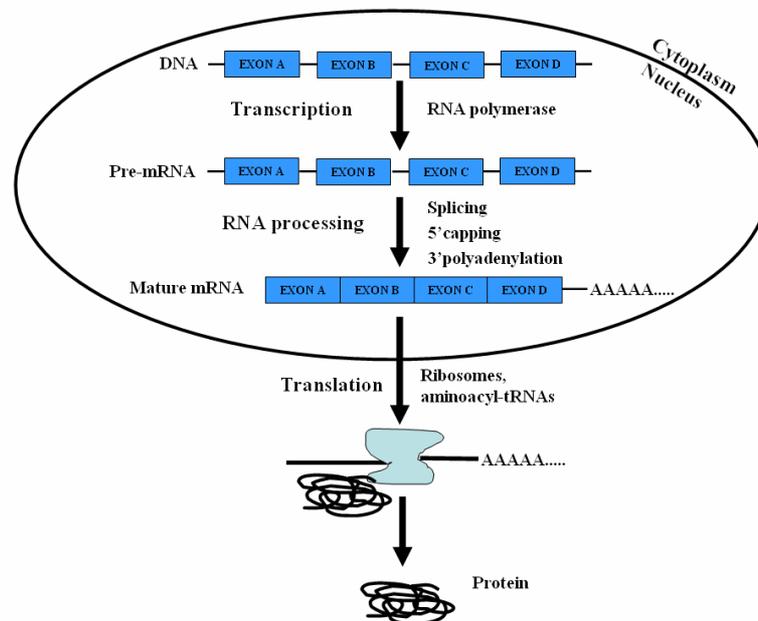


Figure 6.1: A schematic view of eukaryotic gene expression (Adapted from Turner 2005).

The CYP4Z1 gene is a novel cytochrome P450 enzyme that belongs to family 4 and the physiological functions of the CYP4Z1 gene are yet to be clearly characterised. The tissue localisation of CYP4Z1 has been studied in an attempt to identify its functions. Reiger and co-workers reported that CYP4Z1 mRNA and protein are preferentially expressed in breast tumour tissues but low levels are seen in other tumour types (i.e. uterus, prostate, lung and kidney) (Rieger et al. 2004). No significant difference in CYP4Z1 mRNA was found in

the breast tumour types and progression stages. In addition, Downie and colleagues demonstrated that overexpression of CYP4Z1 is associated with a poor prognosis of primary ovarian cancer (Downie et al. 2005). These two research groups suggest that the expression of CYP4Z1 is involved in the carcinogenic process of human breast and ovary.

A study by Savas and co-workers demonstrated that CYP4Z1 mRNA in the breast cancer T47D cells is markedly induced by progesterone or the synthetic glucocorticoid dexamethasone. The induction of CYP4Z1 mRNA by these compounds was suppressed by the antagonist of progesterone and glucocorticoid receptor RU486. In addition, CYP4Z1 mRNA in the breast cancer progesterone receptor negative MCF7 cells is elevated by dexamethasone but not progesterone. However, there was no significant alteration of CYP4Z1 mRNA by the PPAR α agonist pirinixic acid in either the T47D or MCF7 cells (Savas et al. 2005). These findings suggest that the CYP4Z1 gene has cell-type specificity for the induction of mRNA expression.

The physiological role of the splicing variant in the CYP4Z1 gene found in this study is unknown, but it is likely to be involved in the carcinogenic property of the test cell lines. Therefore, the regulation of gene transcription and substrate-specificity of this gene with the occurrence of the alternative splice variant in CYP4Z1 are of interest for further investigation. The main aims of this chapter were to (a) profile mRNA expression of the candidate nuclear receptors (i.e. progesterone receptor; PGR, glucocorticoid receptor; GCR and peroxisome proliferator-activated receptor alpha; PPAR α) for the CYP4Z1 gene, (b) determine the inducibility of the two forms of CYP4Z1 mRNA, and (c) ascertain whether the induction of CYP4Z1 mRNA is regulated at the transcriptional or translational level.

6.2 Materials and Methods

6.2.1 Cell culture and CYP4Z1 induction in cultured cells

6.2.1.1 Induction of CYP4Z1 mRNA by the nuclear receptor agonists

All four CYP4Z1-expressing cell lines (i.e. MCF10A, T47D, MDA-MB-468 and ZR-75-1) and two representative non-CYP4Z1-expressing cell lines (i.e. MCF7 and MDA-MB-231) were used to determine the inducibility of CYP4Z1 mRNA by agonists of the progesterone receptor (PGR), glucocorticoid receptor (GCR) and peroxisome proliferator-activated receptor alpha (PPAR α). The cells were seeded at 5×10^5 cells in a 25 cm² flask, and were grown under appropriate conditions (Section 2.2.3). Cells at ~60% confluence were treated with growth medium containing one of the following nuclear receptor agonists for 21 hours; either 2 μ M progesterone, 1 μ M dexamethasone or 50 μ M PPAR α agonist (pirinixic acid) as previously described (Savas et al. 2005) with the exception that chemicals were dissolved in ethanol instead of dimethylsulphoxide (DMSO). This amendment was made because a previous study had demonstrated that expression of certain cytochrome P450s, particularly members of the CYP1, CYP2 and CYP3 families can be inhibited or induced by DMSO in *in vitro* studies (Busby et al. 1999). The final concentration of ethanol in the culture medium was 0.1% (v/v). Vehicle control cells were exposed to 0.1% (v/v) ethanol and treated in the same manner as those treated with nuclear receptor agonists. Untreated cells were used as an additional control against which to compare the solvent controls.

6.2.1.2 Evaluation of transcriptional and translational regulation of CYP4Z1

The breast cancer T47D and ZR-75-1 cell lines were used to ascertain whether CYP4Z1 mRNA induction is through transcriptional or translational regulation. This is because CYP4Z1 mRNA in the T47D or ZR-75-1 cells was highly inducible by the nuclear receptor agonists according to results obtained from the initial experiment (Section 6.2.1.1). Cells at 60% confluence were pre-treated with either 10 μ g/mL of actinomycin D

or 10 µg/mL of cycloheximide for 30 minutes in order to block the transcriptional or translational regulation respectively. Spent medium containing actinomycin D or cycloheximide was removed, and the cells were washed twice with warm (37°C) serum-free medium to minimise the residue of actinomycin D or cycloheximide. Growth media containing the same concentrations of progesterone, dexamethasone or pirinixic acid (Section 6.2.1.1) were used to treat the cells for 21 hours. Untreated and vehicle control cells were included in each experiment. Three independent experiments were performed in triplicate on cultures of the T47D and ZR-75-1 cells.

6.2.2 RNA extraction and cDNA synthesis

Following treatment of the cells with vehicle control, the nuclear receptor agonists or co-exposure to actinomycin D/cycloheximide with the nuclear receptor agonists (Section 6.2.1), total RNA was isolated using the RNeasy Plus Mini kit. The RNA concentration and purity were quantified by A_{260}/A_{280} ratios. Integrity of the isolated total RNA sample was determined by examination of the 28S and 18S rRNA bands on 1% (w/v) agarose gel electrophoresis (Section 2.3). Synthesis of cDNA was performed under the conditions described in Tables 2.2 and 2.3.

6.2.3 Qualitative RT-PCR analysis

To determine the appropriate receptor for mRNA expression in the test cell lines, the eight cDNA samples from the investigation of the target genes in Chapter 4 were employed. These samples were subjected to routine amplification (Tables 2.3 and 2.4) with previous PGR, GCR and PPAR α published primers (Table 2.7). Primer specificity was confirmed by BLAST analysis. The PCR products were separated by 1.5% (w/v) agarose gels containing ethidium bromide and visualised by ultra-violet light (Section 2.8). To confirm expression of the target genes, the PCR products were digested with specific restriction

enzymes as described previously (Section 2.9). Table 2.10 illustrates the restriction enzymes used and the expected size of the products.

To examine the inducibility of the two forms of CYP4Z1 mRNA by the nuclear receptor agonists in the six cell lines, expression of β_2 -microglobulin and CYP4Z1 mRNA was assessed in each cell line by the qualitative RT-PCR method (Sections 3.3.2 and 3.3.3.4). The PCR products were visualised on 1.5% (w/v) agarose gels containing ethidium bromide.

6.2.4 Semi-quantitative PCR amplification

In order to determine whether CYP4Z1 mRNA induction is through transcriptional or translational regulation, the PCR products obtained from β_2 -microglobulin and CYP4Z1 mRNA in the T47D and ZR-75-1 cells were visualised on 1.5% (w/v) agarose gels containing ethidium bromide. To normalise amplified RNA products and PCR variations, the band intensities of CYP4Z1 mRNA were corrected with the band intensities of β_2 -microglobulin mRNA as the internal standard (Section 4.2.3).

6.2.5 Statistical analysis

Results of CYP4Z1/ β_2 -microglobulin mRNA relative expression for each sample from three independent experiments in the T47D and ZR-75-1 cells were presented as mean \pm standard error of the mean (SEM). Statistical analysis was performed with one-way analysis of variance (ANOVA), followed by Tukey's Honest Significant Difference (HSD) to compare the results from the various experimental groups with their corresponding controls (vehicle control or untreated cells) and the nuclear receptor agonist alone. The differences were considered statistically significant when the *p* value was less than 0.05, 0.01 or 0.001. Data were analysed with SPSS version 13 (SPSS Corporation, Chicago, Illinois, USA).

6.3 Results

6.3.1 Profile of constitutive mRNA of the candidate nuclear receptors

As stated in Section 6.2.3, the eight cDNA samples used were the same set as those investigated for the target genes in Chapter 4. All RNA samples derived from the eight cell lines were shown to have high integrity and purity (Figure 4.1). Reverse transcription efficiency was verified by the presence of β_2 -microglobulin and no major difference in expression was observed (Figure 4.2).

6.3.1.1 Progesterone receptor (PGR) RT-PCR

PGR mRNA was detected in four out of the seven breast cancer cell lines as the correct band at 533 bp but no product was found in the normal breast MCF10A cell line. A higher level of expression was observed in the T47D cells than in the MCF7, ZR-75-1 and MDA-MB-157 cells (Figure 6.2). The expression of PGR mRNA was confirmed by restriction digestion with *AluI* which generated restricted product of the correct size (417 bp and 84 bp, Figure 6.3).

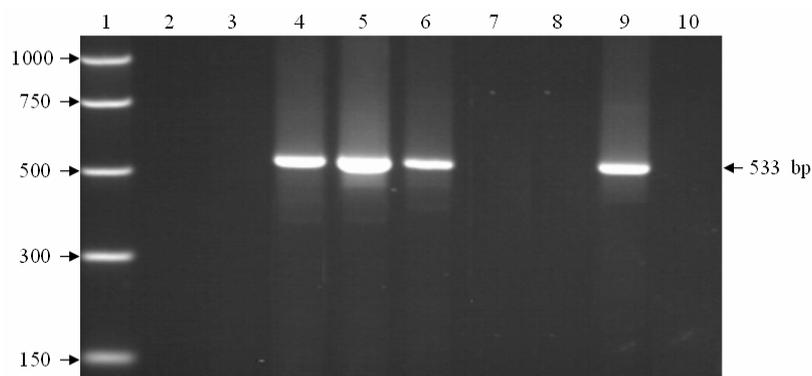


Figure 6.2: PGR RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468.

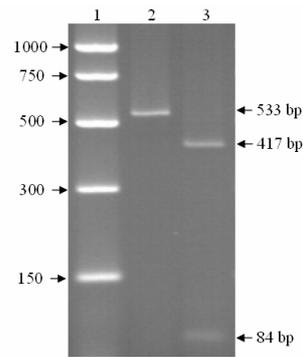


Figure 6.3: Restriction digestion of PGR RT-PCR using *AluI*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

6.3.1.2 Glucocorticoid receptor (GCR) RT-PCR

A band of 360 bp corresponding to the cDNA product size for GCR mRNA was found in three out of the seven breast cancer cell lines and the normal breast cell line. A higher mRNA level was detected in the ZR-75-1 cells than in the MCF10A cells. Very low levels of expression were observed in both the MDA-MB-157 and MDA-MB-468 cells.

However, there is a faint band below 360 bp in the presence of GCR mRNA expression (Figure 6.4). To confirm the GCR mRNA expression, the PCR product was digested with restriction enzyme *AluI* and the correct fragments were found at 172 bp and 148 bp (Figure 6.5).

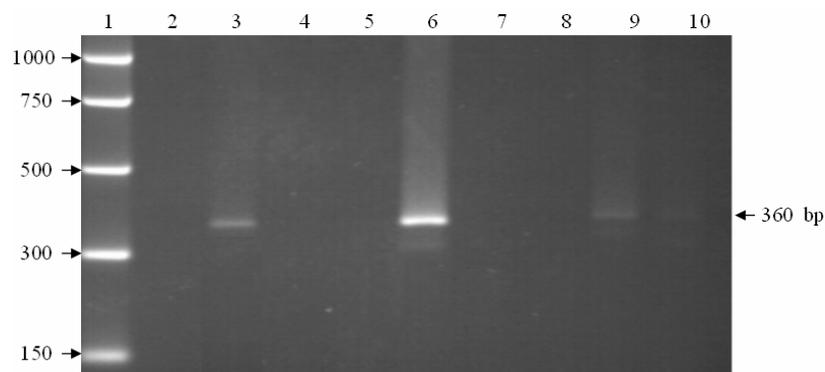


Figure 6.4: GCR RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468.

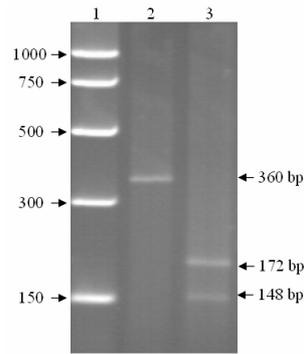


Figure 6.5: Restriction digestion of GCR RT-PCR using *AluI*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

6.3.1.3 Peroxisome proliferator-activated receptor alpha (PPAR α) RT-PCR

A band of 735 bp was observed for PPAR α mRNA in all of the cell lines except MCF7. PPAR α mRNA was expressed in the MCF10A, T47D, ZR-75-1 and MDA-MB-468 cells at higher levels than in the MDA-MB-231 and MDA-MB-157 cells. Only a very weak level was detected in the ZR-75-30 cells (Figure 6.6). PPAR α mRNA was confirmed by restriction digest with *RsaI* which generated digested fragments of the correct size (518 bp and 188 bp) (Figure 6.7).

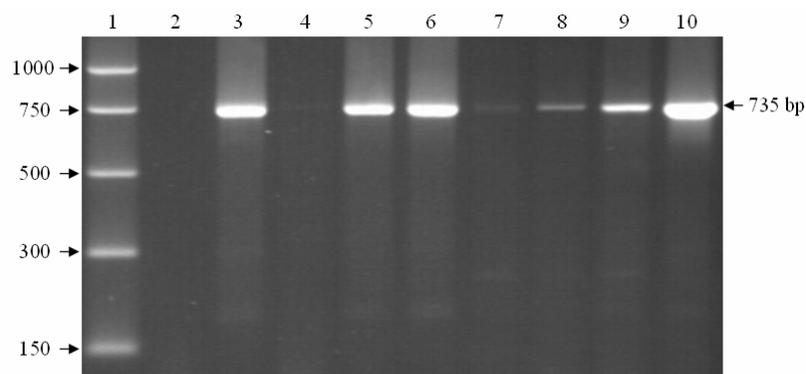


Figure 6.6: PPAR α RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: MCF10A, Lane 4: MCF7, Lane 5: T47D, Lane 6: ZR-75-1, Lane 7: ZR-75-30, Lane 8: MDA-MB-231, Lane 9: MDA-MB-157, Lane 10: MDA-MB-468.

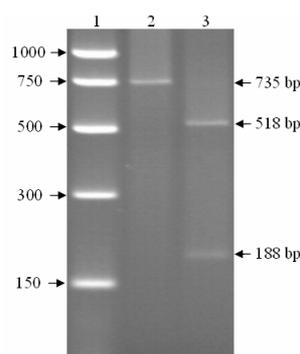


Figure 6.7: Restriction digestion of PPAR α RT-PCR using *RsaI*. Lane 1: 1000 bp PCR markers, Lane 2: undigested product, Lane 3: digested product.

6.3.2 Induction of CYP4Z1 mRNA by the agonists of candidate nuclear receptors

In Chapter 4, CYP4Z1 mRNA was expressed in three out of the seven breast cancer cells (i.e. T47D, MDA-MB-468 and ZR-75-1) and also in the normal breast MCF10A cells (Figure 4.13). To determine which nuclear receptors were involved in regulating the expression of CYP4Z1, agonists against the PGR, GCR and PPAR α were used. The panel of all eight cell lines were used in this investigation and were exposed to the individual agonists over a period of 21 hours. This time period was selected as it was successfully used in a previous study by Savas and colleagues (Savas et al. 2005) who investigated CYP4Z1 regulation in the T47D and MCF7 cells. All cDNA samples were verified by the housekeeping β_2 -microglobulin RT-PCR (319 bp). Little variation of β_2 -microglobulin expression was observed between untreated and treated cells in the MCF7 (Figures 6.8A), MDA-MB-231 (Figures 6.9A) and MCF10A cells (Figures 6.10A). However, β_2 -microglobulin mRNA in the treated cells in the MDA-MB-468 (Figure 6.11A, lane 6), T47D (Figure 6.12A, lane 4) and ZR-75-1 cells (Figure 6.13A, lane 5) was slightly weaker than the other lanes which was taken into account when interpreting the result.

In the non CYP4Z1-expressing cell lines, there was no detectable cDNA product size corresponding to CYP4Z1 mRNA (159 bp or ~250 bp) either in the MCF7 (Figure 6.8B) or MDA-MB-231 cells following exposure to the respective agonists (Figure 6.9B).

In the CYP4Z1-expressing cell lines, the induction of CYP4Z1 mRNA in the normal breast MCF10A cell line was barely detectable (Figure 6.10B). However, it is important to note that the presence of a ~250 bp fragment was only found in the cells treated with dexamethasone and a band of 159 bp was not detected. In the MDA-MB-468 cells, it was found that CYP4Z1 mRNA was slightly altered by the three nuclear receptor agonists in comparison to the untreated and vehicle control cells (Figure 6.11B). In addition, CYP4Z1 mRNA in the T47D cells was induced only by dexamethasone (Figure 6.12B) whereas, CYP4Z1 mRNA in the ZR-75-1 cells was elevated by either dexamethasone or pirinixic acid (Figure 6.13B).

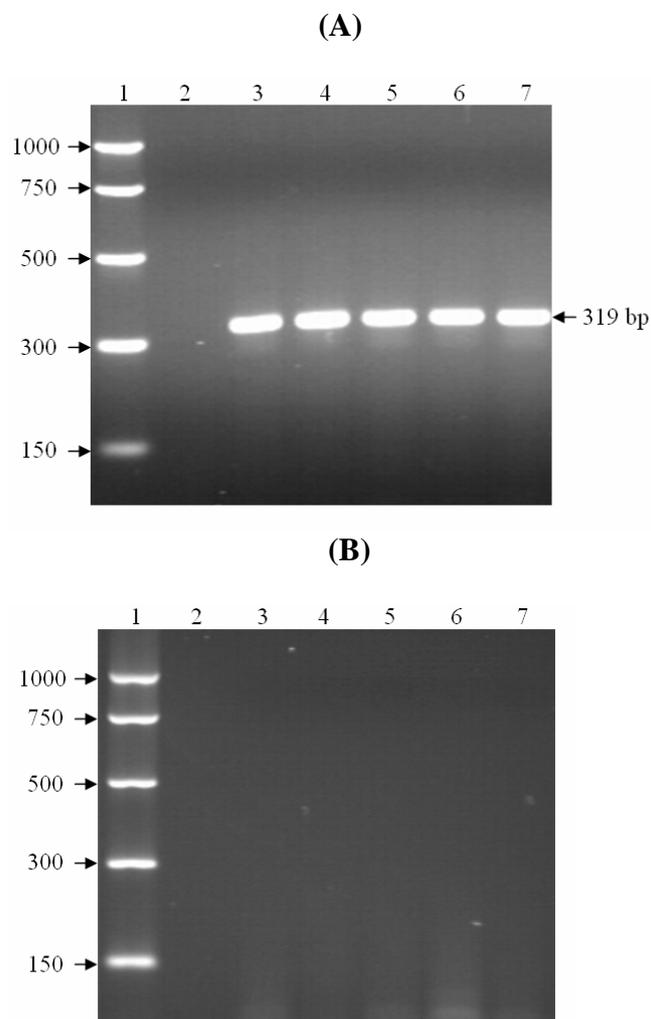


Figure 6.8: Induction of CYP4Z1 in the MCF7 cells. (A) β_2 -microglobulin RT-PCR. (B) CYP4Z1 RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: untreated cells, Lane 4: 0.1% (v/v) ethanol, Lane 5: progesterone (2 μ M), Lane 6: dexamethasone (1 μ M), Lane 7: pirinixic acid (50 μ M).

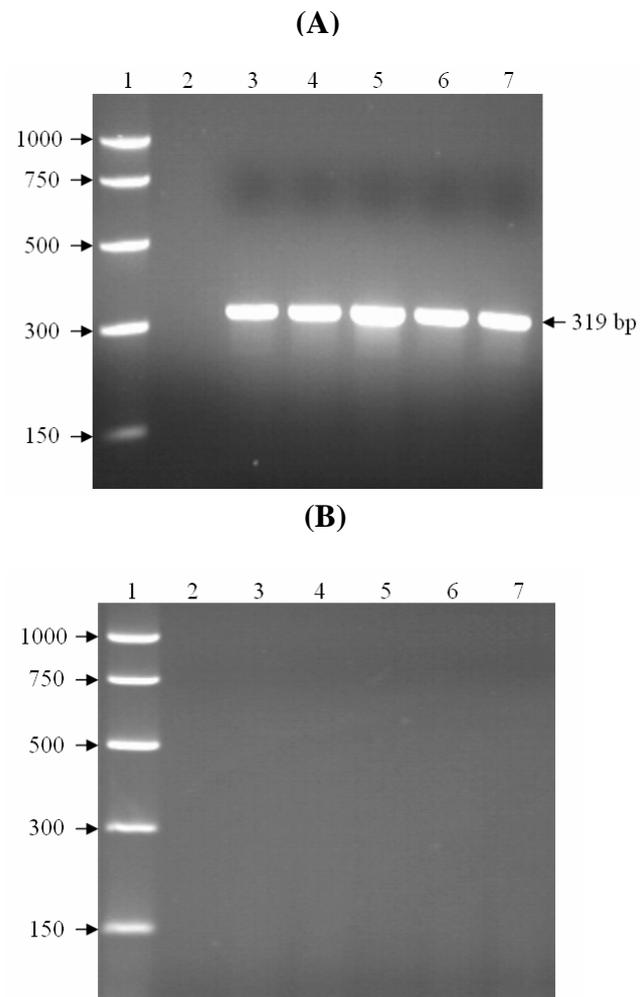


Figure 6.9: Induction of CYP4Z1 in the MDA-MB-231 cells. (A) β_2 -microglobulin RT-PCR. (B) CYP4Z1 RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: untreated cells, Lane 4: 0.1% (v/v) ethanol, Lane 5: progesterone (2 μ M), Lane 6: dexamethasone (1 μ M), Lane 7: pirinixic acid (50 μ M).

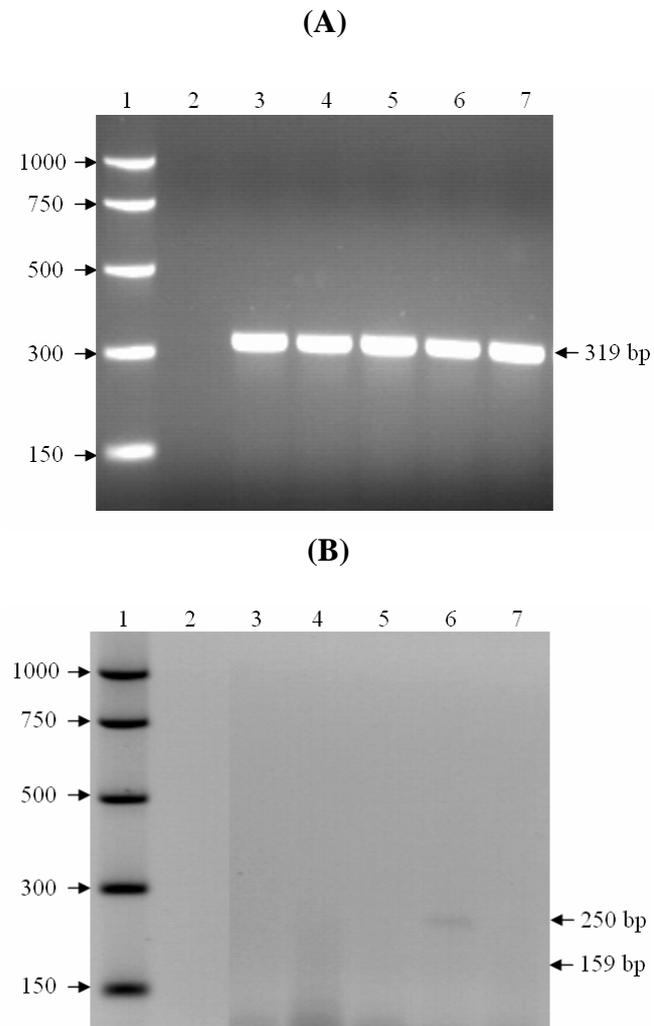


Figure 6.10: Induction of CYP4Z1 in the MCF10A cells. (A) β_2 -microglobulin RT-PCR. (B) CYP4Z1 RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: untreated cells, Lane 4: 0.1% (v/v) ethanol, Lane 5: progesterone (2 μ M), Lane 6: dexamethasone (1 μ M), Lane 7: pirinixic acid (50 μ M).

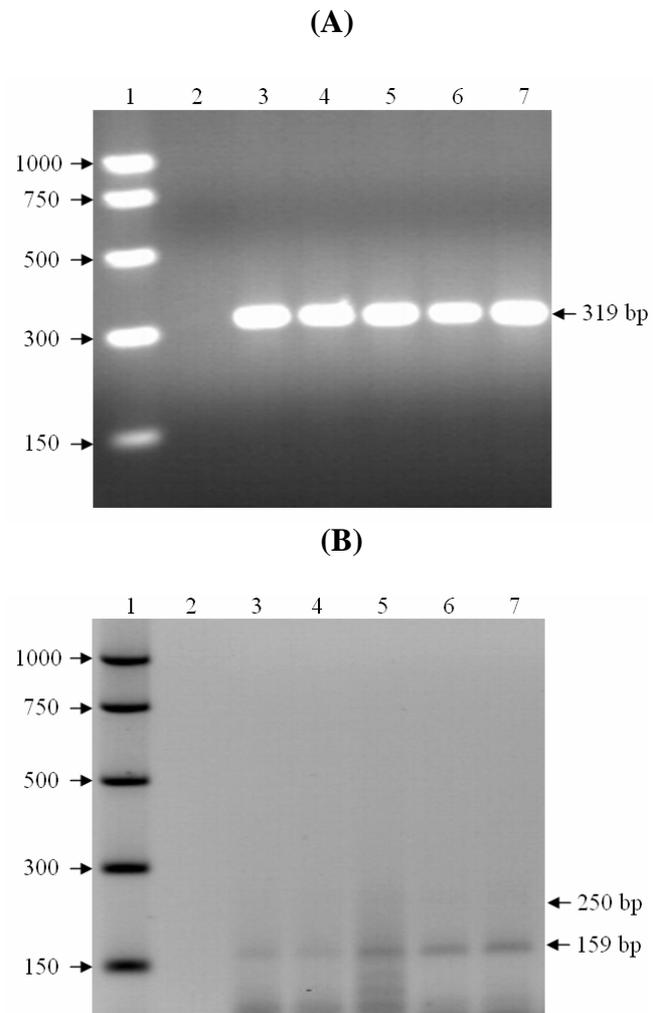


Figure 6.11: Induction of CYP4Z1 in the MDA-MB-468 cells. (A) β_2 -microglobulin RT-PCR. (B) CYP4Z1 RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: untreated cells, Lane 4: 0.1% (v/v) ethanol, Lane 5: progesterone (2 μ M), Lane 6: dexamethasone (1 μ M), Lane 7: pirinixic acid (50 μ M).

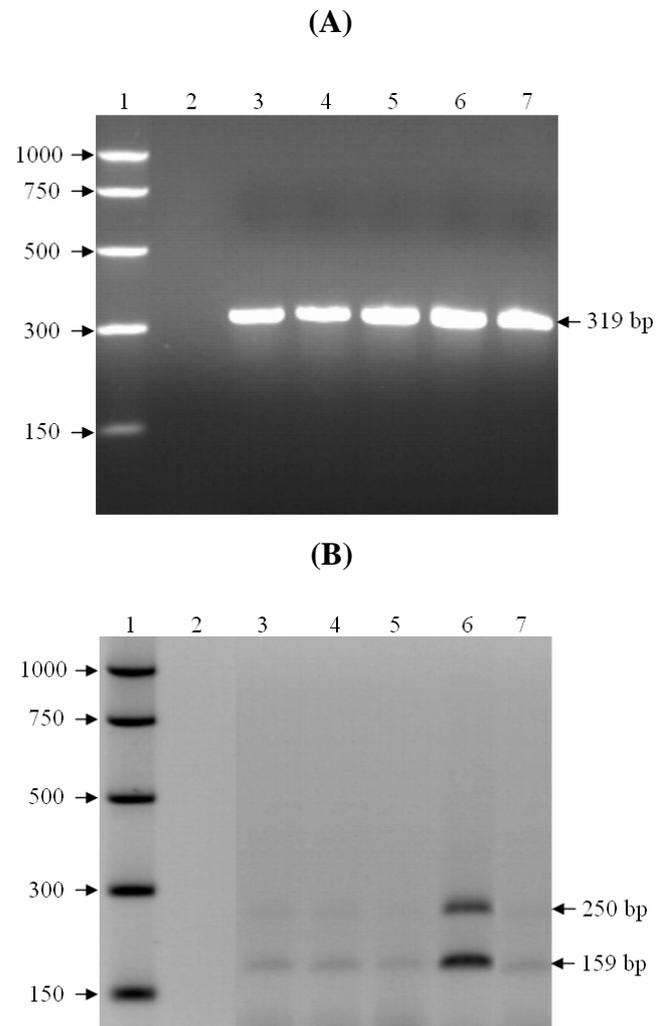


Figure 6.12: Induction of CYP4Z1 in the T47D cells. (A) β_2 -microglobulin RT-PCR. (B) CYP4Z1 RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: untreated cells, Lane 4: 0.1% (v/v) ethanol, Lane 5: progesterone (2 μ M), Lane 6: dexamethasone (1 μ M), Lane 7: pirinixic acid (50 μ M).

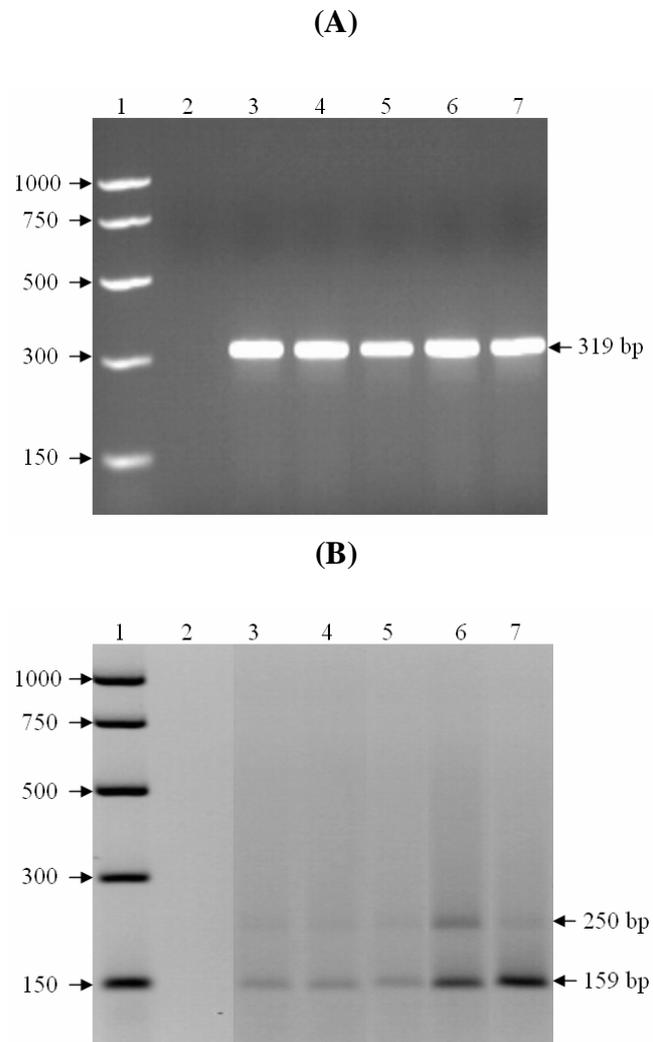


Figure 6.13: Induction of CYP4Z1 in the ZR-75-1 cells. (A) β_2 -microglobulin RT-PCR. (B) CYP4Z1 RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: untreated cells, Lane 4: 0.1% (v/v) ethanol, Lane 5: progesterone (2 μ M), Lane 6: dexamethasone (1 μ M), Lane 7: pirinixic acid (50 μ M).

6.3.3 Evaluation of transcriptional and translational regulation of CYP4Z1

To investigate whether the nuclear receptor agonists are able to increase *de novo* CYP4Z1 mRNA synthesis, expression of CYP4Z1 mRNA was investigated in the T47D and ZR-75-1 cells and this was found to increase following exposure to the nuclear receptor agonists. These experiments were performed in the presence or absence of 10 µg/mL actinomycin D (an RNA synthesis inhibitor) for 30 minutes, which prevents the induction of CYP4Z1 mRNA, followed by exposure to the agonists (i.e. PGR, GCR and PPAR α) for 21 hours. If CYP4Z1 mRNA expression was increased due to *de novo* RNA synthesis, a decrease in the level of CYP4Z1 mRNA following exposure to actinomycin D would be expected (Figure 6.14).

To examine whether *de novo* protein synthesis is required for the induction of CYP4Z1 mRNA, the experiments were performed in the presence or absence of 10 µg/mL cycloheximide (a protein synthesis inhibitor) for 30 minutes, followed by treatment with the nuclear receptor agonists (i.e. PGR, GCR and PPAR α) for 21 hours. If CYP4Z1 mRNA was regulated through *de novo* protein synthesis, an increase in the accumulation of CYP4Z1 mRNA after treatment with cycloheximide would be observed (Figure 6.14).

The effect of the RNA and protein synthesis inhibitors on β_2 -microglobulin expression and CYP4Z1 mRNA induction by the nuclear receptor agonists in the ZR-75-1 and T47D cells is shown in Figures 6.15 and 6.16 respectively. This data is representative of triplicate experiments. The differences in β_2 -microglobulin mRNA expression observed in the samples were taken into account by normalising the CYP4Z1 mRNA levels in each treatment against the equivalent β_2 -microglobulin. This data is presented in Figures 6.17, 6.18 and 6.19. There were no significant differences (p value > 0.05) in CYP4Z1 mRNA between the untreated cells and cells treated with vehicle control 0.1% (v/v) ethanol, suggesting that 0.1% (v/v) ethanol has no effect on CYP4Z1 mRNA expression.

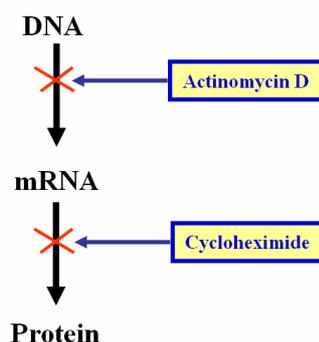


Figure 6.14: A schematic view of the effect of actinomycin D and cycloheximide on mRNA expression.

In the T47D cells, a significant increase of CYP4Z1 mRNA expression was observed in cells treated with either progesterone (p value < 0.05 , Figure 6.17A) or dexamethasone alone (p value < 0.001 , Figure 6.18A), when compared with the vehicle control data. The ZR-75-1 cells showed a significant increase of CYP4Z1 mRNA when induced by either dexamethasone (p value < 0.01 , Figure 6.18A) or pirinixic acid alone (p value < 0.001 , Figure 6.19A).

Co-treatment of the T47D cells with actinomycin D and the individual nuclear receptor agonists resulted in a significant decrease of CYP4Z1 mRNA for progesterone (p value < 0.001 , Figure 6.17A), dexamethasone (p value < 0.001 Figure 6.18A) and pirinixic acid (p value < 0.01 , Figure 6.19A) when compared with cells induced by the nuclear receptor agonist alone. A significant decrease in CYP4Z1 transcription was found when the ZR-75-1 cells were treated with actinomycin D following either dexamethasone (p value < 0.001 , Figure 6.18A) or pirinixic acid (p value < 0.01 , Figure 6.19A).

Pre-treatment of the T47D cells with cycloheximide followed by one of the individual nuclear receptor agonists resulted in a significant decrease of CYP4Z1 mRNA in the cells co-treated with progesterone (p value < 0.001 , Figure 6.17A), dexamethasone (p value < 0.001 , Figure 6.18A) or pirinixic acid (p value < 0.05 , Figure 6.19A). A significant

increase of CYP4Z1 mRNA was observed in the ZR-75-1 cells when the cells were pre-treated with cycloheximide following treatment with progesterone. Cells co-treated with cycloheximide following treatment with either dexamethasone or pirinixic acid showed a significant decrease of CYP4Z1 mRNA.

Considerable variation was observed with the expected CYP4Z1 product size of 159 bp during these experiments and this was also found with the alternative splice variant ~250 bp fragment (CYP4Z1~250 bp). However, the difference between the variations in the expression of two bands was observed. A significant increase of CYP4Z1~250 bp mRNA was found in the T47D cells treated with dexamethasone alone (p value < 0.001 , Figure 6.18B) and in the ZR-75-1 cells induced by either dexamethasone (p value < 0.05 , Figure 6.18B) or pirinixic acid alone (p value < 0.001 , Figure 6.19B) as compared with vehicle control data.

There were no significant differences (p value > 0.05) in CYP4Z1~250 bp mRNA when T47D cells were co-treated with actinomycin D and progesterone or pirinixic acid.

However, a significant decrease of the CYP4Z1~250 bp fragment was found when the cells were treated with actinomycin D followed by dexamethasone (p value < 0.001 , Figure 6.18B), compared with cells treated with dexamethasone alone. Pre-treatment of the ZR-75-1 cells with actinomycin followed by one of the individual nuclear receptor agonists resulted in a significant decrease of CYP4Z1~250 bp in the cells co-treated with either dexamethasone or pirinixic acid (p value < 0.001) was compared with cells treated with the nuclear receptor agonist alone (Figures 6.18B and 6.19B).

A significant increase of CYP4Z1~250 bp was found in the ZR-75-1 cells treated with cycloheximide followed by progesterone (p value < 0.001 , Figure 6.17B), compared to cells treated with vehicle control or progesterone alone. Pre-treatment of the T47D cells

with cycloheximide followed by dexamethasone resulted in a significant decrease of CYP4Z1~250 bp (p value < 0.001, Figure 6.18B) compared to cells treated with dexamethasone alone.

Tables 6.1 and 6.2 summarise the results for the T47D and ZR-75-1 cells demonstrating differences in expression of CYP4Z1 (159 bp and ~250 bp) mRNA against β_2 -microglobulin expression following co-treatment with either the nuclear receptor agonists plus or minus actinomycin D or cycloheximide using Tukey's test.

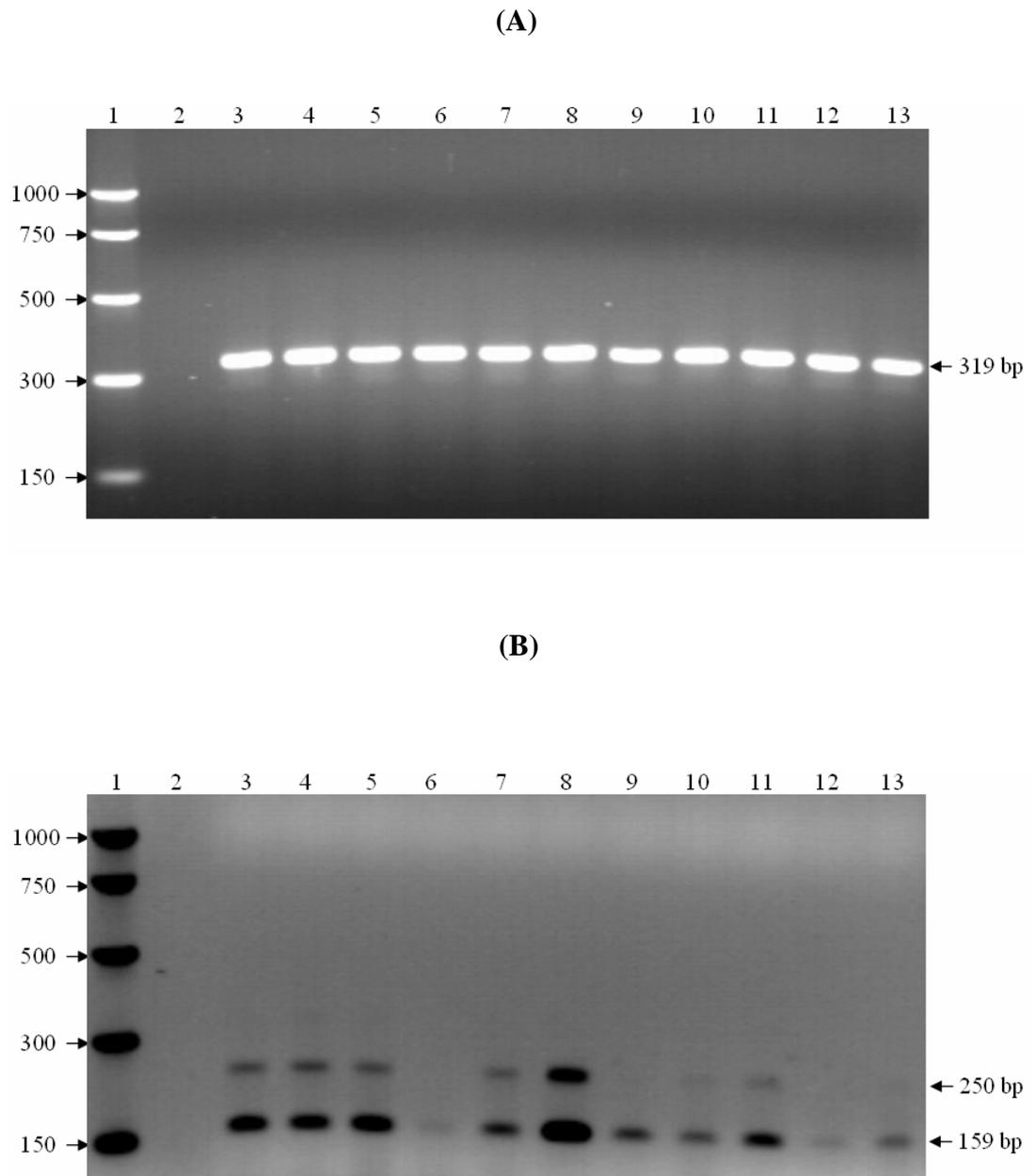


Figure 6.17: Effect of RNA and protein synthesis inhibitors on CYP4Z1 induction in the T47D cells. (A) β_2 -microglobulin RT-PCR. (B) CYP4Z1 RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: untreated, Lane 4: VC, Lane 5: PG, Lane 6: ACTD+PG, Lane 7: CHX+PG, Lane 8: DEX, Lane 9: ACTD+DEX, Lane 10: CHX+DEX, Lane 11: PNX, Lane 12: ACTD+PNX, Lane 13: CHX+PNX.

Abbreviations: VC; 0.1% (v/v) ethanol, PG; progesterone (2 μ M), DEX; dexamethasone (1 μ M), PNX; pirinixic acid (50 μ M), ACTD; actinomycin D (10 μ g/mL), CHX; cycloheximide (10 μ g/mL).

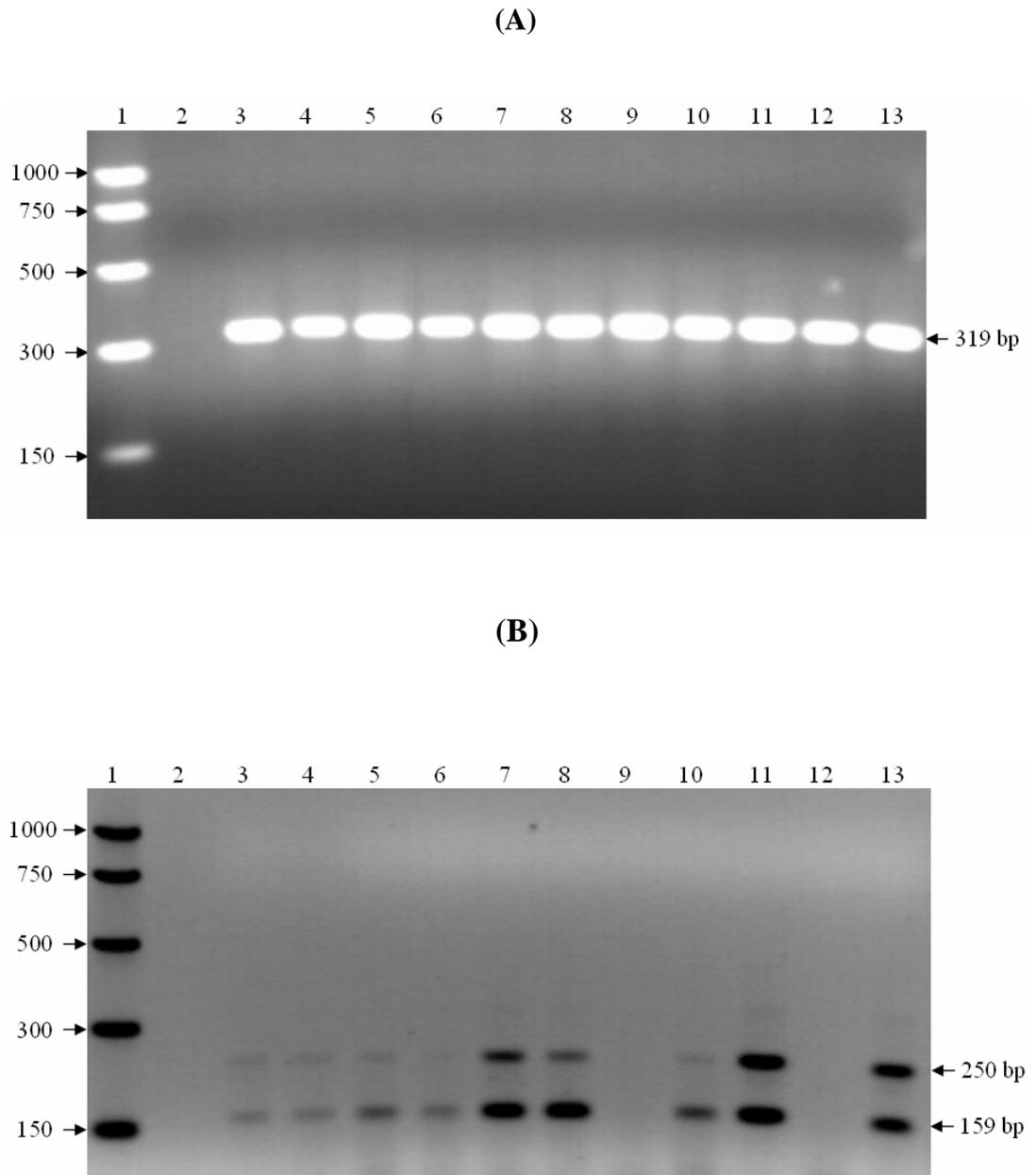


Figure 6.18: Effect of RNA and protein synthesis inhibitors on CYP4Z1 induction in the ZR-75-1 cells. (A) β_2 -microglobulin RT-PCR. (B) CYP4Z1 RT-PCR. Lane 1: 1000 bp PCR markers, Lane 2: negative control, Lane 3: untreated, Lane 4: VC, Lane 5: PG, Lane 6: ACTD+PG, Lane 7: CHX+PG, Lane 8: DEX, Lane 9: ACTD+DEX, Lane 10: CHX+DEX, Lane 11: PNX, Lane 12: ACTD+PNX, Lane 13: CHX+PNX.

Abbreviations: VC; 0.1% (v/v) ethanol, PG; progesterone (2 μ M), DEX; dexamethasone (1 μ M), PNX; pirinixic acid (50 μ M), ACTD; actinomycin D (10 μ g/mL), CHX; cycloheximide (10 μ g/mL).

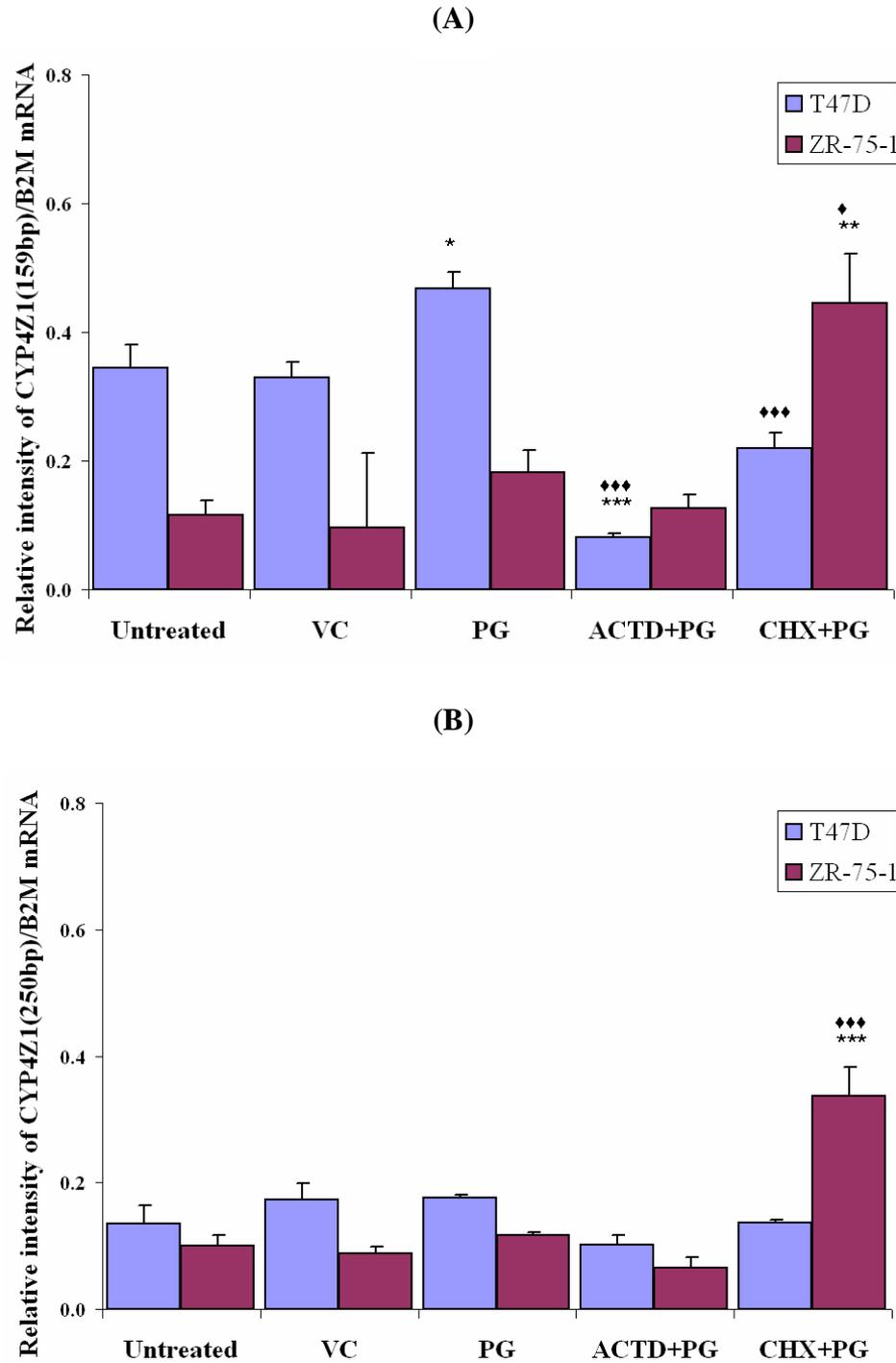


Figure 6.19: Effect of RNA and protein synthesis inhibitors on progesterone-mediated increase in CYP4Z1 mRNA in the T47D and ZR-75-1 cells. (A) CYP4Z1 (159 bp). (B) CYP4Z1 (250 bp). Data represent the mean \pm SEM of three independent experiments for each treatment showing the relative intensity of CYP4Z1 normalised against β_2 -microglobulin mRNA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with VC. $\blacklozenge p < 0.05$, $\blacklozenge\blacklozenge p < 0.01$, $\blacklozenge\blacklozenge\blacklozenge p < 0.001$ compared with PG.

Abbreviations: VC; 0.1% (v/v) ethanol, PG; progesterone (2 μ M), ACTD; actinomycin D (10 μ g/mL), CHX; cycloheximide (10 μ g/mL).

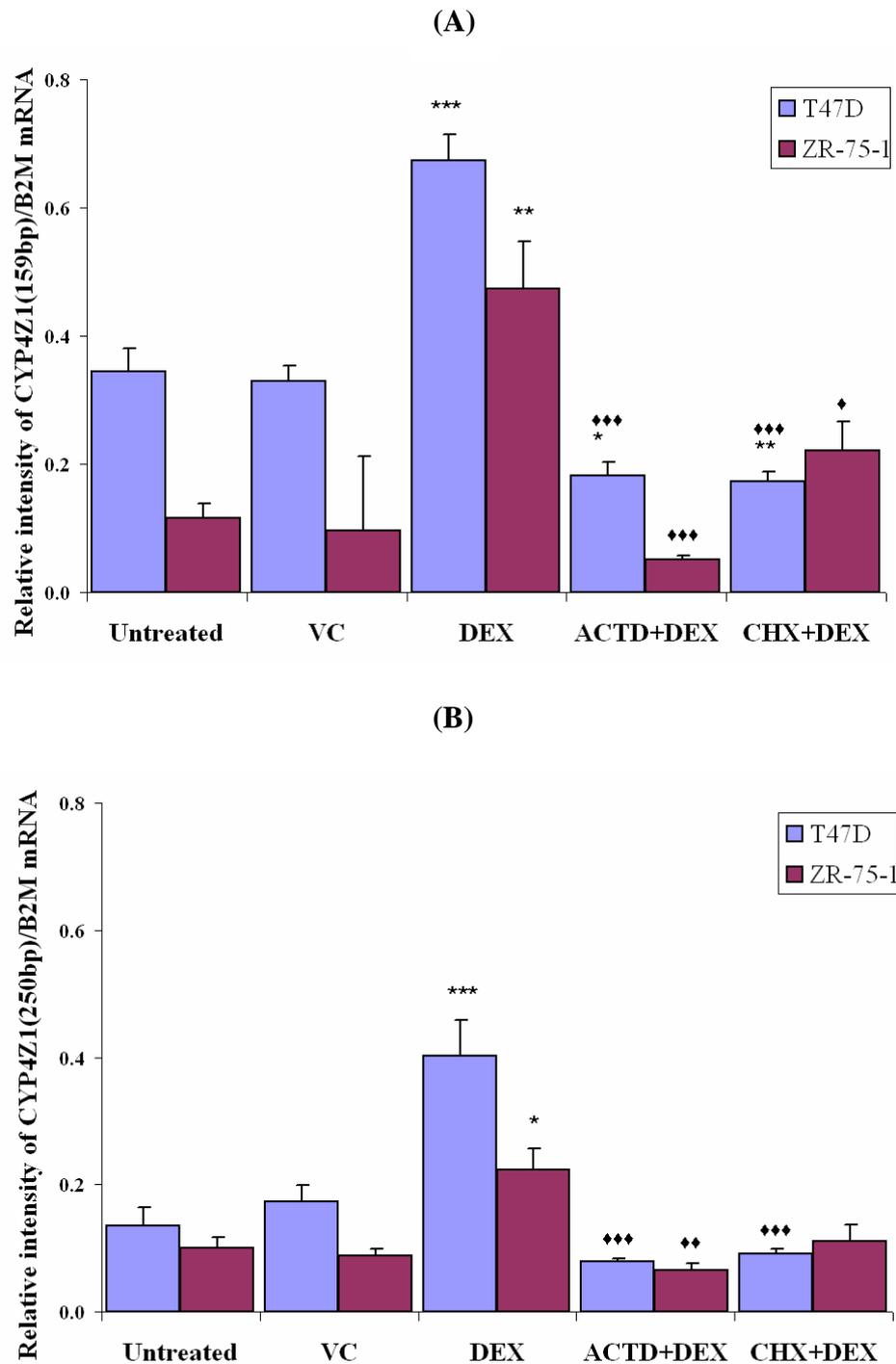


Figure 6.20: Effect of RNA and protein synthesis inhibitors on dexamethasone-mediated increase in CYP4Z1 mRNA in the T47D and ZR-75-1 cells. (A) CYP4Z1 (159 bp). (B) CYP4Z1 (250 bp). Data represent the mean \pm SEM of three independent experiments for each treatment showing the relative intensity of CYP4Z1 normalised against β_2 -microglobulin mRNA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with VC. ♦ $p < 0.05$, ♦♦ $p < 0.01$, ♦♦♦ $p < 0.001$ compared with DEX.

Abbreviations: VC; 0.1% (v/v) ethanol, DEX; dexamethasone (1 μ M), ACTD; actinomycin D (10 μ g/mL), CHX; cycloheximide (10 μ g/mL).

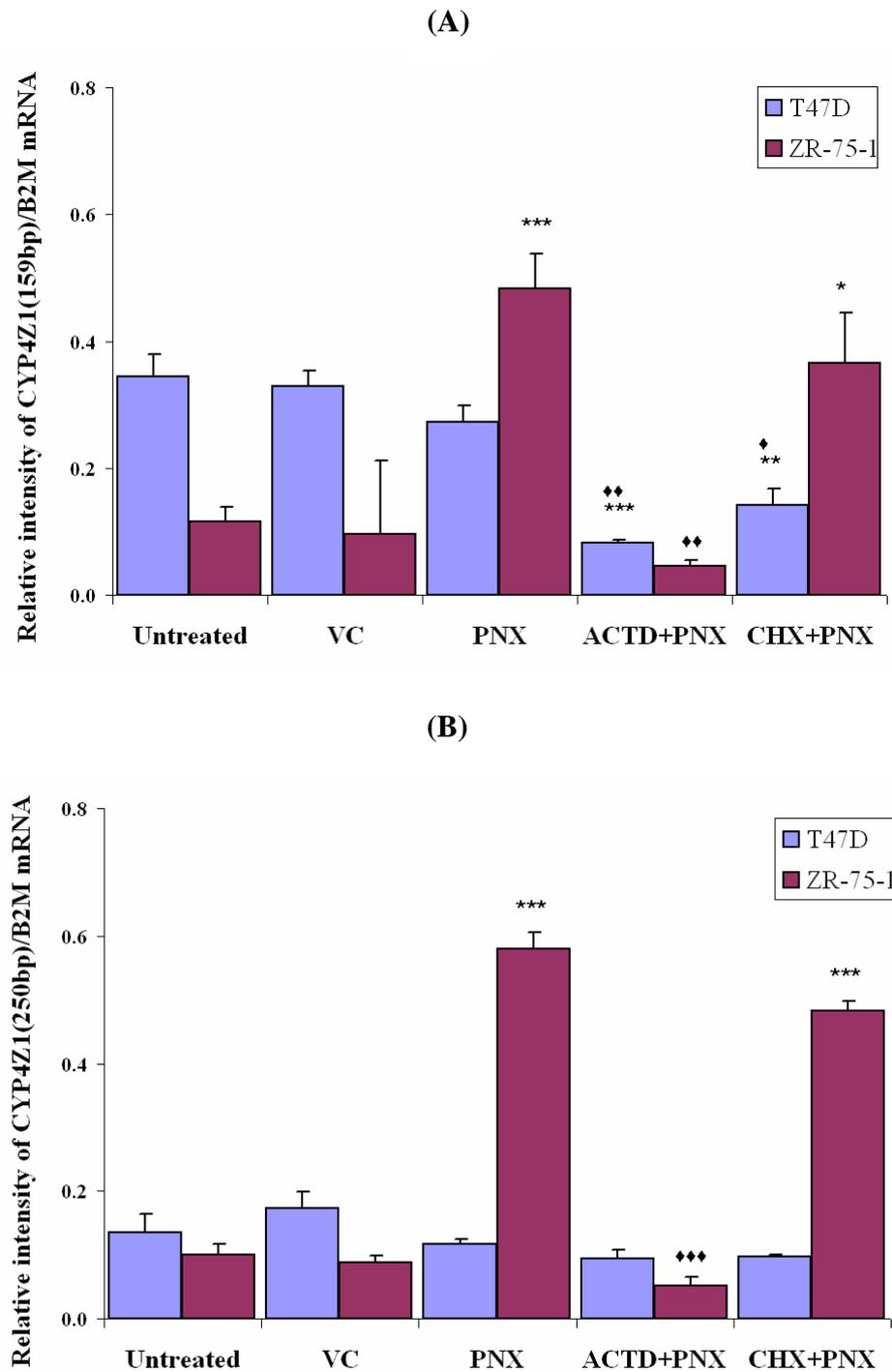


Figure 6.21: Effect of RNA and protein synthesis inhibitors on pirinixic acid-mediated increase in CYP4Z1 mRNA in the T47D and ZR-75-1 cells. (A) CYP4Z1 (159 bp). (B) CYP4Z1 (250 bp). Data represent the mean \pm SEM of three independent experiments for each treatment showing the relative intensity of CYP4Z1 normalised against β_2 -microglobulin mRNA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with VC. ♦ $p < 0.05$, ♦♦ $p < 0.01$, ♦♦♦ $p < 0.001$ compared with PNX.

Abbreviations: VC; 0.1% (v/v) ethanol, PNX; pirinixic acid (50 μ M), ACTD; actinomycin D (10 μ g/mL), CHX; cycloheximide (10 μ g/mL).

Table 6.1: Summary of results for the T47D cells illustrating differences in expression of CYP4Z1 (159 bp and ~250 bp) mRNA/ β_2 -microglobulin following co-treatment with either the nuclear receptor agonists plus or minus actinomycin D or cycloheximide using Tukey's test.

	Untreated		VC		PG		ACTD+PG		CHX+PG		DEX		ACTD+DEX		CHX+DEX		PNX		ACTD+PNX		CHX+PNX	
	159bp	250bp	159bp	250bp	159bp	250bp	159bp	250bp	159bp	250bp	159bp	250bp	159bp	250bp	159bp	250bp	159bp	250bp	159bp	250bp	159bp	250bp
Untreated			NS	NS	NS	NS	↑***	NS	NS	NS	↓***	↓***	↑**	NS	↑**	NS	NS	NS	↑***	NS	↑***	NS
VC	NS	NS			↓*	NS	↑***	NS	NS	NS	↓***	↓***	↑*	NS	↑**	NS	NS	NS	↑***	NS	↑**	NS
PG	NS	NS	↑*	NS			↑***	NS	↑***	NS	↓***	↓***	↑***	NS	↑***	NS	↑**	NS	↑***	NS	↑***	NS
ACTD+PG	↓***	NS	↓***	NS	↓***	NS			↓*	NS	↓***	↓***	NS	NS	NS	NS	↓**	NS	NS	NS	NS	NS
CHX+PG	NS	NS	NS	NS	↓***	NS	↑*	NS			↓***	↓***	NS	NS	NS	NS	NS	NS	↑*	NS	NS	NS
DEX	↑***	↑***	↑***	↑***	↑***	↑***	↑***	↑***	↑***	↑***			↑***	↑***	↑***	↑***	↑***	↑***	↑***	↑***	↑***	↑***
ACTD+DEX	↓**	NS	↓*	NS	↓***	NS	NS	NS	NS	NS	↓***	↓***			NS	NS	NS	NS	NS	NS	NS	NS
CHX+DEX	↓**	NS	↓**	NS	↓***	NS	NS	NS	NS	NS	↓***	↓***	NS	NS			NS	NS	NS	NS	NS	NS
PNX	NS	NS	NS	NS	↓**	NS	↑**	NS	NS	NS	↓***	↓***	NS	NS	NS	NS			↑**	NS	↑*	NS
ACTD+PNX	↓***	NS	↓***	NS	↓***	NS	NS	NS	↓*	NS	↓***	↓***	NS	NS	NS	NS	↓**	NS			NS	NS
CHX+PNX	↓***	NS	↓**	NS	↓***	NS	NS	NS	NS	NS	↓***	↓***	NS	NS	NS	NS	↓*	NS	NS	NS		

Abbreviations: VC; 0.1% (v/v) ethanol, PG; progesterone (2 μ M), DEX; dexamethasone (1 μ M), PNX; pirinixic acid (50 μ M), ACTD; actinomycin D (10 μ g/mL), CHX; cycloheximide (10 μ g/mL), ↓; decreasing, ↑; increasing, NS=not significant, * p <0.05, ** p <0.01, *** p <0.001

Table 6.2: Summary of results for the ZR-75-1 cells illustrating differences in expression of CYP4Z1 (159 bp and ~250 bp) mRNA/ β_2 -microglobulin following co-treatment with either the nuclear receptor agonists plus or minus actinomycin D or cycloheximide using Tukey's test.

	Untreated		VC		PG		ACTD+PG		CHX+PG		DEX		ACTD+DEX		CHX+DEX		PNX		ACTD+PNX		CHX+PNX	
	159bp	250bp	159bp	250bp	159bp	250bp	159bp	250bp	159bp	250bp	159bp	250bp	159bp	250bp	159bp	250bp	159bp	250bp	159bp	250bp	159bp	250bp
Untreated			NS	NS	NS	NS	NS	NS	↓**	↓***	↓**	↓*	NS	NS	NS	NS	↓**	↓***	NS	NS	↓*	↓***
VC	NS	NS			NS	NS	NS	NS	↓**	↓***	↓**	↓*	NS	NS	NS	NS	↓***	↓***	NS	NS	↓*	↓***
PG	NS	NS	NS	NS			NS	NS	↓*	↓***	↓**	NS	NS	NS	NS	NS	↓**	↓***	NS	NS	NS	↓***
ACTD+PG	NS	NS	NS	NS	NS	NS			↓**	↓***	↓**	↓**	NS	NS	NS	NS	↓**	↓***	NS	NS	NS	↓***
CHX+PG	↑**	↑***	↑**	↑***	↑*	↑***	↑**	↑***			NS	↓*	↑***	↑***	NS	↑***	NS	↓***	↑***	↑***	NS	↓**
DEX	↑**	↑*	↑**	↑*	↑**	NS	↑**	**	NS	↓*			↑***	↑**	↑*	NS	NS	↓***	↑***	↑**	NS	↓***
ACTD+DEX	NS	NS	NS	NS	NS	NS	NS	NS	↓***	↓***	↓***	↓**			NS	NS	↓***	↓***	NS	NS	↓**	↓***
CHX+DEX	NS	NS	NS	NS	NS	NS	NS	NS	NS	↓***	↓*	NS	NS	NS			↓*	↓***	NS	NS	NS	↓***
PNX	↑**	↑***	↑***	↑***	↑**	↑***	↑**	↑***	NS	↑***	NS	↑***	↑***	↑***	↑*	↑***			↑***	↑***	NS	NS
ACTD+PNX	NS	NS	NS	NS	NS	NS	NS	NS	↓***	↓***	↓***	↓**	NS	NS	NS	NS	↓***	↓***			↓**	↓***
CHX+PNX	↑*	↑***	↑*	↑***	NS	↑***	NS	↑***	NS	↑**	NS	↑***	↑**	↑***	NS	↑***	NS	NS	↑**	↑***		

Abbreviations: VC; 0.1% (v/v) ethanol, PG; progesterone (2 μ M), DEX; dexamethasone (1 μ M), PNX; pirinixic acid (50 μ M), ACTD; actinomycin D (10 μ g/mL), CHX; cycloheximide (10 μ g/mL), ↓; decreasing, ↑; increasing, NS=not significant, * p <0.05, ** p <0.01, *** p <0.001

6.4 Discussion

The results presented in this chapter provide an in depth look at the mRNA expression profile of candidate nuclear receptors (i.e. PGR, GCR and PPAR α) in the test cell lines. This should facilitate a better understanding of the association between expression of those receptors and CYP4Z1 mRNA. The CYP4Z1 mRNA was not induced in response to the nuclear receptor agonists (i.e. PGR, GCR and PPAR α) in the non CYP4Z1-expressing cell lines (i.e. MCF7 and MDA-MB-231). In addition, CYP4Z1 mRNA in the CYP4Z1-expressing cell lines, particularly the T47D and ZR-75-1 breast cancer cells, seems to be preferentially induced by different substrates and the pattern of expression differs between the cells lines.

The expression of nuclear receptors PGR, GCR and PPAR α in the cell lines was demonstrated and confirmed (Figures 6.2-6.7). The relationship between the basal expression of CYP4Z1 and those receptors is presented in Table 6.3. It is important to note that a positive correlation between CYP4Z1 mRNA and PPAR α expression was found in the T47D, ZR-75-1 and MDA-MB-468 breast cancer cells. However, an inverse relationship between CYP4Z1 expression and PPAR α mRNA was observed only in the normal breast MCF10A cells. PPAR α is involved in the metabolism of endogenous (i.e. fatty acids) and xenobiotic compounds (i.e. fibrate drugs, long-chain polyunsaturated fatty acids) (Hihi et al. 2002) and is highly expressed in the liver (Motojima and Hirai 2006). In mouse mammary gland, PPAR α expression has been shown to be associated with the regulation of steroid hormones during pregnancy and lactation, suggesting that PPAR α may play a role in mammary gland physiology and pathophysiology. The expression of PPAR α mRNA and protein was found to occur in normal breast and breast tumours (Roberts-Thomson 2000).

Table 6.3: The basal mRNA of interest genes

Gene Name	MCF10A	MCF7	T47D	ZR-75-1	ZR-75-30	MDAMB-231	MDA-MB-157	MDA-MB-468
CYP4Z1 (Figure 4.13)	Faint	-	++	+	-	-	-	+++
PGR (Figure 6.2)	-	++	+++	+	-	-	++	-
GCR (Figure 6.4)	Faint	-	-	++	-	-	Faint	Faint
PPARα (Figure 6.6)	++/ +++	-	++	++/ +++	Faint	Faint	+	+++

Note: +: indicates the level of intensity of the cDNA product by comparing in each gene.

In human breast cancer cell lines, Suchanek and colleagues reported that a higher level of PPAR α mRNA expression was found in the breast cancer ER-negative MDA-MB-231 cells than in the breast cancer ER-positive MCF7 cells (Suchanek et al. 2002). In addition, a previous study used quantitative real-time RT-PCR to demonstrate a relationship between ER mRNA and PPAR α expression in the breast cancer ER-positive ZR-75-1, T47D, MCF7 and BT-483 cells. The findings showed a significant (p value < 0.001) inverse relationship between these genes, a high level of ER expression was correlated with a low level of PPAR α mRNA (Faddy et al. 2006). In the current study, the presence of PPAR α mRNA in MDA-MB-231, T47-D and ZR-75-1 is in agreement with the previous studies at a qualitative level (Suchanek et al. 2002, Faddy et al. 2006). However, the current project did not detect the expression of PPAR α mRNA in the MCF7 cells and this does not correlate with the previous two studies. It should be noted that the lack of PPAR α mRNA in the MCF7 cells was correlated with the absence of CYP4Z1 expression in this cell line as shown in Figures 4.13 and 6.6 respectively. This suggests that the

regulation of CYP4Z1 mRNA may have a link with the expression of PPAR α mRNA.

These results are the first to show a positive relationship between CYP4Z1 expression and PPAR α mRNA.

The highest level of PPAR α mRNA was detected in the MDA-MB-468 cells and moderate to low levels of PPAR α expression were found in the MCF10A, ZR-75-1 and T47D cells. However, the induction of CYP4Z1 mRNA by PPAR α agonists was clearly shown only in the ZR-75-1 cells (Figure 6.13), not in the MDA-MB-468, T47D or MCF10A cells. These results may suggest that the presence of PPAR α was not directly associated with the induction of CYP4Z1 mRNA.

PPAR α needs to form a dimerisation with RXR to activate transcription and a PPAR α /RXR heterodimer binds to PPAR response elements (PPREs) in the regulatory region of target genes. Transcriptional activation or repression of target gene requires recruitment of co-activators or co-repressors respectively to the ligand binding domain of the gene. RXR is a heterodimerising partner for several members of the nuclear receptor super family such as PPAR α , PXR, CAR, VDR and RAR (Rastinejad 2001). It should be noted that these nuclear receptors play a crucial role in the regulation of several cytochrome P450 enzymes (i.e. PPAR α for CYP4A, CAR for CYP2B6, PXR for CYP3A, VDR for CYP24A1 and RAR for CYP26). Crosstalk between PPAR α and other members of the nuclear receptor may occur at an intracellular level, as they share DNA response elements in the regulatory region or ligands (i.e. steroid hormones and some xenobiotic compounds) in target genes (Motojima and Hirai 2006).

Evidence of crosstalk between the PPAR α signalling pathway and nuclear hormone receptor signalling pathway has been reported. Keller and colleagues (Keller et al. 1995)

demonstrated inhibitory crosstalk between the ER signalling pathway and expression of PPAR α . Due to competition for oestrogen response element (ERE) binding between PPAR α /RXR and oestrogen receptor, transcriptional activation of PPAR α /RXR heterodimerisation is inhibited by ER in target genes. These observations support the findings of Faddy and co-workers (Faddy et al. 2006) who demonstrated an inverse relationship between PPAR α and ER expression in breast cancer cell lines.

In addition, a previous study reported that PPAR α mRNA was induced by glucocorticoids in rat hepatocyte cultures, suggesting that the transcriptional activation of PPAR α was mediated through GCR (Lemberger et al. 1994). In this research, there was a lack of GCR mRNA (Figure 6.4, lane 5) in the T47D cells but when the cells were induced by the GCR agonist dexamethasone, an increase in CYP4Z1 mRNA was observed (Figure 6.12). A moderate level of PPAR α mRNA expression was detected in the T47D cells (Figure 6.6, lane 5). These findings may suggest that CYP4Z1 induction by the GCR agonist in the T47D cells is mediated through the PPAR α signalling pathway. However, information on protein-protein interaction of the PPAR α signalling pathway with other hormone nuclear receptor signalling pathways would be necessary to identify the regulation of CYP4Z1 expression in breast cancer cells.

To determine whether the nuclear receptor agonists' effect on CYP4Z1 mRNA is at the transcriptional or post-transcriptional level, CYP4Z1 mRNA was further investigated in the T47D and ZR-75-1 cells. These two cell lines were selected as they showed increased CYP4Z1 mRNA expression following treatment with the nuclear receptor agonist for 21 hours. Pre-treatment of the T47D and ZR-75-1 cells with the RNA synthesis inhibitor actinomycin D followed by one of the individual nuclear receptor agonists resulted in a significant decrease in CYP4Z1 mRNA levels, and inhibition of CYP4Z1 induction by

either progesterone, dexamethasone or pirinixic acid (Figures 6.17, 6.18 and 6.19). This indicates decreased transcription of CYP4Z1 and/or reduced stability.

Moreover, the induction of CYP4Z1 mRNA by all three agonists in the ZR-75-1 cells was not prevented by the protein synthesis inhibitor cycloheximide, ruling out the possibility that protein synthesis is required for CYP4Z1 mRNA induction. These findings suggest that the pre-existing expression of PGR, GCR or PPAR α mediated the progesterone, dexamethasone or pirinixic acid respectively for CYP4Z1 mRNA induction. Unlike the ZR-75-1 cells, CYP4Z1 mRNA induction by all three nuclear agonists in the T47D cells was significantly inhibited by cycloheximide (Figures 6.17, 6.18 and 6.19), suggesting that specific proteins are involved in the transcription or stability of CYP4Z1 mRNA by these nuclear receptor agonists.

This research demonstrated that a significant increase of CYP4Z1 mRNA in T47D cells is induced by progesterone and dexamethasone but not pirinixic acid. These observations are in agreement with those of Savas and co-workers who demonstrated CYP4Z1 mRNA induction in the T47D cells by either progesterone or dexamethasone, and suppression of CYP4Z1 induction by RU486 (the antagonist of PGR and GCR) (Savas et al. 2005).

However, the present study is the first to show that progesterone or dexamethasone affect CYP4Z1 mRNA at a pre-translational level. The CYP4Z1 mRNA induction profile has not been previously reported. This research demonstrated that CYP4Z1 mRNA in the ZR-75-1 cells is significantly elevated by either dexamethasone or pirinixic acid. These two agonists affect CYP4Z1 mRNA transcription and/or stability, and protein synthesis is not required.

CYP4Z1 mRNA in the ZR-75-1 cells was inducible by either dexamethasone or pirinixic acid, which is similar to CYP4A11 expression in the human hepatoma HepG2 cell line

(Savas et al. 2003). A study by Savas and colleagues (Savas et al. 2005) also demonstrated that CYP4X1 mRNA was induced by pirinixic acid. The CYP4Z1 gene is located in a cluster that contains CYP4A11 and CYP4X1 genes on chromosome 1p33 (Rieger et al. 2004). CYP4Z1 shares 54% and 52% sequence homology with CYP4X1 and CYP4A11 respectively (Savas et al. 2005). Comparative genomics of CYP4Z1 between human and rodent in a study by Nelson and colleagues (Nelson et al. 2004) has indicated that there are no orthologs expressed in mouse or rat, and CYP4Z1 may be human or primate specific. This could suggest overlapping substrate specificity among these genes, and CYP4Z1 could be responsive to peroxisome proliferator chemicals including the PPAR α mediated pathways. PPAR α is activated by a variety of fatty acids and previous studies demonstrated that high-fat diets are associated with an increase in breast cancer incidence (Lu et al. 1995).

In the T47D and ZR-75-1 cells, considerable variation was observed with the expected product size of 159 bp as well as with the alternative splice variant (~250 bp) (Figures 6.15 and 6.16), and there were differences in the induction profile with some treatments. However, this research has demonstrated that higher levels of CYP4Z1 mRNA induction were observed with the fragment of 159 bp than with the fragment of ~250 bp, particularly in the T47D cells (Figures 6.17, 6.18 and 6.19). As shown in Figure 6.1, removal of introns from pre-mRNAs is an essential process in eukaryotic gene expression. Alternative splicing of pre-mRNA generates the production of multiple mature mRNAs obtained from a single gene encoding different protein. Alternative pre-mRNA splicing and its regulation has an important role in human diseases including cancer and this observation is being exploited to identify novel targets for cancer therapeutics and diagnostic markers (Ben-Dov et al. 2008). Alternative splicing events can cause down regulation of a gene by producing a pre-mature stop codon in the mRNA, promoting the degradation of mRNA (Fedor 2008).

In conclusion, this chapter provides evidence of potential nuclear receptors regulating the CYP4Z1 gene, and the inducibility of CYP4Z1 in non CYP4Z1-expressing and CYP4Z1 expressing cell lines. Differential regulation of the PCR products in different cell lines and in response to different nuclear receptor agonists suggests alternative splicing may be under specific controls. Further investigation into protein expression in these cell lines, both the wild-type and alternative splice variant, is warranted as this may provide a novel target for cancer therapy.

Chapter 7

General Discussion

7.1 Introduction

The work presented in this thesis represents the first large scale study to characterise cytochrome P450 mRNA and cytochrome P450-regulatory nuclear receptor mRNA expression in a wide range of human breast cancer cell lines and compare this profile with a normal breast cell line. The relationship between cytochrome P450 profile and specific tumour characteristics (i.e. oestrogen receptor status, invasive phenotype and ethnicity) was investigated in Chapter 4. Of particular interest in the profile obtained was the CYP4Z1 gene as findings from the validation (Chapter 3) and its expression in a panel of the cell lines (Chapter 4) raised the possibility of alternative splicing. The results in Chapter 5 demonstrates intron retention at the boundary of exon 5 and exon 6 in the CYP4Z1 gene using specific primer sets within the regions of both intron and exon. In addition, this study has established *in vitro* models for the study of regulatory mechanisms of the CYP4Z1 gene using the T47D and ZR-75-1 cells to determine whether the CYP4Z1 gene is regulated at a transcriptional or translational level. Alteration of the CYP4Z1 expression pattern after induction and combination treatment with RNA or protein synthesis inhibitor was also determined with regard to the presence of intron retention (Chapter 6).

7.2 Profile of cytochrome P450 mRNA in breast cancer cell lines

As mentioned previously, the results in Chapter 4 provide detailed information on differential expression of cytochrome P450s in human breast tumour cultured cells of different tumour origins. Results showed that mRNA from six cytochrome P450s, CYP1B1, CYP2D6, CYP2J2, CYP2R1, CYP2U1 and CYP4X1, was found in all normal

breast and breast cancer cell lines. However, the CYP2J2 and CYP2U1 mRNA expression profiles are probably the most exciting as lower levels of mRNA expression were observed in the normal breast cells than in almost all of the breast cancer cells. If their protein expression shows the same trend as their mRNA expression, this may suggest that CYP2J2 and CYP2U1 exhibit tumour-specific expression in breast cancer cells and they can be used as prognostic biomarkers. This research also suggests that CYP2J2 and CYP2U1 may represent new targets for the development of cancer therapeutics. To date, the physiological role of CYP2J2 and CYP2U1 in anticancer drug metabolism remains unknown.

Although no difference in cytochrome P450 mRNA expression was observed between the different ethnic groups used in this study. Some trends were suggested in particular a potential inverse relationship of cytochrome P450 mRNA (i.e. CYP2A6, CYP2C8, CYP2C18, CYP2F1 and CYP4Z1) between ER status and ethnic groups (i.e. ER-positive Caucasian and ER-negative Afro-Caribbean) was highlighted. In addition, CYP2A6 and CYP2C8 mRNA were found to be primarily expressed in the non-invasive Caucasian and invasive Afro-Caribbean breast cancer cells in these preliminary studies.

CYP2A6 and CYP2C8 are involved in the inactivation of anticancer drugs letrozole and paclitaxel respectively. The potent aromatase inhibitor letrozole is used as adjuvant hormonal therapy for postmenopausal women with ER-positive tumours (Goldhirsch et al. 2006). Paclitaxel is widely used as adjuvant chemotherapy in first-line treatment in women with ER-negative tumours (Goldhirsch et al. 2007). In the current investigation, CYP2A6 mRNA was not detected in the ER-positive Afro-Caribbean breast cancer cells and no CYP2C8 mRNA was found in the ER-negative Caucasian breast cancer cells. This variability in letrozole and paclitaxel metabolism between different ethnic groups may help to explain unpredictability of clinical outcome. This research suggests potential

similarities in the ER-positive Caucasian and ER-negative Afro-Caribbean breast cancer cell lines which warrants further investigation.

It is known that the hepatic CYP2B6 enzyme plays an important role in the conversion of anticancer prodrug cyclophosphamide to its active form (phosphoramidate mustard and acrolein) which is then delivered to tumour cells via blood circulation (Stoff-Khalili et al. 2006). Cyclophosphamide is one of the drugs in the TAC regimen which is commonly used for women with metastatic breast cancer who have ER-negative tumours (Ahluwalia et al. 2005). To minimise toxic effects to normal cells, MetXia which encodes human CYP2B6 activating cyclophosphamide in tumours, is injected directly into the malignant cells prior to treatment with cyclophosphamide (Braybrooke et al. 2005). In the present study, it was surprising that CYP2B6 mRNA was detected in the ER-positive Caucasian/Afro-Caribbean and the ER-negative Afro-Caribbean breast cancer cells but not in the invasive ER-negative Caucasian MDA-MB-231 breast cancer cell line. Future studies should investigate CYP2B6 protein expression in breast cancer cell lines presenting CYP2B6 mRNA. This preliminary study highlights the possibility that if the expression of protein correlates well with mRNA expression, a clinician in future may have to adjust the dosage of cyclophosphamide/MetXia.

7.3 Characterisation of CYP4Z1

Results in Chapter 5 indicate the possibility of intron retention between exon 5 and exon 6 in the CYP4Z1 gene. A similar pattern of expression of CYP4Z1 (i.e. 159 bp and ~250 bp) was found in all four cell lines. As described previously, the purified DNA of these fragments was analysed by restriction digest and DNA sequencing to confirm that the additional band was not a PCR artefact.

Alternative splicing is associated with alterations of transcriptional regulation for a certain gene. Alternative splice variants of genes can be found in a specific type of tissue and may play a crucial role in transcriptional regulation of certain proteins. Variability in drug metabolising enzymes including cytochrome P450s in a specific tissue has major clinical consequences, and has been associated with adverse drug side effects and susceptibility to cancer (Stamm et al. 2005). A study by Christmas and colleagues (Christmas et al. 2001) demonstrated intron and exon splice junctions in the CYP4F3 gene in the liver and neutrophils. These findings indicate tissue-specific splicing expression (Christmas et al. 1999), and an alteration in the regulation of CYP4F3, affecting its substrate specificity. This may also occur in other members of the cytochrome P450 family 4, including the CYP4Z1 gene, given that they have at least 40% amino acid sequence homology and overlapping properties (Nebert et al. 1989).

The current investigation demonstrated the presence of CYP4Z1 mRNA in the ER-positive Caucasian ZR-75-1 and the ER-negative Afro-Caribbean MDA-MB-468 breast cancer cells and those are novel observations. Particularly as CYP4Z1 mRNA expression appears to be specific to the non-invasive phenotype in both ethnic groups. These preliminary findings may also suggest a relationship between CYP4Z1 and ethnic differences with regard to ER status and stage of breast cancer.

7.4 Regulation of CYP4Z1

As mentioned in Chapter 6, this project is the first study to establish *in vitro* cell models for study of the regulatory mechanisms of CYP4Z1. Breast cancer T47D and ZR-75-1 cell lines were used to evaluate whether CYP4Z1 mRNA induction is regulated at transcriptional or translational level, because CYP4Z1 mRNA in these cells was highly inducible by the nuclear receptor agonists according to results from induction experiments. In T47D, a significant increase of CYP4Z1 mRNA was observed in either the cells induced

by progesterone (p value < 0.05) or dexamethasone alone (p value < 0.001). ZR-75-1 showed a significant increase of CYP4Z1 expression in the cells treated with either dexamethasone (p value < 0.01) or pirinixic acid (p value < 0.001). These findings suggest that there are different receptor mechanism involved in CYP4Z1 mRNA induction in T47D and ZR-75-1.

In conjunction, pre-treatment of these two cell lines with the RNA synthesis inhibitor actinomycin D followed by the nuclear receptor agonists resulted in a significant decrease in (p value < 0.05) CYP4Z1 mRNA levels and inhibited CYP4Z1 induction by either progesterone, dexamethasone or pirinixic acid, indicating that these nuclear receptor agonists have effects on CYP4Z1 mRNA stability. In contrast, the protein synthesis inhibitor cycloheximide did not inhibit CYP4Z1 mRNA induction by these agonists in the ZR-75-1 cells so protein synthesis is not involved. These results suggest that CYP4Z1 mRNA induction in T47D and ZR-75-1 is mediated through differential cell type specific regulatory mechanisms. The detection of CYP4Z1 induction in the T47D cells is in agreement with a study by Savas and colleagues (Savas et al. 2005). The results in the ZR-75-1 cells are a novel observation, suggesting that the ZR-75-1 cells are suitable to use as a model to investigate the regulation of CYP4Z1.

In terms of alternative splicing in CYP4Z1, comparative analysis of CYP4Z1 mRNA between the fragments of 159 bp and ~250 bp was performed to clarify whether this event changes the regulation of the gene or not. A difference in the induction patterns was observed between two bands. These observations suggest that the alternative splice variant of CYP4Z1 may have a different regulation mechanism. It is essential to further investigate CYP4Z1 protein expression in these cells to clarify the mechanisms involved.

7.5 Conclusions

The main points emerging from the current study are:

(a) The relationships between cytochrome P450 mRNA expression profile and specific tumour characteristics are observed in the current study:

- CYP2B6 which plays a crucial role in the activation of cyclophosphamide was not detected in the invasive ER-negative Caucasian breast cancer cells.
- Some of the findings in this study are of a preliminary nature and warrant further investigation particularly as to whether the individual cytochrome P450 protein is expressed. But also as to whether the trends observed in this thesis are confirmed in clinical patient samples (at both mRNA and protein level). The trends in cytochrome P450 expression suggested in this thesis are detailed as follows:
 - An inverse relationship of CYP2A6, CYP2C8, CYP2C18, CYP2F1 and CYP4Z1 mRNA between ethnicity and ER status (ER-positive Caucasian and ER-negative Afro-Caribbean breast cancer cell lines).
 - An inverse relationship of CYP2A6 and CYP2C8 mRNA between ethnicity and invasive phenotype (Caucasian non-invasive and Afro-Caribbean invasive breast cancer cell lines).
 - CYP4Z1 and CYP2F1 mRNA were specific for non-invasive phenotype.

(b) Two distinct bands obtained from the CYP4Z1 PCR product were elucidated by specific primer sets, and this project does suggest potential intron retention at the boundary of exon 5 and exon 6 in the CYP4Z1 gene.

(c) Two out of the four CYP4Z1-expressing cell lines, T47D and ZR-75-1, seem to be preferentially induced by different substrates and the pattern of expression differs between the cells lines:

- CYP4Z1 mRNA in T47D was induced only by the PGR or GCR agonist whereas CYP4Z1 mRNA in ZR-75-1 was elevated by either the GCR or PPAR α agonist.

(d) CYP4Z1 induction by progesterone, dexamethasone or pirinixic acid is inhibited by actinomycin D, suggesting that these agonists have effects on CYP4Z1 mRNA transcription or stability. However, CYP4Z1 induction in the ZR-75-1 cells is not prevented by cycloheximide, indicating that CYP4Z1 mRNA induction by these agonists is unlikely to involve protein synthesis. These suggest that the inducibility of CYP4Z1 mRNA is mediated through differential cell type specific regulatory mechanisms and there is evidence for differential regulation of the splice variants (CYP4Z1~250 bp).

7.6 Future work

This investigation has provided comprehensive information on the differential cytochrome P450 mRNA expression among breast cancer cell lines with different characteristics, the exploration of alternative splicing in CYP4Z1, and clarification of the regulatory mechanism of the CYP4Z1 gene in the breast cancer cell lines. These findings raise a number of questions though, and further study in these areas is likely to lead to a better understanding of the role of cytochrome P450 enzymes in breast cancer cell.

The expression of CYP2A6, CYP2B6, CYP2C8, CYP2J2, CYP2F1, CYP2U1 and CYP4Z1 mRNA should be investigated by looking at their protein expression in test cell lines using western blotting or immunocytochemistry (ICC) followed by cytochrome P450 expression in clinical samples. It is known that mRNA expression does not always correlate with protein expression, for example the expression of CYP1B1 in normal tissues (i.e. breast, brain and testis) (Murray et al. 1997). Therefore, a demonstration of protein expression in these cell lines is required. If protein expression is detected in these cell lines, they could be established as a model system for the development of novel cancer therapeutics. Alternatively, they could be of benefit in the design of more effective drug regimens for breast cancer patients.

The data presented here provides a clearer understanding of the presence/absence of cytochrome P450 mRNA using qualitative and semi-quantitative RT-PCR. The use of a quantitative PCR method would be useful in further investigations as it would increase the sensitivity of the method.

Detection and evaluation of an intron retention event in the CYP4Z1 gene could be achieved through the establishment of a specific cell line stably expressing the human CYP4Z1 gene, identification of specific region of intron retention and investigation of protein expression. This would provide a better understanding of the presence of abnormal transcripts and a functional gene of CYP4Z1.

In order to investigate whether alternative splicing in CYP4Z1 is specific for breast cancer or other types of cancer, determination of this event in a variety of tumour cell lines would allow a clearer understanding of the involvement of CYP4Z1 expression in the carcinogenic process.

The regulation of CYP4Z1 in the T47D and ZR-75-1 cells is mediated through different receptors. The current study was carried out using a single set of concentrations of nuclear receptor agonists and a 21-hour of treatment as described previously by Savas and co-workers (Savas et al. 2005). Varying concentrations of agonists and incubation time would be a useful approach to elucidate the regulatory mechanisms of CYP4Z1. The present study employed semi-quantitative RT-PCR method; it would be more accurate if quantitative real-time PCR was instead used to determine mRNA level. Correlation between mRNA and protein expression would be required to explain the mechanism of regulation in breast cancer cells.

Transcriptional regulation of CYP4Z1 by nuclear receptors requires further examination to clarify the factors involved in the regulatory mechanisms and establish whether CYP4Z1 induction is connected with the expression of PGR, GCR or PPAR α . Identification of a molecular mechanism of functional cross-talk between nuclear receptors would provide a theoretical basis for development of novel therapeutic strategies and design of novel compounds for treatment of breast cancer.

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Appendix A

Table A.1: List of chemicals and reagents.

Supplier	Chemical/reagent (Catalogue number)
Fisher Scientific Ltd. (Leicestershire, UK)	<ul style="list-style-type: none"> ▪ Agarose (BPE1356-100) ▪ Ethylenediaminetetraacetic acid (EDTA) (BPE120-500) ▪ Tris base (T/P/630/53)
Invitrogen Ltd. (Paisley, UK)	<ul style="list-style-type: none"> ▪ 0.4% (w/v) trypan blue stain (15250-061) ▪ DMEM/Ham's F12 with GlutamaxTMI (31331-093) ▪ Dulbecco's Modified Eagle's Medium (DMEM) with GlutamaxTMI (61965-026) ▪ Epidermal growth factor (13247-051) ▪ Foetal bovine serum (FBS) (10108-165) ▪ Horse serum (26050-070) ▪ Insulin, human recombinant Zinc (12585-014) ▪ Leibnitz's (L15) with GlutamaxTMI (31415-086) ▪ Low melting point agarose gel (15517-014) ▪ Mixtures of enicillin (10,000 U/mL) and streptomycin (10,000 µg/mL) solution (15140-122) ▪ RPMI 1640 with (61870-010) ▪ Sodium pyruvate MEM (11360-039) ▪ Trypsin-EDTA (0.05% w/v trypsin with 0.53 mM EDTA•4Na) (25300-054)
Promega Ltd. (Southampton, UK)	<ul style="list-style-type: none"> ▪ PCR markers 50-1000 bp (G3161)
Qiagen Ltd. (Crawley, UK)	<ul style="list-style-type: none"> ▪ RNeasy Plus Mini Column kit (74134)
Quadrantech Diagnosis Ltd. (Surrey, UK)	<ul style="list-style-type: none"> ▪ Cholera toxin (101B)
Roche Diagnostics Ltd. (Lewes, UK)	<ul style="list-style-type: none"> ▪ <i>Taq</i> DNA polymerase (11418432001) ▪ Deoxynucleotide triphosphates (dNTPs) (11581295001) ▪ High pure PCR product purification kit (11732668001)
Sigma Aldrich Ltd. (Poole, UK)	<ul style="list-style-type: none"> ▪ 3 M sodium acetate buffer solution (S7899) ▪ Actinomycin D (A9415) ▪ Boric acid (B6768) ▪ Cycloheximide (C1988) ▪ Dexamethasone (D4902) ▪ Diethyl pyrocarbonate (DEPC) (D5758) ▪ Dimethylsulphoxide (DMSO) (D5879) ▪ Ethanol (E7023) ▪ Ethidium bromide (E1385) ▪ Hydrocortisol solution (H6909) ▪ Isopropanol (I9516) ▪ Moloney Murine Leukemia virus (M-MLV) reverse transcriptase (M1302) ▪ Phosphate buffered saline (PBS) tablets (P4417) ▪ Pirinixic acid (C7081) ▪ Restriction endonuclease: <i>Alu</i>I (R6885), <i>Eco</i>RI (R6265), <i>Hae</i>III (R56280), <i>Msp</i>I (R4506), <i>Pst</i>I (R7023), <i>Rsa</i>I (R4756), <i>Ssp</i>I (R6759) and <i>Xho</i>I (R6379) ▪ β-mercaptoethanol (M6250) ▪ Progesterone (P8783)
Sigma-Genosys Ltd. (Haverhill, UK)	<ul style="list-style-type: none"> ▪ All the specific oligonucleotide primers ▪ Oligo P(dT)₁₃ primers

Appendix B

B.1 Preparation of RNase-free solution

Ribonuclease (RNase) is a major concern in molecular biology experiments as it is a nuclease that catalyses the hydrolysis of RNA into smaller components, leading to a short lifespan for any RNA. Diethyl pyrocarbonate (DEPC), a histidine-specific alkylating agent, is an effective nuclease inhibitor. DEPC treated (RNase-free) solution is routinely used for handling RNA, effectively reducing the risk of RNA being degraded by environmental RNases. Typically a 0.1% solution is used to inactivate the environmental RNases by the covalent modifications of the histidine residues.

All solutions used in this study for PCR or molecular biology techniques were pre-treated 0.1% (v/v) DEPC. A 0.1 mL aliquot of DEPC was added into 100 mL of the solution to be treated. As DEPC is extremely viscous so the mixture was stirred for 2 hours to bring the DEPC into solution prior to autoclaving for 15 minutes at 15 lb/in² to inactivate traces of DEPC

Appendix C

Table C.1: Components of the digestion buffers.

Buffer for restriction enzymes	Component (1x)
Buffer SA (For <i>AluI</i>)	<ul style="list-style-type: none"> ▪ 33 mM Tris-acetate ▪ 66 mM Potassium acetate ▪ 10 mM Magnesium acetate ▪ 0.5 mM dithiothreitol (DTT) ▪ pH 7.9
Buffer SB (For <i>BamHI</i>)	<ul style="list-style-type: none"> ▪ 10 mM Tris-HCl ▪ 100 mM NaCl ▪ 5 mM Magnesium chloride ▪ 1 mM 2-Mercaptoethanol ▪ pH 8.0
Buffer SH (For <i>EcoRI</i> , <i>PstI</i> and <i>SspI</i>)	<ul style="list-style-type: none"> ▪ 50mM Tris-HCl ▪ 100 mM NaCl ▪ 10 mM MgCl₂ ▪ 1 mM dithioerythritol ▪ pH 7.5
Buffer SL (For <i>RsaI</i> and <i>MspI</i>)	<ul style="list-style-type: none"> ▪ 10 mM Tris-HCl ▪ 10 mM MgCl₂ ▪ 1 mM dithioerythritol (DTE) ▪ pH 7.5
Buffer SM (For <i>HaeIII</i>)	<ul style="list-style-type: none"> ▪ 10 mM Tris-HCl ▪ 50 mM NaCl ▪ 10 mM MgCl₂ ▪ 1 mM dithioerythritol (DTE) ▪ pH 7.5

