Chemotherapeutic drugs, 5-fluorouracil and cisplatin, differentially affect expression of drug metabolising enzyme genes in an oesophageal cancer cell line

by

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Declaration

I hereby certify that this dissertation submitted by me for the M.Sc. Med (Human Genetics) degree at the University of Cape Town is based on my original work and has not been previously submitted for another degree at this or any other university. I waive copyright of the dissertation in favour of the University of Cape Town. I declare that this work is not plagiarised and that each contribution to and quotation in this thesis from the work of others has been cited and referenced.

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ABSTRACT

Cancer is a leading cause of death worldwide. Oesophageal cancer in particular is the sixth most common cause of cancer deaths globally and its incidence and mortality rates in Southern Africa are among the highest in the world. One of the major challenges with cancer treatment is the vast variability in patient response to chemotherapy, which is predominantly due to genetic variability. The most relevant genes in this context encode the CYP and GST drug metabolising enzymes (DMEs) as these enzymes metabolise up to 90% of clinically-prescribed medication. Patients are also exposed to a variety of other compounds that along with chemotherapeutic drugs may alter DME gene expression. Changes in DME gene expression influence the therapeutic outcomes for patients; thus, understanding the effects of drugs and compounds on the expression of DMEs is crucial for the advancement of personalised medicine.

The aim of this study was to determine the effects of two commonly-used chemotherapeutic drugs, as well as a *CYP*-inducing compound, on the differential expression of four pharmacogenetically relevant DME-encoding genes, CYP1A1, 1A2, 1B1 and GSTP1, in a human oesophageal cancer cell line.

WHCO1 cells (derived from a human oesophageal squamous cell carcinoma) were treated with the chemotherapeutic drugs 5-fluorouracil (5-FU), cisplatin (CDDP), and the model *CYP*-inducer benzo[a]pyrene (BP). Total RNA and proteins were extracted from the cells at various time points following treatment. Changes in *CYP1A1, CYP1A2, CYP1B1* and *GSTP1* gene expression were determined by quantitative real-time PCR, and the corresponding changes in protein expression were determined by western blotting.

In most cases, *CYP* mRNA expressions were slightly but significantly enhanced by 5-FU but down-regulated by CDDP exposures alone, and as predicted, BP led to a robust increase in expression of all three *CYP* genes. The changes in mRNA levels observed with 5-FU or CDDP alone were mostly not reflected at the protein level. Additionally, the induction levels of *CYP1A1* and *CYP1A2* mRNA caused by BP were lower in the presence of 5-FU or CDDP, particularly 24

hours following drug treatment, which were reflected at the protein levels. On the other hand, *CYP1B1* mRNA and protein expressions caused by BP were lower at earlier time points in the presence of 5-FU, but enhanced in the presence of CDDP. *GSTP1* mRNA expression was significantly affected by both 5-FU and CDDP, whilst GSTP1 protein expression remained unaffected by the treatment with any of the compounds. Additionally, combining either 5-FU or CDDP with BP induced early apoptosis in the WHCO1 cells.

The commonly-used chemotherapeutic drugs 5-FU and CDDP influenced the mRNA expression levels, and in some cases, the corresponding protein expression levels, of four pharmacogenetically important DMEs, both individually and in the presence of BP. This has important clinical consequences for oesophageal cancer patients undergoing chemotherapy, who are prescribed drugs including 5-FU and CDDP, and are also likely to be exposed to *CYP*-inducing medications and compounds on a daily basis. Furthermore, the observed induction of tumour cell apoptosis by the combination treatments may suggest a potential mechanism for improving current chemotherapy. These findings provide the basis for future pharmacogenetics research, which is key to the progression towards personalised medicine, a field that holds promise as a means to ensure greater drug safety and efficacy.

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All my family, especially my loving husband Waseem and my precious little sisters Adhila and Saadiyah, who have nurtured, supported and motivated me without fail and in ways that nobody else could.

Dedication

This work is dedicated to my beloved parents, Ebrahim and Shenaaz Mayet, who taught me that knowledge is something no one can take away from me. Thank you for sacrificing so many things in your lives so that I wouldn't have to sacrifice anything in mine.

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Abbreviations

3′	3 prime
5′	5 prime
5-FU	5-fluorouracil
α	alpha
β	beta
ºC	degree Celsius
µg	micrograms
µL	microlitres
µM	micromolar
%	percentage
ADRs	adverse drug reactions
AhR	arylhydrocarbon receptor
ARNT	arylhydrocarbon receptor nuclear translocator
ASR	age-standardised rate
BP	benzo[a]pyrene
BLAST	Basic Local Alignment Search Tool
bp	base pairs
CDDP	cis-diamminedichloroplatinum(II)
Cdk2	cyclin-dependent kinase 2
cDNA	complementary DNA
cm	centimetres
CMV	CDDP + Methotrexate + Vinblastine
CO_2	carbon dioxide
Ct	threshold cycle
CYP	cytochrome P450

DCF	Docetaxel + CDDP + 5-FU
DEPC	diethylpyrocarbonate
dH_2O	distilled water
DHFU	dihydrofluorouracil
DME	drug metabolising enzyme
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DPD	dihydropyrimidine dehydrogenase
DRE	dioxin response element
dTT	dithiothreitol
ECF	Epirubicin + CDDP + 5-FU
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EH	epoxide hydrolase
et al.	*et alia* (and others)
F	forward
FdUMP	fluorodeoxyuridine monophosphate
FdUTP	fluorodeoxyuridine triphosphate
FUTP	fluorouridine triphosphate
g	gram
GAPDH	glyceraldehyde-3-phosphate
GST	glutathione-S-transferase
GSTP1	glutathione-S-transferase-π
HCl	hydrochloric acid
HMGB1	high-mobility group box-1
HRP	horseradish peroxidase

hrs	hours
HSP90	heat shock protein 90
IARC	International Agency for Research on Cancer
IC_{50}	inhibitory concentration (half maximal)
IgG	immunoglobulin G
IV	intravenous
L	litres
m^2	cubic metres
MCF	Methotrexate + Cyclophosphamide + 5-FU
mg	milligrams
mL	millilitres
mM	millimolar
mm	millimetres
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NLS	nuclear localisation signal
nm	nanometres
Nrf2	NF-E2 p45-regulated factor
OD	optical density
OPRT	orotate phosphoribosyltransferase
P value	probability value
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline-Tween-20
PCR	polymerase chain reaction
pH	percentage hydrogen
PM	poor metaboliser
pmol	picomoles

PRPP	phosphoribosyl pyrophosphate
Pt	platinum
qRT-PCR	quantitative real-time polymerase chain reaction
R	reverse
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RR	ribonucleotide reductase
SDS	sodium dodecyl sulphate
SNP	single nucleotide polymorphism
TBE	tris-borate-EDTA
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TK	thymidine kinase
Tris	tris hydroxymethyl aminomethane
TS	thymidylate synthase
TTP	thymidine triphosphate
UK	uridine kinase
UM	ultra-rapid metaboliser
UP	uridine phosphorylase
UTP	uridine triphosphate
UV	ultraviolet
V	volts
v	version
W	age-standardised rate

1. INTRODUCTION

1. 1 Cancer epidemiology and treatment

Cancer is a leading cause of death worldwide, and has been identified by the World Health Organisation as one of the four primary threats to human health and development. There were 14.1 million new cancer diagnoses across the world in 2012, responsible for 8.2 million deaths. It has been estimated that by the year 2030, there will be 22.2 million new cancer cases and over 13.1 million cancer deaths annually. Currently, cancers of the breast, prostate, lungs, colorectum and cervix constitute the most common cancers globally and account for more than half of all cancer deaths (GLOBOCAN 2012, IARC).

About 65% of all cancer deaths occur in low to middle-income countries. In Africa, the burden of cancer has been estimated to increase from 715,000 new cases and 542,000 deaths in 2008, to double by the year 2028 (Bray *et al*., 2012). The incidence and mortality patterns of cancers in Africa vary greatly across regions. In Southern Africa, oesophageal cancer is among the top five most common cancers (Figure 1.1).

1.1.1 Oesophageal cancer in Southern Africa

Oesophageal cancer is the fourth most common cancer among males and the fifth most common among females, in Southern Africa (Figure 1.1). Furthermore, it is the sixth most common cause of cancer deaths globally, with an estimated 400,000 deaths in 2012. Generally, oesophageal cancer exhibits a higher frequency in males than in females, typically 2-4 fold higher in most countries.

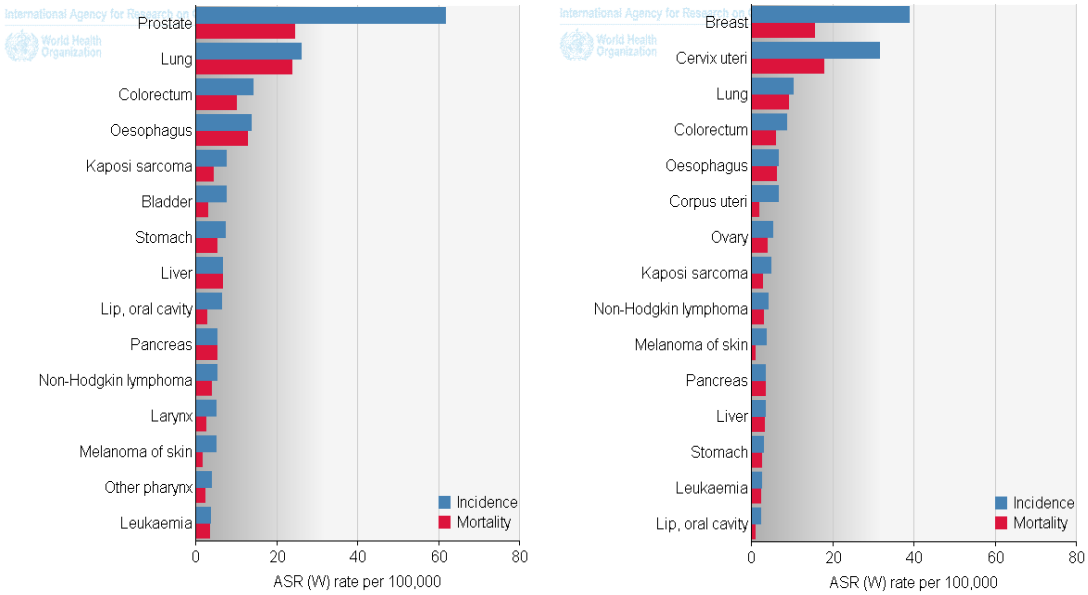


Figure 1.1 Ranking of cancers by incidence and mortality in Southern Africa. Oesophageal cancer ranks as the fourth most common cancer in males (left) and the fifth most common cancer in females (right). Age-standardised rate (ASR or W) refers to the number of new cases or deaths per 100,000 people per year, in a population with a standard age structure. (Adapted from http://www.globocan.iarc.fr, GLOBOCAN 2012, IARC)

Southern Africa exhibits some of the highest mortality rates for oesophageal cancer worldwide (Figure 1.2), with a rate of 12.8 (per 100,000) in men, second only to Eastern Asia (14.1), and a rate of 6.2 in women, second to Eastern Africa (7.3). Compared to other parts of Africa, the incidence and mortality rates of oesophageal cancer in Southern Africa are over 7 times higher among men and 4 times among women. In particular, the East Cape Province (former Transkei area) of South Africa has recorded exceptionally high incidence and mortality rates for oesophageal cancer, accounting for as much as 80% of all cancer deaths (Somdyala *et al*., 2010). As is the case in many Western countries, tobacco-smoking and alcohol consumption have been linked to the high incidence of oesophageal cancer in the Eastern Cape; however, these factors are not sufficient to explain the high incidence on a global scale (Cook, 1971). Dietary deficiencies are also thought to be responsible for the development of oesophageal cancer in this region, and additionally, fungal toxins from home-grown maize (Gelderblom *et al.*, 1988; Marasas *et al.*, 1988;

Pacella-Norman *et al.*,2002). The survival rate of oesophageal cancer when compared to other cancers is particularly poor, with a 5-year survival rate of just 5-10%, with 75% of patients dying within one year of diagnosis (Hiyama *et al.*, 2007). There is therefore an urgent need for new and more effective treatment regimens to improve the outcome for these patients.

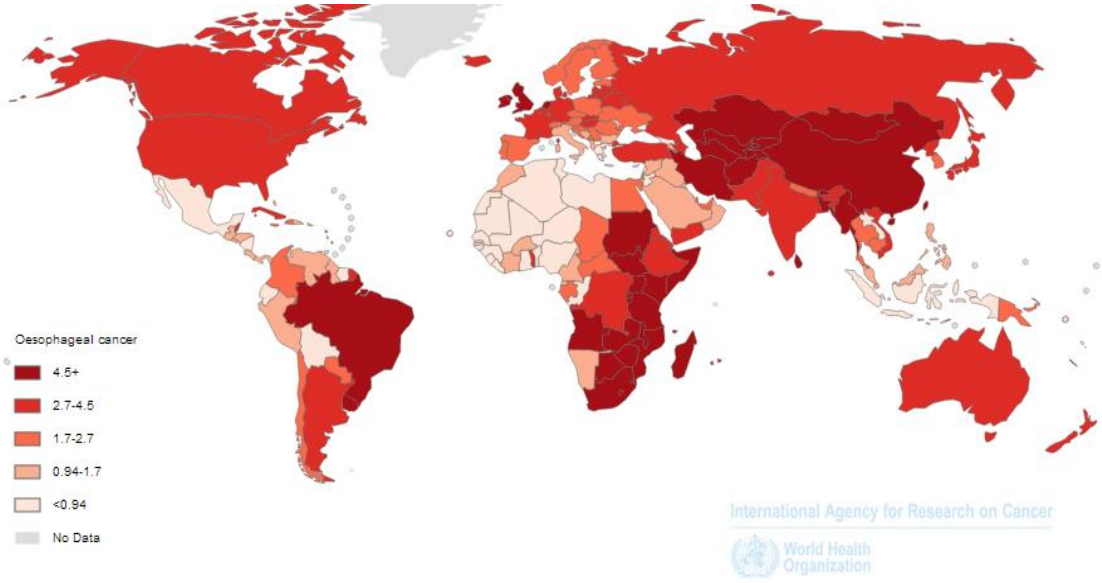


Figure 1.2 Map depicting the global burden of oesophageal cancer. Southern Africa displays some of the highest mortality rates for oesophageal cancer worldwide. Rates are age-standardised and are given per 100,000 people. (Adapted from http://www.globocan.iarc.fr, GLOBOCAN 2012, IARC)

The modality of treatment is specific to the type and stage of the cancer (e.g. type and stage of cancer, solid tumours vs. haematological malignancies) as well as a variety of patient factors (e.g. age, concomitant disease) and socioeconomic factors (e.g. access to healthcare). In most cancer cases, early detection significantly improves prognosis and outcomes for the patient. Once the patient has been through a complete evaluation, which includes the patient's history, physical examination, and various other procedures, the type of cancer and stage is diagnosed. Based on the diagnosis, there are numerous possible options for treatment (Pollock and Morton, 2000). For most solid tumours, surgery represents the oldest form of cancer treatment, and, though not without its

own risks, has a high success rate in the eradication of localised primary tumours. Radiation therapy is also highly effective at targeting both localised tumours and non-solid cancers such as leukaemia, although, depending on the specific cancer being treated, surrounding tissues and organs may be damaged by the ionising radiation resulting in harmful side-effects for the patient (Urruticoechea *et al.*, 2010) . Radiation therapy is often administered in combination with surgery, and/or chemotherapy. Targeted therapies (e.g. imatinib, a tyrosine-kinase inhibitor, used for the treatment of leukaemia), immunotherapy (e.g. rituximab, a chimeric monoclonal antibody against CD20 of B cells, used for the treatment of leukaemia and lymphoma) and angiogenesis inhibitors (e.g. bevacizumab, a chimeric monoclonal antibody against vascular endothelial growth factor A, used for the treatment of various solid tumours) are all examples of modes of cancer treatment that show varying levels of success and each exhibit their own set of side effects or complications for the patient. The sections which follow will focus on chemotherapy, and more specifically, on 5-fluorouracil and cisplatin, two chemotherapeutic agents used in the treatment of oesophageal cancer.

1.1.2 Chemotherapy

Cancer chemotherapy first emerged in the 1960s, prior to which surgery and radiation therapy formed the only means of cancer treatment. Since the advent of chemotherapy, over 50 different drugs have become available. Chemotherapeutic drugs are cytotoxic agents that target rapidly dividing cells, a characteristic of cancer cells, through various mechanisms. These drugs are administered by a number of different methods, including oral, topical and intravenous (IV) administration, and are usually given over different time cycles and in combinations to form specific treatment regimens and schedules. Generally, chemotherapy can be given alone or in a neo-adjuvant (pre-surgery), adjuvant (post-surgery) or palliative manner. The nature of chemotherapeutic drugs is such that it presents with extremely high toxicity to non-cancerous tissues, has a 30% success rate and varies greatly in patient response. Currently, the majority of chemotherapeutic drugs available are associated with serious adverse drug reactions (ADRs) (Dos Santos *et al.*, 2012). Additionally, deciding on the correct drug dose to achieve maximal

therapeutic proficiency while minimising adverse side effects is a great challenge to oncologists, due to the fact that each patient is unique and responds differently to the chemotherapeutic drugs. As a result, palliative care becomes vitally important in ensuring that the patient is able to cope with ADRs which include nausea, vomiting, diarrhoea, fatigue and pain. More severe side effects, including cardiac dysfunction and nephrotoxicity, can be fatal, and may only manifest years after chemotherapy. Table 1.1 shows some commonly used chemotherapeutic drugs, their commercial names, targeted cancers and some of their known side effects.

Table 1.1 Examples of commonly-used chemotherapeutic drugs. The commercial names, targeted cancers and some known side effects of the drugs are also listed. (Adapted from http://www.cancer.org/treatment/treatmentsandsideeffects/guidetocancerdrugs)

Drug	Commercial name(s)	Cancer	Side effects
5-Fluorouracil	Adrucil®, Efudex®, Fluoroplex®	Breast, gastrointestinal (including oesophagus), head	Diarrhoea, dermatitis, mouth ulcers, neutropenia, cardiotoxicity
Cisplatin	Platinol®, Platinol-AQ®	Oesophagus, bladder, ovary, testicles	Nausea, vomiting, hearing loss, peripheral neuropathy, nephrotoxicity, fertility impairment
Doxorubicin	Adriamycin®, Rubex®	Breast, oesophagus, lymphoma, multiple myeloma	Nausea, vomiting, neutropenia, cardiotoxicity, fertility impairment
Cyclophosphamide	Cytoxan®, Neosar®	Breast, ovary, lymphoma	Nausea, vomiting, neutropenia, lung and bladder damage, cardiotoxicity
Methotrexate	Folex®, Mexate®, Amethopterin®	Breast, lung, oesophagus, leukaemia, lymphoma	Nausea, vomiting, mouth ulcers, neutropenia, nephrotoxicity, hepatotoxicity, seizures
Paclitaxel	Taxol®, Abraxane®	Breast, ovary, lung	Nausea, vomiting, neutropenia, peripheral neuropathy, hepatotoxicity

1.1.3 5-Fluorouracil

5-Fluorouracil (5-FU) is one of the most commonly-used chemotherapeutic drugs for the treatment of a wide variety of malignancies, including breast, gastrointestinal, oesophageal, cervical, skin and lung cancer (Poorter *et al*., 1995; Oguri *et al*., 2005; Ofverholm *et al*., 2010). The drug was synthesised over 50 years ago, upon the discovery that fluorine markedly inhibited tumours in mice (Heidelberger *et al*., 1957).

5-FU is administered orally, topically, by IV injection or by slow IV infusion, either alone or in combination with other drugs (Matsuyama *et al*., 2008). For example, 5-FU forms part of the ECF (Epirubicin + Cisplatin + 5-FU) regimen for pre- and post-operative chemotherapeutic treatment of oesophageal cancer patients, and part of the MCF (Methotrexate + Cyclophosphamide + 5-FU) regimen, one of the oldest regimens for breast cancer (Cunningham *et al*., 2006 and 2008). Dosage is based on the type of cancer and the method of administration, and can vary from 25 mg/m^2 (body surface area) orally, to 2000 mg/m^2/day over a 96-hour slow IV infusion (McAuley, 2012). The pharmacokinetics of 5-FU is characterised by a high drug clearance rate and a short distribution period, with an elimination half-life of 8 to 14 minutes (Schilsky, 1998).

One of the predominant toxic effects of 5-FU in patients is myelo-suppression, which manifests as neutropenia, anaemia or thrombocytopenia, consequently raising the risks of infection and bleeding. Other adverse reactions include cardiotoxicity, dermatitis, mucositis of the gastrointestinal tract, and long-term negative impact on cognition (Kimura and Okuda, 1999; Alter *et al*., 2006; Wigmore *et al*., 2010).

1.1.4 Mechanisms of action and metabolism of 5-FU

For 5-FU to exert its effects, it must be anabolised to its active forms by the sequential actions of a series of enzymes, including orotate phosphoribosyltransferase (OPRT) with phosphoribosyl pyrophosphate (PRPP) as the cofactor, uridine kinase (UK), uridine phosphorylase (UP), ribonucleotide reductase (RR) and thymidine kinase (TK). The three main active metabolites are

fluorouridine triphosphate (FUTP), fluorodeoxyuridine triphosphate (FdUTP) and fluorodeoxyuridine monophosphate (FdUMP), and each one utilises different cytotoxic mechanisms (Longley *et al.*, 2003).

The action of FUTP demonstrates the rationale behind the development of 5-FU: the "decoy molecule". As the name of the drug denotes, the chemical structure of 5-FU is analogous to that of the important pyrimidine derivative, uracil (Figure 1.3).

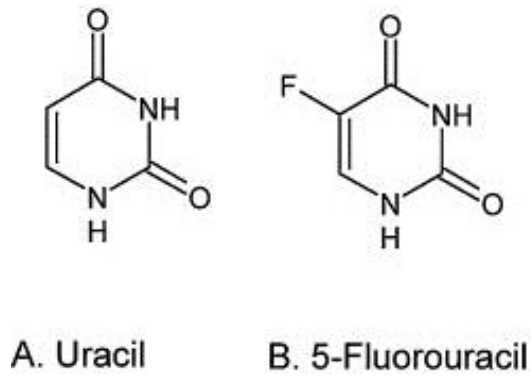


Figure 1.3 Structural differences between uracil and 5-FU. (Adapted from Peer *et al*., 2012)

Consequently, during RNA synthesis, FUTP is incorporated instead of UTP, resulting in profound inhibitory effects on the synthesis, processing and stability of RNA. The other active metabolite FdUTP can be incorporated into DNA to a certain extent, which causes a halt in DNA elongation during DNA replication and leads to fragmentation of the strand (Diasio and Schuetz, 1985). Although the decoy concept originally formed the basis for the synthesis of 5-FU as a chemotherapeutic agent, 5-FU is now known primarily as a thymidylate synthase (TS) inhibitor. TS is responsible for the synthesis of thymidine triphosphate (TTP), one of the four nucleotide triphosphates involved in *in vivo* DNA synthesis. The third 5-FU active metabolite, FdUMP, is able to inhibit TS activity resulting in TTP depletion and hence stunted DNA synthesis and impaired cell growth. This property of 5-FU has been described as its most significant anti-tumour effect (Van Kuilenburg *et al*., 2004).

Whilst the anabolic pathway of 5-FU leads to anticancer effects, the catabolic pathway is responsible for approximately 85% of 5-FU metabolism (Ezzeldin and Diasio, 2004). The dihydropyrimidine dehydrogenase (DPD) enzyme is key to regulating the bioavailability of 5-FU and converts it to the inactive form dihydrofluorouracil (DHFU). In both normal and tumour cells, the activity of 5-FU is thus dependant on the amount of DHFU present in the cell (Longley *et al*., 2003). The anabolic and catabolic pathways for 5-FU are summarised in Figure 1.4.

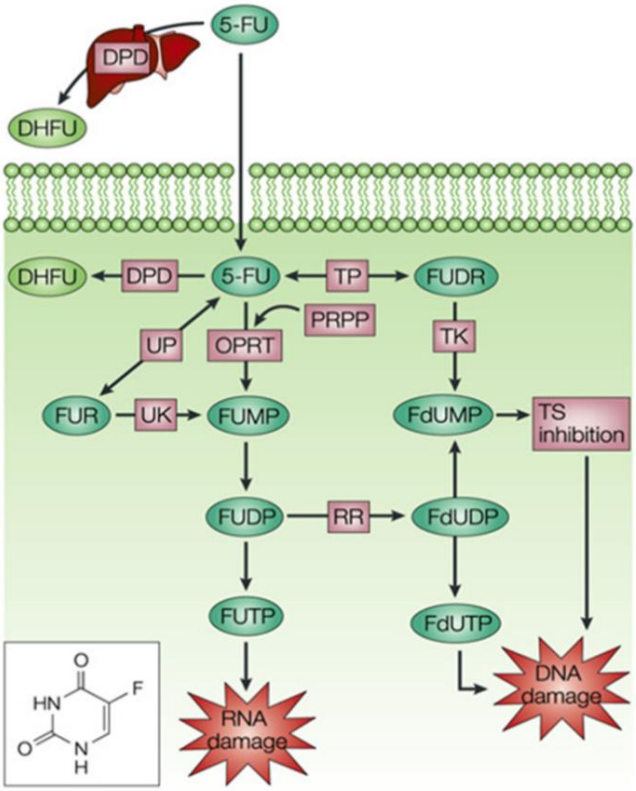


Figure 1.4 Metabolic pathway of 5-FU. 5-FU is primarily eliminated by conversion to inactive DHFU by DPD in the liver. The remainder of the 5-FU is converted by a series of enzymes into three main active metabolites, FUTP, FdUTP and FdUMP. These metabolites result in RNA damage, DNA damage and DNA damage through TS inhibition, respectively. (Obtained from Longley *et al.*, 2003)

1.1.5 Cisplatin

Cisplatin, also known as cis-diamminedichloroplatinum(II) (CDDP) is an alkylating agent that was the first member of a class of platinum-containing chemotherapeutics, which now also includes carboplatin and oxaliplatin. CDDP compound was first described in 1845, and then accidentally found to be effective against cell proliferation during a study on *E.coli* growth 20 years later (Rosenberg *et al*., 1965). Today, CDDP is highly effective in the treatment of a variety of cancers, including testicular, ovarian, oesophageal, bladder, lung, gastric and cervical tumours.

CDDP is administered by short IV infusion or by direct abdominal infusion in the case of gastric tumours. It can be used alone or in combination with other drugs, for example, the CMV (CDDP + Methotrexate + Vinblastine) neo-adjuvant regimen for bladder cancer, the DCF (Docetaxel + CDDP + 5-FU) regimen, or the CDDP + 5-FU regimen combined with radiotherapy, both for the treatment of oesophageal cancer (Van Cutsem *et al*., 2006). The compound has a half-life of 20-30 minutes, and as is the case with most chemotherapeutic drugs, CDDP dosage ranges greatly (20-150 mg/m^2) depending on patient and tumour-related factors (Candelaria *et al*., 2006; Helm and States, 2009).

Although CDDP is considered an extremely potent anti-neoplastic agent, its clinical application is limited by severe nephrotoxicity (Townsend *et al*., 2003; Pabla and Dong, 2008). Other adverse effects include hearing loss, hepatotoxicity, neurotoxicity and myelo-suppression (Wang and Lippard, 2005).

1.1.6 Mechanisms of action and metabolism of CDDP

CDDP is a small molecule that consists of a platinum (Pt) ion, two chloride ions and two ammonia molecules (Figure 1.5). The mechanism of action of CDDP entails the cross-linking of DNA through aquation, i.e. when the molecule enters the cell, one of the chloride ions is displaced by

water. This allows the Pt atom to bind to guanine bases in the DNA strand, eliciting DNA repair mechanisms, which in turn triggers apoptosis and cell cycle arrest due to the inability of the cell to repair the DNA damage (Reedijk, 1987).

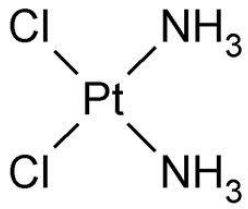


Figure 1.5 Structure of a CDDP molecule. (Adapted from Lovejoy *et al.*, 2008).

The metabolic fate of CDDP has not been as well characterised as that of other chemotherapeutic drugs such as 5-FU (Dos Santos *et al*., 2012). Thus, the general pathways for the metabolism of all Pt-containing drugs will be discussed.

The influx of Pt-containing compounds into the cell is controlled by the membrane-bound protein SLC31A1, and the efflux by ABCC2, ABCG2, ATP7A and ATP7B (Figure 1.6). Once the Pt-containing compound is inside the cell nucleus, its main anti-tumour mechanism is the formation of Pt-DNA adducts, which results in cell cycle arrest and apoptosis. These Pt-DNA adducts are recognised by the high-mobility group box-1 (HMGB1) protein, which activates cellular responses to the adducts. These responses includes the signalling of mismatch repair enzymes such as MLH1 and MSH6, as well as nucleotide excision repair enzymes such as XRCC1, ERCC1, ERCC2 and XPA. These enzymes detect breaks in the DNA and remove proteins from the DNA helix, rendering the helix more accessible to repair enzymes such as POLH and POLB. Many of the above-mentioned repair enzyme genes have known variants that lead to successful repair of the cross-linked DNA, thus causing decreased drug efficiency (Sakano *et al*., 2006; Van der Straaten *et al*., 2006). Additionally, there are numerous enzymes that are responsible for the detoxification of Pt-containing drugs in the cell, and thus play a key role in cellular drug resistance (Figure 1.6).

Among these is the glutathione-S-transferase-π (GSTP1) enzyme, which forms part of a group of enzymes referred to as drug metabolising enzymes (DMEs) and which will be discussed in more detail below.

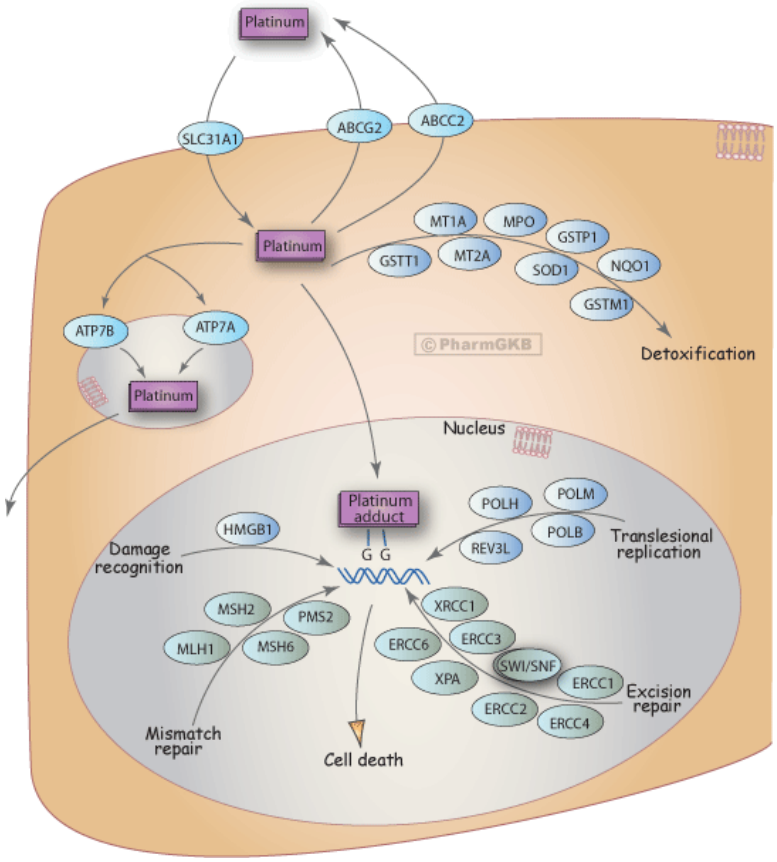


Figure 1.6 Metabolic pathway of Pt-containing drugs. Once Pt enters the cell, it exerts its anti-proliferative effect by forming DNA adducts. A number of different mismatch repair and nucleotide excision repair enzymes attempt to repair the damaged DNA, once the damage has been recognised by the HMGB1 protein. Pt is detoxified by enzymes such as GSTP1. (Obtained from Sharon *et al*., 2009)

1.2 Drug metabolising enzymes: role in drug response and effects of chemotherapeutics

There are several factors that affect cancer patient response to treatment, and chief among them is the variability in the metabolism of chemotherapeutic drugs. Most of the currently used chemotherapeutics are substrates for DMEs, particularly the cytochrome P450 (CYP) enzymes, which form the basis of this study. These DMEs activate, deactivate and detoxify foreign

compounds in the human body, through catalysing various biochemical modifications of xenobiotics (e.g. drugs), as well as endogenous chemicals (e.g. hormones). Through their activities, DMEs are therefore able to convert drugs into pharmacologically active metabolites and hence influence the susceptibility of tissues and organs to the therapeutic and toxic effects of these drugs (Li and Bluth, 2011).

The DMEs exhibit broad substrate selectivity, but the genes encoding them exhibit genetic polymorphisms which affect their expression and activity, which in turn leads to variability in the response to chemotherapeutic drugs. This genetic variability accounts for up to 90% of the wide range in therapeutic response observed from patient to patient (Wrighton and Stevens, 1992; Scripture *et al*., 2005; Code *et al*., 1997). Genetic variations in DME genes have been shown to lead to decreased intracellular enzyme concentrations, dysfunctional or structurally altered enzymes with functional consequences. Subsequently, the metabolism of drugs is compromised or altered, and the efficacy of treatment may be affected (Ekhart *et al*., 2009). As an example, 40-50% of patients experiencing severe toxicity to 5-FU have been shown to possess a deficiency in the DME DPD, as a result of a single nucleotide polymorphism (SNP) in the *DPD* gene (Ezzeldin and Diasio, 2004). Additionally, severe toxicities induced by the anticancer drug irinotecan have been shown to be linked to a genetic polymorphism in the uridine diphosphate glucuronosyltransferase DME gene (Fakih *et al*., 2007). Therefore, genetic variations in DMEs which alter metabolic capacity can be responsible for some patients being poor metabolisers (PMs) and experiencing toxic build-up of the drug, or for other patients to be ultra-rapid metabolisers (UMs) and experiencing decreased efficacy of treatment.

Considering the importance of DMEs in predicting therapeutic outcomes, it is crucial to understand how drugs may affect DME gene expression levels. Increased expression of a particular DME gene will result in increased drug clearance and decreased efficiency; conversely, decreased expression of the DME will lead to decreased drug clearance and increased efficiency, but could also lead to toxicity due to elevated drug concentrations. The outcome is dependent on the specific function of the DME, and this outcome will vary between patients due to differential expression. For example,

an improved response to the chemotherapeutic drug docetaxel, used in the treatment of breast cancer, was correlated with low mRNA and protein expression level of the DME CYP3A4, which functions to deactivate this drug (Iwao-Koizumi *et al*., 2005). In some cases the opposite effect is expected, such as when medications are administered as pro-drugs. A pro-drug is a compound that is administered to the body in an inactive or less than fully active form, requiring activation by metabolic enzymes to become functional, for example codeine, which requires DME activation to form the functional morphine (Zanger and Schwab, 2013). Figure 1.7 shows the consequences of under- and over-expression of a *CYP* gene for a hypothetical patient administered a drug and a pro-drug metabolised by the CYP. These examples serve to illustrate the importance of DME gene expression in treatment outcome and emphasise the intricacy of optimising individual patient's drug response.

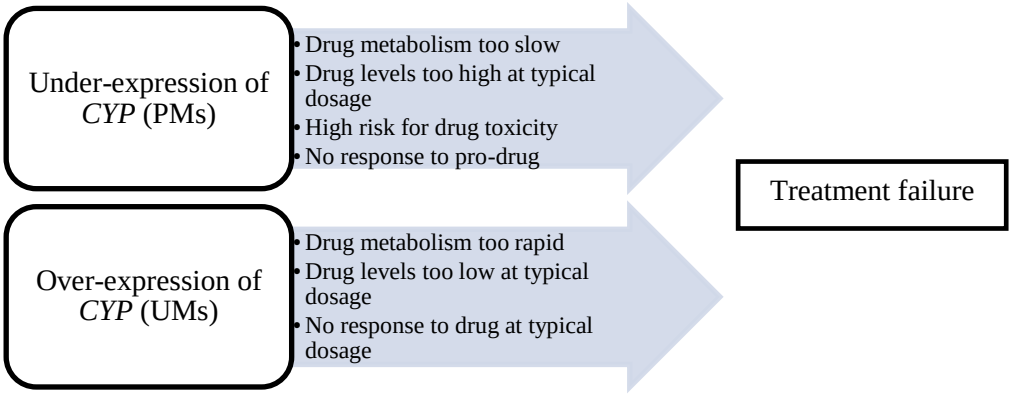


Figure 1.7 Consequences of under- and over-expression of CYP genes. When *CYP* is under-expressed, patients can be poor metabolisers (PMs) and when *CYP* is over-expressed, patients can be ultra-rapid metabolisers (UMs). In both cases, this results in treatment failure, illustrating the importance of DME expression in drug response.

While the variability of drug response can be explained, at least partially, by the genetic variations in the genes coding for DMEs, the effects of the chemotherapeutic drugs themselves on the expression of DMEs have been poorly studied. Such effects are vital in understanding patient

response. As an example, the drug itself may affect its own metabolism by increasing or decreasing DME gene expression, depending on the dosage used. Alternatively, a chemotherapeutic agent may influence the gene expression of DMEs required to detoxify other drugs or chemicals within the patient's body. In the case of 5-FU in particular, there are few studies on the effects of the drug on DME gene expression, especially the CYP1 DMEs, which will be discussed in more detail in section 1.4.1. Using mouse models, Afsar *et al*. (1996) has shown that 5-FU treatment down-regulates hepatic CYP2C11 and CYP3A isoenzymes at the protein level, and Yoshisue *et al*. (2001) has demonstrated that exposure to 5-FU causes a decrease in small intestinal CYP1A1/2 and CYP3A isoenzyme levels. Another study has shown that 5-FU has little or no inhibitory effect on CYP-catalysed reactions in human liver microsomal preparations (Park and Kim, 2003). Generally, most studies using human cells focus on the effects of 5-FU on the expression of *DPD*, *TS* and *OPRT* (Yoshisue *et al*., 2001; Takechi *et al*., 2002; Oguri *et al*., 2005; Oeda *et al*., 2006; Li *et al*., 2007; Li *et al*., 2013), as these are the genes involved in 5-FU metabolism. However, as the CYP DMEs contribute to the metabolism of over 90% of clinically-prescribed drugs, the potential effects of 5-FU on the *CYP*s need to be elucidated, and this has yet to be fully described (Motawi *et al*., 2013).

In the case of CDDP, a previous study using rat kidney cortex cells has shown that CDDP is capable of reducing intracellular glutathione, as well as several CYP enzyme levels (Bompart, 1989). However, the majority of studies have since focussed on CYP2E1, as elevated levels of this enzyme have been shown to enhance CDDP-induced hepatotoxicity through the production of reactive oxygen species (ROS) (Liu *et al.*, 2002; Liu and Baliga, 2003; Lu and Cederbaum, 2007; Martins *et al*., 2008). Masek *et al*. (2009) performed a study showing a minor inhibition of CYP2C9 and CYP2B6 enzyme levels in response to CDDP treatment. In addition, the GSTP1 DME is of interest as increased expression levels of this protein may have a protective effect against CDDP-induced cytotoxicity, but may also lead to cellular resistance to the drug (Yellin *et al*., 1994; La Pensee *et al*., 2009; Sharon *et al*., 2009; Townsend *et al*., 2009; Sahu *et al*., 2013). This is because GSTP1 is one of the main enzymes responsible for the detoxification of CDDP,

resulting in lowered concentrations of the drug in the cells (Peklak-Scott *et al.*, 2008). As is the case with 5-FU, more human cell studies on the effects of CDDP on DME expression, especially the CYP1 enzymes, are required in order to better understand patient response to this widely-utilised chemotherapeutic drug.

1.3 Administered drugs and other compounds as substrates, inducers or inhibitors of DMEs

In addition to drugs, it is important to understand the potential effects on DME genes caused by substances that a cancer patient would likely be exposed to on a daily basis through the patient's immediate environment or lifestyle habits. Whilst oesophageal cancer has many genetic risk factors, including somatic mutations and polymorphisms in the DME genes, there is also a host of environmental risk factors that have been linked to oesophageal tumour development. One of the most well-characterised of these factors is tobacco-smoking, due to exposure to benzo[a]pyrene (BP) (Wogan *et al.*, 2004; Ye and Xu, 2010). It has been shown that many head and neck cancer patients continue to smoke or are exposed to secondary tobacco smoke during treatment; in some cases, 30% of patients continue cigarette smoking after diagnosis (Duffy *et al.*, 2007; Chen *et al.*, 2011; Kashigar *et al.*, 2013). For an oesophageal cancer patient that is exposed to BP in cigarette smoke, it is important to understand the effects of chemotherapeutic drugs on DME expression in the presence of BP, as the ratio of activity between the CYP enzymes, particularly CYP1A1 and CYP1B1 which metabolise BP, and the detoxification enzymes such as GSTP1, is crucial for the prevention of toxic build-up of reactive BP intermediates (Liang *et al.*, 2003). Whilst BP is a substrate of CYP activity, it also acts as an inducer of CYP activity by activating the arylhydrocarbon receptor (AhR) signalling pathway (Figure 1.8), to induce transcription of genes such as *CYP1A1*, *CYP1A2* and *CYP1B1*. The AhR pathway will be briefly explained. The first step in the pathway is the interaction of a foreign compound with the ligand-activated AhR in the cytosol of the cell (Figure 1.8, step 1). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and the commonly-used drug omeprazole depicted in Figure 1.8, are also examples of potent inducers of *CYP1* expression (Yoshinari *et al.*, 2008). This ligand binding triggers a conformational change in the AhR, which in turn exposes a nuclear localisation signal (NLS) allowing translocation of the

bound AhR from the cytosol to the nucleus (step 2). Heat shock protein 90 (HSP90) and ligand both remain bound to the AhR during this translocation. Once in the nucleus, the AhR dimerizes with AhR nuclear translocator (ARNT) to form an active transcription factor that recognises the dioxin response element (DRE) motif in the promoter region of the target genes (step 3). The final steps in the pathway entail an increase in mRNA of these genes (step 4), and ultimately, increased production and exportation of the corresponding proteins (step 5). Increases in *CYP1* mRNA and subsequent increases in CYP1 protein are often used as markers of AhR activation (Israel and Whitlock, 1983; Hankinson, 1994; Long *et al*., 2006).

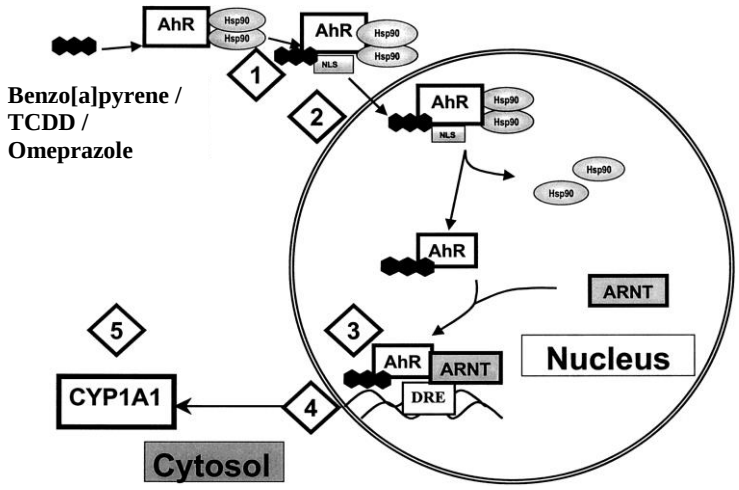


Figure 1.8 Schematic representation of the AhR/ARNT signalling pathway. The AhR is a ligand-activated transcription factor. Ligand binding to the AhR (step 1) triggers transport of the AhR to the cell nucleus, with the aid of HSP90 and NLS (step 2), followed by dimerization of the AhR with ARNT (step 3) to recognise DREs and up-regulate transcription of toxic response genes such as *CYP1A1* (step 4). This results in increased production of the relevant proteins and their exportation from the cell (step 5). (Adapted with modification from Soprano and Soprano, 2003)

Therefore, it is important to include BP in this study not only due to its presence in substances that a patient may be exposed to, like tobacco, but also as a model inducer of CYP expression. This is because inducers of CYPs are in abundance in the environment. BP is classified as a polycyclic aromatic hydrocarbon (PAH), and the PAHs form a group of one of the most widespread organic

pollutants, found not only in cigarette smoke but also in processed fossil fuels (e.g. motor exhaust fumes), meat and fish cooked at high temperatures (e.g. grilling or barbecuing) and a variety of culinary oils (e.g. coconut oil) (Alomirah *et al*., 2010; Perumal *et al*., 2012). Therefore, there are numerous environmental PAHs other than BP that a cancer patient may be exposed to and that may affect DME expression. In addition to the PAHs, dietary substances also present with potential effects on *CYP* expression. A well-studied example is grapefruit juice, from which bio-active compounds have been shown to result in rapid, irreversible and sustained inhibition of *CYP3A4* and in some studies, varying levels of inhibition of *CYP1B1* (Ohta *et al*., 2002; Girennavar *et al*., 2006). In fact, it is well-established that ADRs occur in patients regularly consuming grapefruit juice whilst taking benzodiazepines, which are largely metabolised by CYP3A4 and are administered to treat anxiety, insomnia or even as pre-medication for certain medical procedures (Ozdemir *et al*., 1998; Evans, 2000). Other strong exogenous inducers or inhibitors of DMEs have been found in numerous commonly-used herbal products that are sold over-the-counter, such as St. John's Wort, which is a well-known a *CYP3A4* inducer that is used for the treatment of depression, and Gingko biloba, which acts as a *CYP1A2* inducer and is used for the general enhancement of cognitive functions such as memory and concentration (Hellum *et al*., 2007). Thus, it is plausible that a cancer patient may be consuming foods or nutritional/herbal supplements that contain compounds capable of influencing DME expression.

Although many studies focus on the effect of one drug at a time, in reality, cancer patients are administered several chemotherapeutic drugs simultaneously, as well as other medication to treat side effects and possibly unrelated acute or chronic conditions. The drugs themselves can act not only as substrates for DMEs, but also as inducers or inhibitors of DME gene expression. For an individual who is administered multiple chemotherapeutic drugs and/or other concurrent medication prescribed for the treatment of other conditions, this may have significant clinical consequences. For example, adverse reactions have arisen from interactions between the chemotherapeutic drug capecitabine (the orally-active pro-dug of 5-FU) and the anticoagulant warfarin, when 5-FU down-regulates *CYP2C9* expression, which is essential for warfarin

metabolism (Giunta, 2010). Another example is lidocaine, a commonly-used local anaesthetic and antiarrhythmic agent, which is predominantly metabolised by the CYP1A2 enzyme. The anti-depressant fluvoxamine is a potent inhibitor of *CYP1A2*, hence when these drugs are administered together, the inhibition of *CYP1A2* by fluvoxamine considerably reduces lidocaine metabolism, thus increasing the risk of lidocaine toxicity (Isohanni *et al*., 2006). These two classic examples of conflicting effects of co-administered drugs on *CYP* expression are illustrated in Figure 1.9.

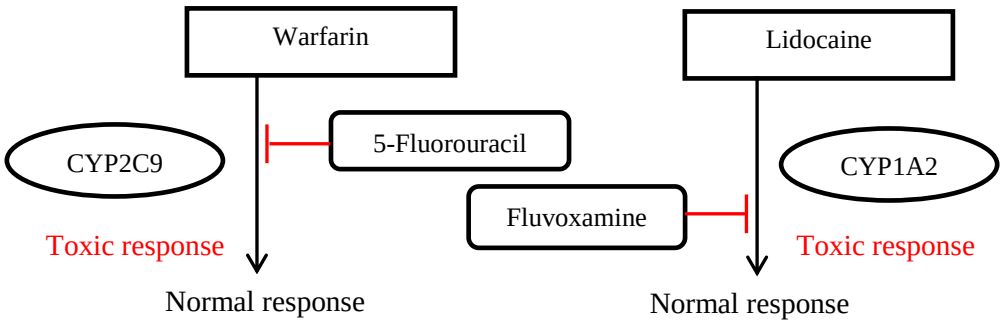


Figure 1.9 Examples of the effects of co-administered drugs on *CYP* expression. Warfarin is an anticoagulant drug metabolised by CYP2C9, and when the chemotherapeutic drug 5-fluorouracil is taken at the same time, *CYP2C9* is inhibited, resulting in toxic accumulation of warfarin (left). Similarly, lidocaine is an anaesthetic and antiarrhythmic drug metabolised by CYP1A2, and the co-administration of the anti-depressant lidocaine inhibits *CYP1A2* expression, causing lidocaine-induced toxicity (right).

Therefore, the use of BP in this study is not only due to its presence in the environment, but also due to its utility as a model CYP-inducing compound, from which inferences can be made about other CYP-inducing compounds that a cancer patient on chemotherapy may be exposed to, such as environmental substances (e.g. PAHs), dietary compounds (e.g. nutritional supplements like Gingko biloba) and drugs (e.g. the anticoagulant warfarin). Many conventional studies do not include the interrogation of the possible influence of these other exogenous compounds on the metabolism of clinical drugs. Nevertheless, it is important that the possible combinatorial effect of chemotherapeutic drugs and other exogenous DME inducers/inhibitors be taken into account when

undertaking studies of this nature, in order to better understand clinical implications for patients. This approach will provide better insight into the potential for improving existing cancer therapies.

1.4 DMEs to be focussed on in this study

1.4.1 Cytochrome P450s

The human CYP super-family represents the most important system responsible for catalysing the oxidation of most drugs (Nelson, 2004; Guengerich, 2006). The CYPs are also key enzymes to consider in cancer treatment as well as cancer formation, because they catalyse the activation and deactivation of anticancer drugs as well as the metabolic activation of pro-carcinogens such as the previously-mentioned BP (Rodriguez-Antona andIngelman-Sundberg, 2006). In addition to the liver, CYP proteins are also found in almost every extra-hepatic organ in humans, and expression levels of *CYP* genes have been found to vary up to 60-fold from one individual to the next as well as between tissue types (Nebert and Dalton, 2006; Sulem *et al*., 2011).

In this study, the focus will be on three *CYP* genes belonging to the CYP1 family, namely *CYP1A1*, *CYP1A2* and *CYP1B*. The highly-conserved *CYP1A1* (MIM #108330) and *CYP1A2* (MIM #124060) genes map to chromosome 15q24.1, and span 6 kb and 7.8 kb respectively, with *CYP1A1* encoding a 512-amino acid protein, whilst *CYP1A2* is 516 amino acids long (Spurr *et al*., 1987). *CYP1B1* (MIM #601771) is a 12 kb gene located at chromosome 2p22.2, encoding a 543 amino acid protein, making it the largest human CYP (Murray *et al*., 2001). *CYP1A2* is one of the most highly-expressed constitutive *CYP* genes detectable in the liver, whilst *CYP1A1* and *CYP1B1* are mainly expressed in extra-hepatic tissues.

As previously explained, the expression of the *CYP1* genes is inducible via the AhR pathway (Figure 1.8), and there are many substances, including clinically-used drugs, that are known *CYP1* inducers. For example, the broad-spectrum anthelmintic drug albendazole, which is used for treating a variety of parasitic worm infestations, is a known inducer of *CYP1A1*/*1A2* (Asteinza *et*

al., 2000; Bapiro *et al.*, 2002). Additionally, carbamazepine is administered for the treatment of epileptic seizures and is known to be an inducer of *CYP1A2* expression, whilst the chemotherapeutic drug doxorubicin is a *CYP1B1* inhibitor (Rochat *et al.*, 2001; Parker *et al.*, 1998). In some cases, such as in the case of BP, the inducer may also be a CYP1 enzyme substrate. As explained in detail in section 1.2, some studies have investigated the effects of the chemotherapeutic drugs 5-FU and CDDP on the expression of the *CYP*s, but many questions remain to be answered, particularly, what the effects of these drugs on the important DMEs are in human cancer cells, and furthermore, how these effects may vary in the presence of compounds which alter *CYP* expression. It is important to understand these effects because they may have an impact on the metabolism of co-medication such as the examples given above. Furthermore, ADRs may arise from the interactions of co-administered drugs that affect *CYP* expression, as mentioned in section 1.3 (Figure 1.9). This highlights the significance of investigating the effects of 5-FU and CDDP on the expression of the *CYP* genes.

1.4.2 Glutathione-S-transferases

Another DME which will be investigated in this study is a member of the glutathione-S-transferase (GST) super-family, GSTP1. This family of detoxification enzymes, classically viewed as part of the cell's defence against harmful chemicals produced endogenously and in the environment, catalyse the conjugation of glutathione to a range of hydrophobic and electrophilic compounds, resulting in water-soluble metabolites for excretion from the body. The *GSTP1* gene (MIM #134660) is located on chromosome 11q13.2, spans 3 kb and encodes a 210 amino acid protein. *GSTP1* represents the major *GST* expressed in extra-hepatic tissues such as the oesophagus and lungs, with minimal expression in the liver (Rowe *et al*., 1997; Sherratt *et al*., 1997; De Bruin *et al*., 2000). As is the case with *CYP1B1* expression, *GSTP1* exhibits over-expression in malignant tissues in comparison with their matched normal tissues (McIlwain *et al*., 2006).

The gene expression level of *GSTP1* is an important determinant of patient response to drugs. Over-expression of *GSTP1* is thought to confer resistance to chemotherapy, as has been

demonstrated in breast cancer (Su *et al*., 2003; Arai *et al*., 2008), and elevated expression levels is also associated with poor prognosis in a variety of other cancers including colorectal cancer (Mulder *et al*., 1995; Sutoh *et al*., 2000). This is because over-expression of *GSTP1* results in accelerated detoxification of the drug, and therefore a decrease in drug effectiveness, or acquired resistance. However, the detoxifying action of GSTP1 is critical for protection against pro-carcinogenic compounds such as BP. It is therefore of obvious importance to know the effects of chemotherapeutic drugs, as well as concomitant medication or other harmful exogenous substances, on *GSTP1* expression. In the case of the drugs in this study, it has been shown that CDDP is detoxified by GSTs (Goto *et al*., 1999), however for both CDDP and 5-FU, more studies on the effects of these drugs on *GSTP1* gene expression are needed. The majority of research is directed toward associations of *GST* polymorphisms with cancer incidence and prognosis (Goekkurt *et al*., 2006; McIlwain *et al*., 2006; Dhawan *et al*., 2013; Lai *et al*., 2013) and not the direct influence of these drugs on the expression levels of *GSTP1*, which has a significant clinical impact on patient response.

1.5 Aims and objectives

Determining the effects of commonly-used drugs on the critically important DMEs is of great relevance. The aim of this study was to investigate the role of the chemotherapeutic drugs 5-FU and CDDP in the differential expression of four DME genes, *CYP1A1*, *1A2*, *1B1* and *GSTP1*, using BP as a model *CYP*-inducer and a positive control for induction of *CYP* expression, in an oesophageal cancer cell line. This is an important study since most chemotherapeutic drugs have on average 30% success rate, and oesophageal cancer is the sixth most common cause of cancer deaths globally. To the best of our knowledge, no studies have looked at the effects of 5-FU, CDDP and the combinatorial effects of BP and 5-FU, and of BP and CDDP, on the *CYP1* and *GSTP1* genes in oesophageal cancer cells. This study will provide baseline information for future pharmacogenetic research, whereby the effects observed in this study may be evaluated in other tumour cell lines. The aims of this project will be met by the following objectives:

- To investigate the effects of treating the human oesophageal cancer cell line WHCO1 with chemotherapeutic drugs 5-FU and CDDP, as well as the *CYP*-inducer BP.
- To analyse the morphological changes in the cancer cells in response to treatment, using light microscopy.
- To analyse mRNA and protein expression of the DME genes *CYP1A1, CYP1A2, CYP1B1* and *GSTP1*, in response to drug treatment.

2. MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 Cell line and media

The WHCO1 cell line, originally established from surgical biopsies of primary oesophageal squamous cell carcinoma in South Africa (Veale and Thornley, 1994) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, USA) containing 10% foetal calf serum (Biochrom, Germany), 1% penicillin-streptomycin (Sigma-Aldrich, USA) and 1% GlutaMAX™ (Life Technologies, UK), at 37 ºC in a humidified atmosphere of 5% CO_2.

Cryovials containing cells in freezing medium (Appendix A) were removed from liquid nitrogen storage and thawed for 2 minutes in a 37 ºC water bath. The vial contents were then transferred into a 10 cm tissue culture dish containing 10 mL of DMEM, previously warmed to 37 ºC in the water bath. The dish was agitated gently and then incubated at 37 ºC.

2.1.2 Sub-culturing

Cells were sub-cultured when they reached ~80% confluency, and 10X trypsin-EDTA was purchased from Sigma-Aldrich (USA). The culture dish was removed from the incubator and media suctioned off. The remaining medium was washed off using 5 mL 1X PBS (Appendix A) and thereafter the cells were lifted by adding 5 mL of 1X trypsin-EDTA (Appendix A) and incubating at 37 ºC for 7-10 minutes or until the cells were detached from the base of the plate. Thereafter the trypsin was inactivated by the addition of 5 mL of culture medium, and the cell resuspension was transferred to a 15 mL tube and pelleted by centrifugation at 1000 rpm for 3 minutes. Following the removal of the supernatant by suction, the pellet was resuspended in 6 mL DMEM and either counted using a haemocytometer, or plated again at a 1:3 dilution.

2.1.3 Freezing

Following trypsinisation and pelleting of cells as described above, the pellet was resuspended in 6 mL of complete DMEM and the cells were counted using a haemocytometer. Pre-chilled freezing medium was added to the cells to reach a final concentration of 1 X 10^6 cells/mL and mixed gently. The suspension was aliquoted 1 mL per cryovial and the vials were slow-cooled to -80º C overnight. Thereafter, the vials were transferred into a liquid nitrogen tank for long-term storage.

2.1.4 *Mycoplasma* testing

In order to ensure that the growth of any *Mycoplasma* contaminants being suppressed by the antibiotics could be detected, the WHCO1 cells were grown on a sterile glass cover slip in 2 mL antibiotic-free medium, in a 35 mm tissue culture dish for at least 48 hours or to ~60% confluence. Without removing the growth medium, 1 ml of fixing solution (Appendix A) was added to the cells and incubated for a few seconds. The fixing solution was removed, and the incubation and removal of fixing solution repeated in the same manner. The cells were then washed several times with sterile dH_2O to ensure complete removal of any residual fixative, and left to dry by inverting the dish. This was followed by staining of the cells for 30 seconds with 500 µL of 0.5 µg/mL Hoechst solution (Sigma-Aldrich, USA). The stain was washed off with dH_2O and the cover slip placed face down onto a microscope slide with a drop of mounting fluid. The cells were visualised using fluorescent microscopy.

2.2 Cell Treatments

2.2.1 MTT assay

This assay was performed to determine the IC_{50} value, i.e. the concentration of drug required to kill 50% of the cells in a specific cell line, for 5-FU. Cells were seeded in a 96-well plate at 5 X 10^3 cells in a final volume of 90 µL culture medium per well. After 24 hours, the cells were treated with 5-FU at a concentration gradient (0; 0.5; 1; 5; 10; 20; 50 and 100 µM) and each concentration

was done in triplicate. The control wells (0 µM 5-FU) contained a final concentration of 0.4% dimethyl sulphoxide (DMSO) (Sigma-Aldrich, USA), which is representative of the DMSO concentration in all the wells, because DMSO was the diluent used to dissolve 5-FU. After an incubation period of 24 hours, 10 µL of MTT reagent (Appendix A) was added to each well, followed by a further 4 hours of incubation at 37 ºC. Thereafter 200 µL of solubilisation reagent (Appendix A) was added to each well and the plate was left in the 37 ºC incubator overnight. The spectrophotometric absorbance (OD) of the samples was measured at 595 nm using a microtiter plate reader and these values were used to calculate the IC_{50} of 5-FU for WHCO1 cells using GraphPad PRISM® v 5.0 software.

2.2.2 Cell treatment

The 5-FU, CDDP and BP used for cell treatments were all purchased from Sigma-Aldrich (USA). The WHCO1 cells were seeded in 6 cm dishes at 5 X 10^5 cells/well in 5 mL of DMEM and allowed to adhere. The cell culture medium was replaced with serum-free DMEM 16 hours prior to the start of treatment. These cell treatments involved the addition of one of the following reagents at concentrations determined either through the MTT assay (IC_{50} determination) or the literature: 0.2 µM 5-FU; 10 µM BP, a combination of 0.2 µM 5-FU and 10 µM BP; and 0.04% DMSO for the control, as this was the maximum concentration of DMSO present at the IC_{50} concentrations used. For the CDDP treatment, the experiment was performed in the same manner except with 9.2 µM CDDP instead of the 5-FU. The treatments were carried out for 6, 12 and 24 hours.

2.2.3 Microscopy

The morphology of the cells were observed and photographed subsequent to exposure to chemical reagents using a light microscope (Olympus CKX41 with SC30 camera) at 10X and 20X magnification.

2.3 Quantitative-Real time PCR

2.3.1 RNA isolation

Prior to RNA isolation, all plasticware and glassware to be used for the isolation were treated with DEPC to reduce the likelihood of degradation by RNAses. Buffers and solutions were made up with DEPC-treated sterile dH_2O (Appendix A). Total RNA was isolated from the cells using the Roche High Pure RNA Isolation kit (Germany) as per the manufacturer's instructions. Briefly, the cells were trypsinised and pelleted by centrifugation, and then resuspended in 200 µL 1X PBS. To this cell suspension, 400 µL lysis/binding buffer was added and the solution vortexed for 15 seconds. The entire sample was transferred to a High Pure filter tube inserted into a collection tube, then centrifuged for 15 seconds. All centrifugation steps were carried out at 10 000 rpm. Next, the flow-through was discarded and a buffer containing DNase I was applied to the sample-bound membrane and incubated at room temperature for 15 minutes. Thereafter, the membrane was washed with 500 µL of wash buffer I and II subsequently, followed by a final wash with 200 µL wash buffer II. The RNA was eluted by centrifugation in 60 µL of elution buffer in a new sterile collection tube. All RNA samples were quantified using the NanoDrop® ND-1000 Spectrophotometer (Inqaba Biotec, South Africa) and the 260/280 and 260/230 ratios noted as a measure of sample purity. The samples were stored at -80 ºC until used.

2.3.2 RNA integrity using agarose gel electrophoresis

To verify the integrity of the RNA, 1 µg of each RNA sample was prepared in DEPC-treated sterile RNase-free dH_2O with 2X RNA loading buffer (Appendix A) in a total volume of 30 µL. The samples were denatured by heating at 55 ºC for 5 minutes, prior to loading onto a 1% agarose gel containing ethidium bromide (0.5 µg/mL) in a previously bleach-treated tank using 1X TBE (Appendix A) as running buffer. The gel was electrophoresed at 60 V/cm for 1 hour, and visualised with a UV-transilluminator.

2.3.3 Synthesis of cDNA

First strand cDNA was synthesised using 1 µg total RNA and the Maxima H Minus First Strand cDNA Synthesis kit (Thermo Scientific, USA) according to the manufacturer's instructions. Table 2.1 shows the components that were added to a sterile nuclease-free tube on ice. Prior to the addition of 5X RT buffer and RevertAid™ Premium Enzyme Mix the mixture was mixed by gentle pipetting, centrifuged briefly and incubated at 65 ºC for 5 minutes. Thereafter the samples were chilled on ice for a further 5 minutes, centrifuged again and placed on ice. The final components were then added to the tube on ice.

Table 2.1 Components of the Maxima H Minus First Strand cDNA Synthesis kit

Component	Volume (µL)
1 µg Total RNA	x
25 pmol Oligo$(dT)_{18}$ primer	0.25
25 pmol Random hexamer primer	0.25
10 mM dNTP mix	1
Nuclease-free dH_2O	to 15
5X RT buffer	4
RevertAid™ Premium Enzyme Mix	1
Total volume	20

The samples were mixed again by gentle pipetting, centrifuged briefly and incubated for 10 minutes at 25 ºC followed by 15 minutes at 50 ºC. The reaction was terminated by heating at 85 ºC for 5 minutes. The cDNA was stored at 4 ºC until used.

2.3.4 Quantitative real time PCR

Primer sequences and product sizes are listed in Table 2.2. The glyceraldehyde-3-phosphate (*GAPDH*) gene was used as an internal control in the quantitative real-time PCR (qRT-PCR) reactions. Primer specificity was verified by the BLAST program (http://www.ncbi.nlm.nih.gov).

Table 2.2 Primers used in quantitative real-time PCR.

Target gene	Primer sequence (5′ to 3′)	qPCR product size (bp)	Reference
CYP1A1	F:TGGATGAGAACGCCAATGTC R: TGGGTTGACCCATAGCTTCT	392	Huang *et al.,* 1996
CYP1A2	F:AACAAGGGACACAACGCTGAAT R :GGAAGAGAAACAAGGGCTGAGT	456	Rodriguez-Antona *et al.,* 2001
CYP1B1	F:CACTGCCAACACCTCTGTCTT R:CAAGGAGCTCCATGGACTCT	380	Huang *et al.,* 1996
GSTP1	F:CCTGTACCAGTCCAATACCATCCT R:TCCTGCTGGTCCTTCCCATA	72	Joshi *et al.,* 2005
GAPDH	F:GCCTGCTTCACCACCTTC R:GGCTCTCCAGAACATCATCC	192	Van Rooyen *et al.,* 2013

The qRT-PCR reaction was performed in a 96-well plate format and each sample was done in triplicate, using the SYBR FAST Universal qRT-PCR kit from KAPA Biosystems (South Africa). The reaction components are listed in Table 2.3.

Table 2.3 Components of the qRT-PCR reaction

Component	Volume (µL)
cDNA	1
1 µM Forward primer	0.2
1 µM Reverse primer	0.2
2X SYBR FAST mix	5
Sterile dH_2O	3.6
Total volume	10

The reaction was carried out by the Roche Lightcycler 480 II, and the conditions were as follows:

95 ºC: 3 minutes (pre-incubation)

95 ºC: 10 seconds
62 ºC: 20 seconds
72 ºC: 5 seconds
} 40 cycles

For all experiments, qRT-PCR data were analysed using the comparative critical threshold (Ct) method, whereby the amount of gene of interest was normalised to the amount of housekeeping gene (*GAPDH*) and expressed as fold induction, relative to the mean value of control samples using the equation $2^{-\Delta\Delta Ct}$ (Hundley *et al*., 2006).

2.3.4.1 Specificity of qRT-PCR primers

The specificity of the primers was verified by analysis of the melting peaks generated after the qRT-PCR reaction, as well as both agarose gel electrophoresis and cycle sequencing of the qRT-PCR products. The cycle sequencing method will be briefly explained below.

2.3.4.2 Cycle sequencing

First, clean-up of qRT-PCR products was set up as shown in Table 2.4, in order to remove excess primer as well as unincorporated ddNTPs. The shrimp alkaline phosphatase and exonuclease I enzymes were purchased from Thermo Scientific, USA. The reaction tubes were incubated at 37 ºC for 1 hour, and then at 75 ºC for 15 minutes.

Table 2.4 Components of the qRT-PCR clean-up reaction

Component	Volume (µL)
qRT-PCR product	5
Shrimp-alkaline phosphatase	1
Exonuclease I	0.1
Nuclease-free dH_2O	13.9
Total volume	20

Thereafter, the cycle sequencing reaction was prepared using the ABI Prism® BigDye™ Terminator v3.1 kit (Applied Biosystems, USA), as per the manufacturer's instructions. Briefly, the components of the kit were set up for each primer (forward and reverse) as shown in Table 2.5.

Table 2.5 Components of the cycle sequencing reaction

Component	Volume (µL)
10 pm Primer (either forward or reverse)	1
qRT-PCR product (post-clean-up)	1
1X Sequencing buffer	2
0.5X Terminator mix	1
Nuclease-free dH_2O	5
Total volume	10

The cycle sequencing reaction was performed using the Bio-Rad T100™ Thermal Cycler, under the following conditions:

98 ºC: 5 minutes (pre-incubation)

96 ºC: 10 seconds
50 ºC: 15 seconds
60 ºC: 4 minutes
} 30 cycles

Thereafter, a further clean-up step as well precipitation of the sequencing product was required. Firstly, 22 µL absolute ethanol and 1 µL 5 M sodium oxaloacetate (pH 4.5) was added to each cycle sequencing product, followed by brief mixing and storage at -20 ºC for 2 hours. The samples were centrifuged at 10 000 rpm for 5 minutes and the supernatant decanted. Next, 40 µL 70% ethanol was added and the samples were centrifuged for another 5 minutes. Once the supernatant was discarded, the samples were left to air-dry for 1 hour at room temperature. The sequencing products were resuspended in 10 µL sterile dH_2O and half of each sample was aliquoted into the wells of a 96-well plate, followed by the addition of 5 µL Hi-Di™ Formamide (Applied Biosystems, USA) to each well. The samples were analysed on an ABI 3130*xl* Genetic Analyzer (Applied Biosystems, USA), and computational analysis of the results was performed using the BioEdit Sequence Alignment Editor v 7.0.0 (Tom Hall, Isis Pharmaceuticals Inc.). To verify the specificity of the sequences, the online BLAST tool (http://www.ncbi.nlm.nih.gov) was utilised.

2.4 Western blot analysis

2.4.1 Isolation of total soluble protein

10X RIPA buffer and protease inhibitor cocktail were purchased from Cell Signalling Technology® (USA) and Roche (Germany), respectively. The culture dishes were placed in ice, the culture medium was suctioned off and excess medium was washed off using ice-cold sterile 1X PBS. The cells were then scraped off the dish in 1X RIPA buffer containing protease inhibitor cocktail (Appendix A). The lysate was transferred to a sterile 1.5 mL tube and incubated on ice for 30 minutes, prior to centrifugation at 13 000 rpm for 20 minutes at 4 ºC to pellet the cell debris. Next, the supernatant containing the total soluble proteins was transferred into a new tube and stored at

-20 ºC until use. Aliquots were used for protein quantification using the BCA Protein Quantification kit (Thermo Scientific, USA), according to the manufacturer's instructions.

2.4.2 SDS-PAGE

A 1.5 mm thick 8-12% resolving gel (Appendix A) and a 5% stacking gel (Appendix A) were prepared in a Bio-Rad Mini PROTEAN© 3 casting apparatus as per the manufacturer's instructions. Table 2.6 shows the protein sample preparation components and volumes. Samples were denatured by heating at 95 ºC for 10 minutes before loading.

Table 2.6 Components of the protein sample preparation tubes for SDS-PAGE

Component	Volume (µL)
20 µg protein	x
5X SDS loading buffer	6
100 mM dTT	1
1X RIPA buffer	1
Nuclease-free dH_2O	to 35

The prepared gel was placed into a Bio-Rad running tank containing 1X running buffer (Appendix A). The samples were loaded into the wells, as well as 5 µL of the PageRuler™ Prestained Protein Ladder (Thermo Scientific, Europe). The apparatus was connected to the Bio-Rad Power pack 200 and the gel was electrophoresed at 100 V/cm for 75 minutes.

2.4.3 Protein transfer onto nitrocellulose membrane

The 1X transfer buffer (Appendix A) was prepared prior to the transfer and chilled at 4 °C. A Hybond-ECL membrane (Amersham Biosciences, UK) was cut to match the gel size and left to soak in the transfer buffer for at least 15 minutes. After completion of electrophoresis, the stacking gel was removed and the resolving gel containing the separated proteins was assembled under

transfer buffer into a "sandwich" as indicated in the diagram below (Figure 2.1) and placed within a gel holder cassette.

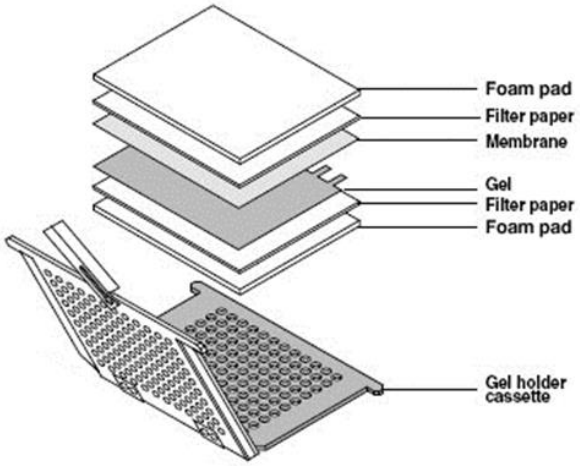


Figure 2.1 Diagram illustrating the assembling of a protein transfer sandwich. (Adapted with modification from http://www.bio-rad.com/en-id/product/trans-blot-cell)

The cassette was then placed into the transfer unit of the Bio-Rad Mini PROTEAN© 3 transfer tank containing transfer buffer. An ice pack was placed in the tank to prevent over-heating. The transfer apparatus was connected to the Bio-Rad Powerpack 200 and protein transfer took place at 100 V for 90 minutes.

2.4.4 Antibody incubation and western blot detection

The primary antibodies used were as follows: goat polyclonal IgG CYP1A1 (sc-9828), mouse monoclonal IgG CYP1A2 (sc-53241) and rabbit polyclonal IgG CYP1B1 (sc-32882) from Santa Cruz Technologies (Europe); rabbit polyclonal IgG GSTP1 (AB8902) from Merck Millipore (Germany); and rabbit anti-p38 MAP kinase IgG (MO800) from Sigma-Aldrich (Germany). The secondary antibodies used were blotting grade affinity purified goat anti-mouse IgG horseradish peroxidase (HRP) conjugate (170-6516) and goat anti-rabbit IgG HRP conjugate (170-6515) (Bio-Rad, USA) as well as donkey anti-goat IgG HRP conjugate (Santa Cruz Technologies, Europe). The antibody dilutions used are listed in Table 2.7.

Table 2.7 Antibody dilutions used for western blot analysis.

Protein	Primary antibody dilution	Secondary antibody dilution
CYP1A1	1:2000	1:3000 (goat anti-mouse)
CYP1A2	1:1000	1:3000 (goat anti-mouse)
CYP1B1	1:1000	1:3000 (goat anti-rabbit)
GSTP1	1:5000	1:5000 (donkey anti-goat)
p38	1:10 000	1:5000 (goat anti-rabbit)

After the transfer was completed the membrane was removed from the sandwich and washed twice with 1X PBS containing 0.1% Tween-20 (PBST, Appendix A) and then blocked with 5% fat-free milk in PBST (Appendix A) for 1 hour at room temperature, with gentle shaking. After blocking, the membranes were incubated with primary antibody, diluted to the desired concentration in blocking solution. This incubation was done overnight at 4 ºC, with gentle shaking. The next day the membrane was washed in PBST (2 X 10 minutes followed by 2 X 5 minute washes) and incubated with the appropriate HRP-conjugated secondary antibody in blocking solution, for 2 hours at room temperature with gentle shaking. The membrane was then washed as before in PBST and visualised by enhanced chemiluminescence using the Bio-Rad Clarity Western ECL Substrate (Thermo Scientific, Europe) as per the manufacturer's instructions. Membranes were exposed to x-ray film and the resulting chemiluminescent signal captured by developing and fixing the film.

To strip the antibody from the blot, the stripping buffer (Appendix A) was warmed to 50 ºC and the blot was incubated in the buffer at 50 ºC for 30 minutes, with brief agitation every 10 minutes. Thereafter, the membrane was washed twice for 10 minutes each with PBST. Each blot was then reused from the blocking stage as described previously, in order to detect the internal loading control protein p38. For analysis of the western blots, the x-ray films were scanned and the intensity of the protein bands quantified and normalised to p38 using Image Studio Lite software. Each western blot was performed twice to confirm reproducibility of the results.

3. RESULTS

The findings of the study are reported in the sections below.

3.1 WHCO1 cells tested negative for *Mycoplasma*

The *Mycoplasmas* constitute a large group of prokaryotic organism that are resistant to most antibiotics used in tissue culture laboratories and frequently contaminate cell cultures. It is important to test for these organisms because although they can easily go undetected, their presence can alter numerous cell functions and characteristics, including cell growth rate and morphology, thus affecting experimental data derived from infected cells (Garner *et al.*, 2000; Drexler and Uphoff, 2002). WHCO1 cells were regularly tested for *Mycoplasma* infection using a method based on Hoechst DNA staining (see section 2.1.4) and the cells were visualised using fluorescent microscopy. Aside from the nuclei of the WHCO1 cells, no other fluorescent bodies could be observed which indicates that the cells were *Mycoplasma*-negative (Figure 3.1). Infected cells would exhibit fluorescing nuclei as well as extra-nuclear fluorescence of *Mycoplasma* DNA with the appearance of small filamentous bodies or granules, as has been shown in by Battaglia *et al* (1994).

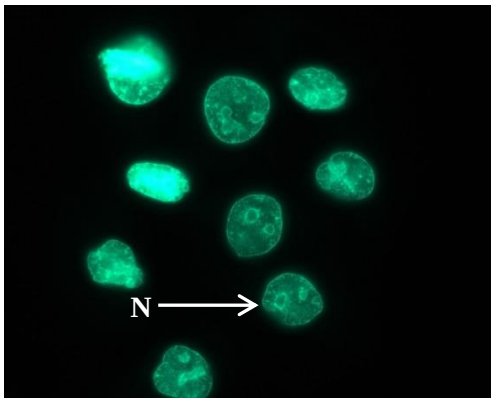


Figure 3.1 Fluorescent microscopy image showing Hoechst-stained cell cultures for *Mycoplasma* testing. WHCO1 cells tested negative for *Mycoplasma* with only nuclear staining (arrow with label N).

3.2 Effects of 5-FU and BP on WHCO1 cells

3.2.1 An IC_{50} of 0.2 µM 5-FU was obtained in WHCO1 cells

For drug treatment experiments, a fixed concentration of each drug was required; thus, an IC_{50} for each drug was calculated or obtained from literature. The MTT cell proliferation assay was used to do this and the methodology is described in section 2.2.1. Resultant assay data analysis was done using GraphPad PRISM® v 5.0 software to generate a sigmoidal dose-response curve as shown in Figure 3.2. The details on how the software calculates the IC_{50} value from this curve are given in Appendix B (Figure B1). The IC50 value for 5-FU was calculated to be 0.2 µM and this concentration was used for all subsequent 5-FU treatments. However, after completion of this set of experiments, a publication by Kaschula *et al.* (2012) reported an IC_{50} for 5-FU in WHCO1 cells of 7.9 µM, which is different to what was observed in this study. This is a cause for concern and further studies are needed to confirm the cause of this variation, but likely reasons are a difference in cell passage numbers, as well as the age and activity of the drug.

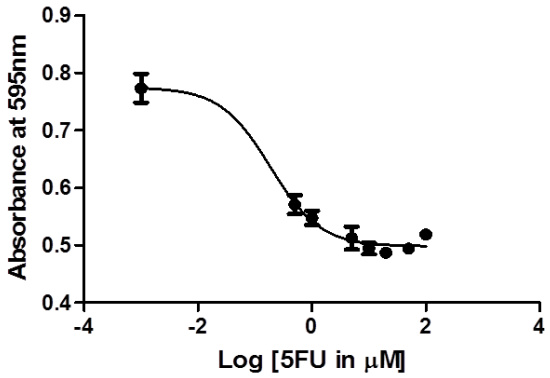
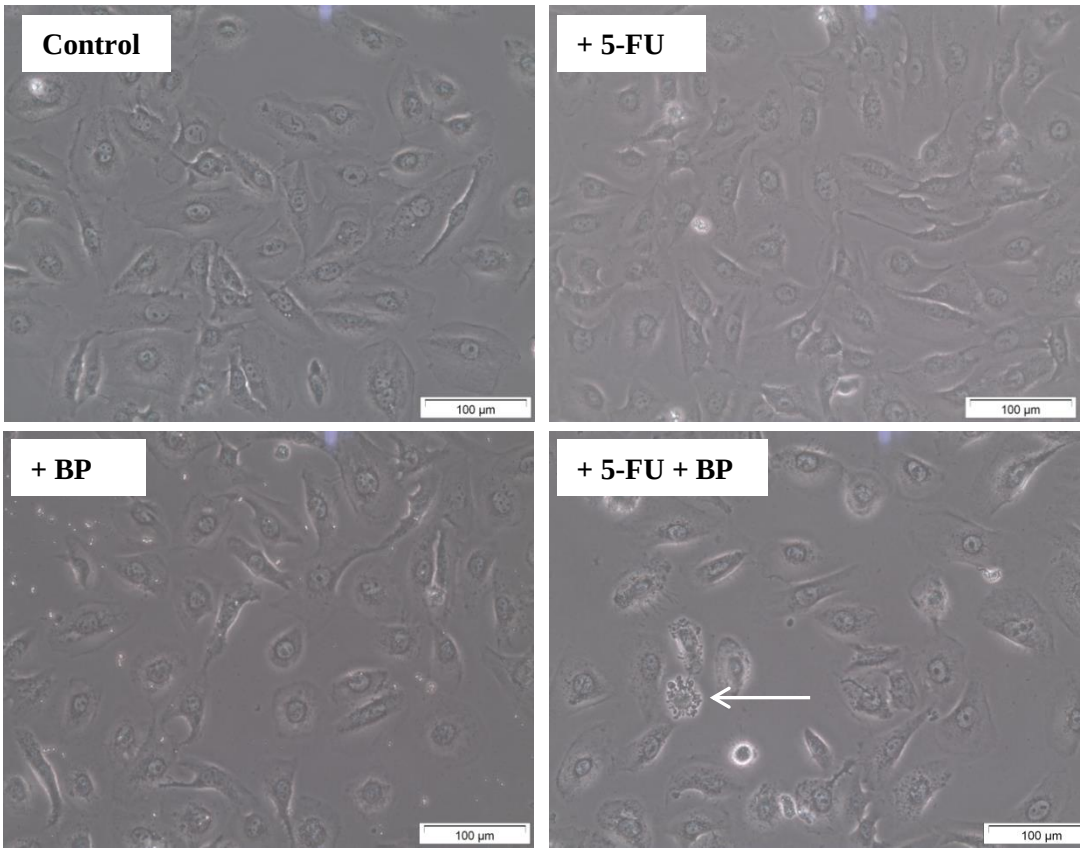


Figure 3.2 Sigmoidal dose-response curve for 24 hour 5-FU treatment of WHCO1 cells. The IC_{50} value of 5-FU in WHCO1 cells was calculated to be 0.2 μM as determined using GraphPad PRISM® v 5.0 software.

3.2.2 Co-treatment of WHCO1 cells with 5-FU and BP induced early apoptosis

The morphology of WHCO1 cells was observed and photographed subsequent to exposure to all drug treatments, using a light microscope (Figure 3.3). The concentration of BP used to treat cells was obtained from the literature (Plant *et al.*, 1985; Barhoumi *et al.*, 2000). No morphological changes were observed when 5-FU and BP were used alone, however, at 24 hours, blebbing of the cell membranes was observed in less than 1% of the cells when a combination of 5-FU and BP was used, indicating early stage apoptosis (Figure 3.3 A).



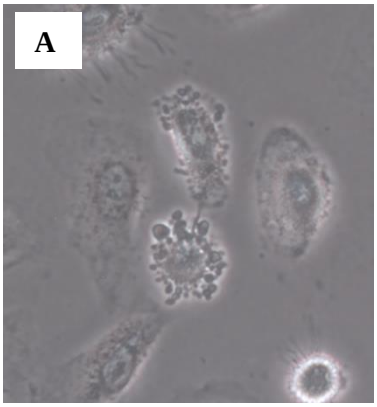
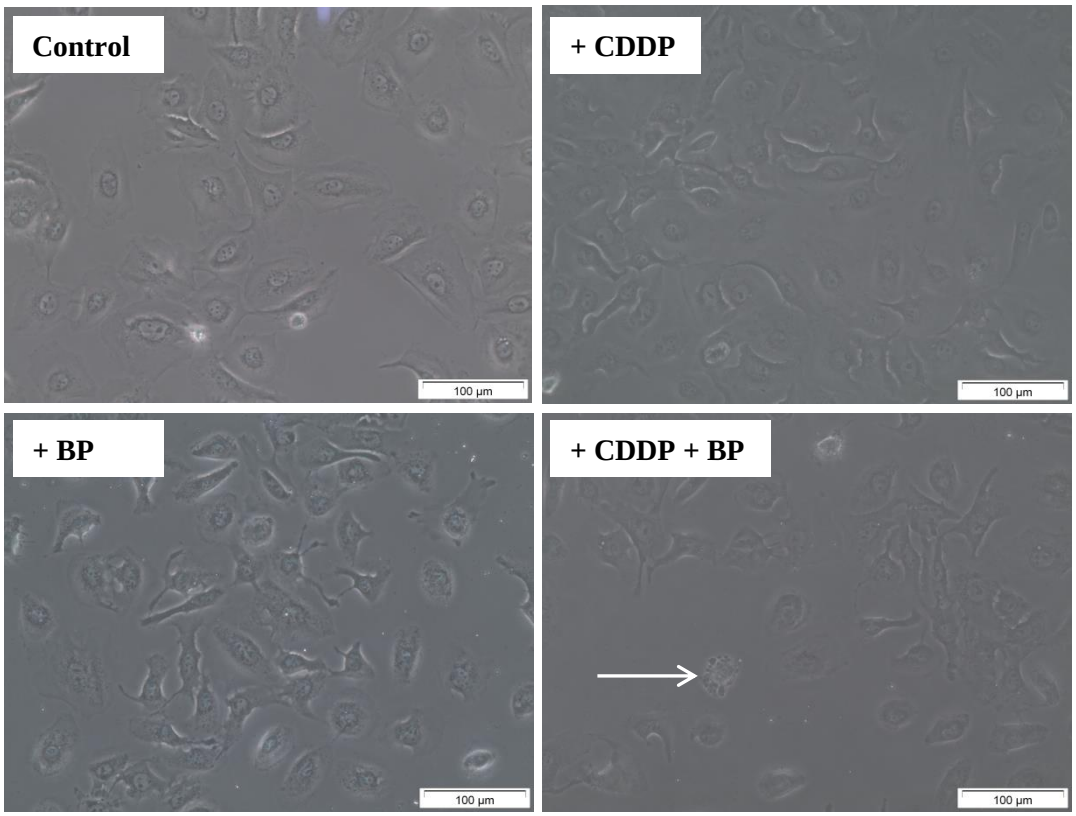


Figure 3.3 Light microscopy images showing WHCO1 cell morphology in response to 5-FU/BP treatment. All images shown were taken after 24 hours of treatment. Blebbing of the cell membrane was observed in the cells treated with 5-FU and BP together (shown by the arrow), which is an indicator of apoptosis. Photograph A (right) is an enlarged image of these apoptotic cells. Control cells were treated with 0.04% DMSO.

3.2.3 Co-treatment of WHCO1 cells with CDDP and BP induced early apoptosis

A concentration of 9.2 µM CDDP was used to treat the WHCO1 cells since the literature reports this concentration to be the IC_{50} for CDDP in this cell line (Kaschula *et al.*, 2012). Similar to the 5-FU and BP co-treatment, CDDP and BP in combination also induced blebbing of the WHCO1 cell surfaces in less than 1% of the cells and this was noticed after 24 hours, indicating early stage apoptosis (Figure 3.4).



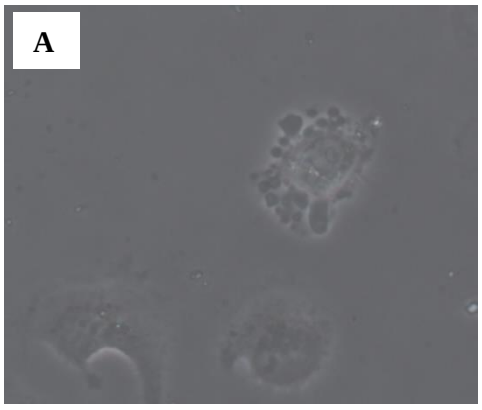


Figure 3.4 Light microscopy images showing WHCO1 cell morphology in response to CDDP/BP treatment. All images shown were taken after 24 hours of treatment. Blebbing of the cell membrane was observed in the cells treated with CDDP and BP together (shown by the arrow), which is an indicator of apoptosis. Photograph A (right) is an enlarged image of these apoptotic cells. Control cells were treated with 0.04% DMSO.

3.3 RNA extraction and qRT-PCR

3.3.1 The extracted RNA was suitable for cDNA synthesis

In order to determine the gene expression profiles associated with observed changes when undertaking cell culture experiments, qRT-PCR was performed, which requires the extraction of RNA. The quality of the RNA must be validated to ensure that the downstream experiments, i.e. cDNA synthesis and qRT-PCR, yield results that are as accurate as possible and not influenced by poor quality of RNA. For each treatment, the overall quality and integrity of the extracted RNA was verified by electrophoresis on an agarose gel. Figure 3.5 shows an example of a gel where the RNA samples showed acceptable overall quality and integrity; sharp 28S and 18S ribosomal RNA bands, with no low molecular weight smears. Furthermore, the amount and purity of the RNA samples were validated by spectrophotometry, where all samples used had 260/280 and 260/230 ratios of ~1.8 and 2.2, respectively.

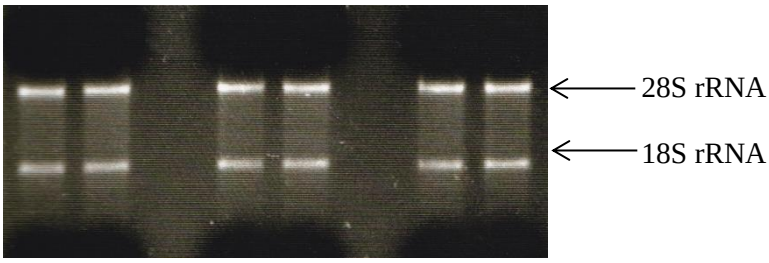


Figure 3.5 Validation of overall RNA quality and integrity by agarose gel electrophoresis. Sharp 28S and 18S rRNA bands were observed after electrophoresis in a 1% agarose gel, as indicated by the arrows.

3.3.2 The primers for CYP1A1, 1A2, 1B1, GSTP1 and GAPDH were specific

When performing qRT-PCR, it is vital to ensure that the primers used amplify only the desired product, and no other non-specific products, and that no primer dimers are formed. One method of verifying this is through melt curve analysis. The melt curve is also referred to as a dissociation curve and provides a measurement of the temperature at which 50% of the double-stranded DNA molecules are dissociated into single strands, which is quantified by the fluorescence of the DNA-intercalating dye in the reaction, such as SYBR green. The melting temperature is unique to the qRT-PCR product, which means that a single peak shows amplification of a single product, whilst multiple different peaks are indicative of co-amplification of extra products. Smaller additional peaks can also indicate the presence of primer dimers. The three identical peaks observed for each product in the melt curves generated by each primer pair were indicative of a single product (in triplicate), with no primer dimers. An example of the melt curve for CYP1B1 is shown in Figure 3.6, and an example for each of the other target genes (including GAPDH) is shown in Appendix C (Figures C1 – C4).

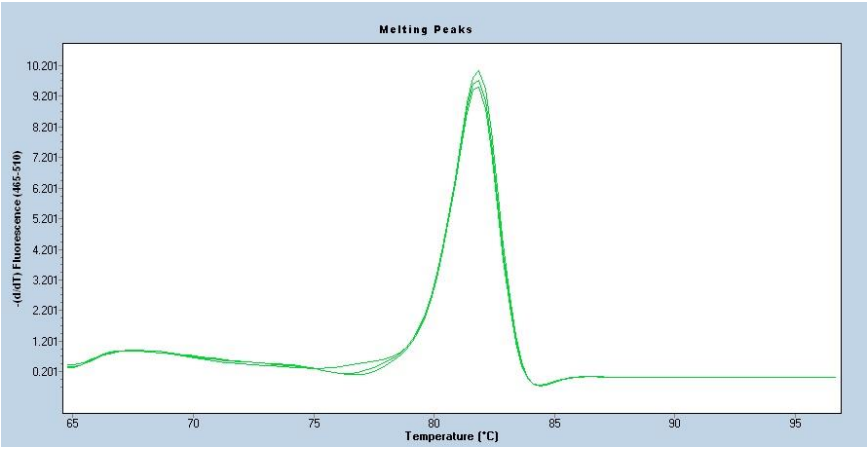


Figure 3.6 Melt curve for CYP1B1 product after qRT-PCR. Three matched peaks can be observed, indicating that the primers amplified a single product (in triplicate), in this case, CYP1B1, and confirming that no primer dimers were formed.

Additionally, the amplification of a single product of the correct size for each primer pair (including GAPDH) was verified by agarose gel electrophoresis of the sample after qRT PCR, as shown in Appendix D (Figure D1). Furthermore, the qRT-PCR products were sequenced and the online BLAST tool (http://www.ncbi.nlm.nih.gov) was used for confirmation (data not shown).

3.4 The effects of 5-FU, CDDP and BP on the mRNA and protein expression of CYP1A1, CYP1A2, CYP1B1 and GSTP1

The main aim of this study was to investigate the effects of chemotherapeutic drug treatment (5-FU and CDDP) alone and in the presence of BP, on the WHCO1 cancer cell line by evaluating changes in mRNA and protein expression of four DMEs (CYP1A1, 1A2, 1B1 and GSTP1). This was achieved using qRT-PCR and western blot analyses and the results are shown below. For each experiment, cells were treated for 6, 12 and 24 hours. All qRT-PCR data are represented as fold induction relative to the control samples (treated with DMSO only), after normalisation to the housekeeping gene *GAPDH*. The result obtained for the control in each qRT-PCR experiment was taken as the reference and represented as having a fold expression of one, thus any bars above one indicate an increase in gene expression whilst those below one indicate a decrease in gene expression, relative to the control. All densitometric analyses of western blots were performed using LI-COR® Image Studio Lite software v 3.1.4 and are represented as the ratio of the amount of DME protein detected to the amount of loading control protein (p38) detected. As was the case with the qRT-PCR results, the result obtained for the control (i.e. DMSO-only treatment) in each western blot experiment was taken as the point of reference and represented as having a fold expression of one.

3.4.1 CYP1A1 mRNA expression was significantly induced by 5-FU

The treatment of the WHCO1 cells with 5-FU triggered small but significant and reproducible increases in *CYP1A1* mRNA expression in comparison with the control, with a maximal increase of ~2-fold at 24 hours. As expected, exposure to BP resulted in robust increases in *CYP1A1* mRNA of 6-fold, 9-fold and 11-fold *CYP1A1* expression at 6, 12 and 24 hour respectively (Figure 3.7). The induction in response to BP was also observed at the protein level at 12 hours (20-fold) and more readily at 24 hours (70-fold, Figure 3.8). When cells were treated with a combination of 5-FU and BP, a response similar to what was observed for the BP only treatment was produced at 6 and 12 hours (~6-fold and ~9-fold, respectively). However, at 24 hours, the increase was not as large, with a 4-fold induction of *CYP1A1* mRNA. The protein expression trend was similar at 24 hours; whilst BP exposure alone led to a 70-fold increase in CYP1A1 protein compared to the control, BP and 5-FU combined resulted in just a 40-fold increase in expression, compared to the control, as shown in Figure 3.8.

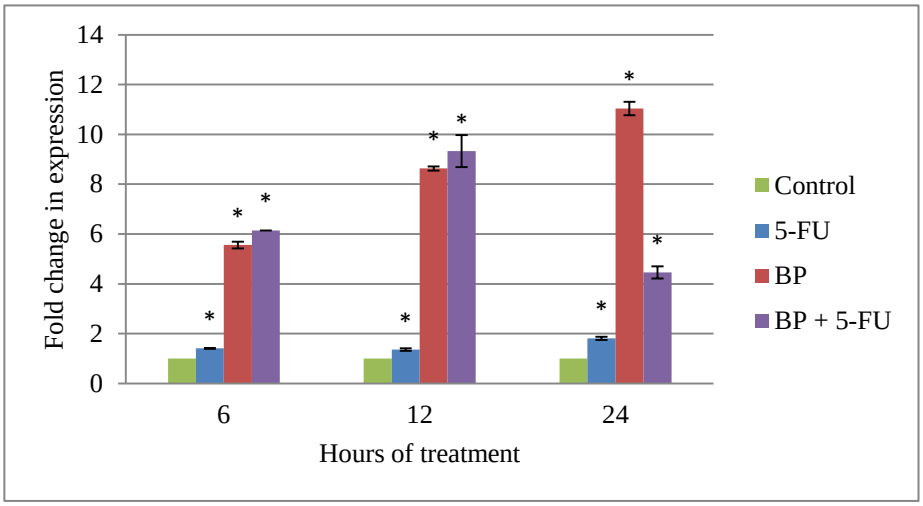


Figure 3.7 *CYP1A1* mRNA expression in response to 5-FU and/or BP treatment. Cells were treated with 5-FU, BP and 5-FU + BP in combination. Control cells were treated with 0.04% DMSO and all control mRNA expression levels are represented as one. The bar graph represents mRNA expression derived from qRT-PCR data and the fold changes are in reference to the control which has been normalised to one. The data are representative of three independent experiments. The * indicates significant differences ($p \leq 0.05$) compared to control cells.

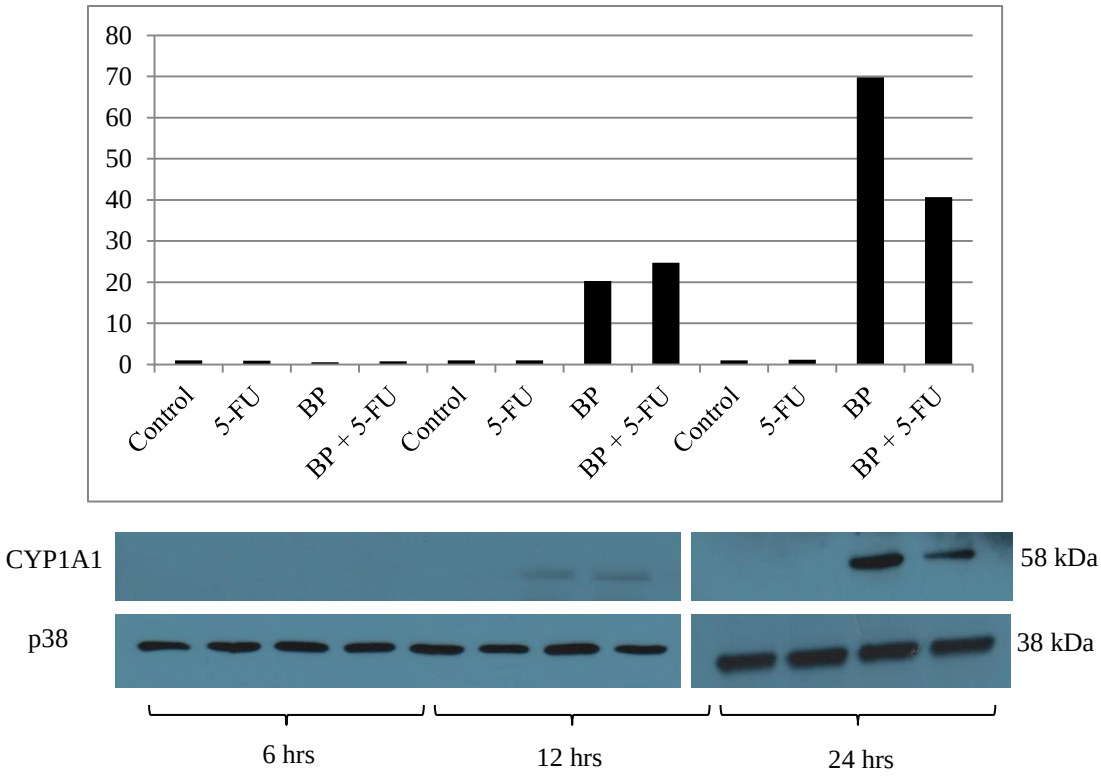


Figure 3.8 CYP1A1 protein expression in response to 5-FU and/or BP treatment. Cells were treated with 5-FU, BP and 5-FU + BP in combination. Control cells were treated with 0.04% DMSO. The bands shown (lower panel) were obtained by western blot analysis using the CYP1A1 antibody and the p38 antibody (loading control), and the corresponding bar graph derived from densitometric analyses of the western blots (upper panel) represents the expression level of CYP1A1 protein normalised to the internal loading control protein p38.

3.4.2 CYP1A2 mRNA expression was differentially altered by 5-FU

Unlike what was observed for CYP1A1, no significant difference was observed with respect to CYP1A2 mRNA expression after 5-FU treatment at 6 hours, however, a significant decrease (0.5-fold) was noted at the 12 hour time point, while a significant increase was observed at 24 hours (~2-fold). Similar to CYP1A1, and as expected, BP alone resulted in much more pronounced CYP1A2 mRNA and protein induction, especially at 12 and 24 hours: for mRNA expression, a 1.5-fold, 4-fold and 8-fold induction was observed at 6, 12 and 24 hours respectively, and for protein induction, a ~5-fold, ~20-fold and ~90-fold was observed (Figure 3.9). At 6 hours, contrary to *CYP1A1*, the combination of 5-FU and BP led to much more enhanced expression of *CYP1A2*, compared to when treated with BP alone. But the effect observed at 24 hours with the combination treatment was similar to that observed for CYP1A1, where the increase was not as large (~5-fold) as that observed for BP alone (~8-fold), and this was reflected at the protein levels (Figure 3.10).

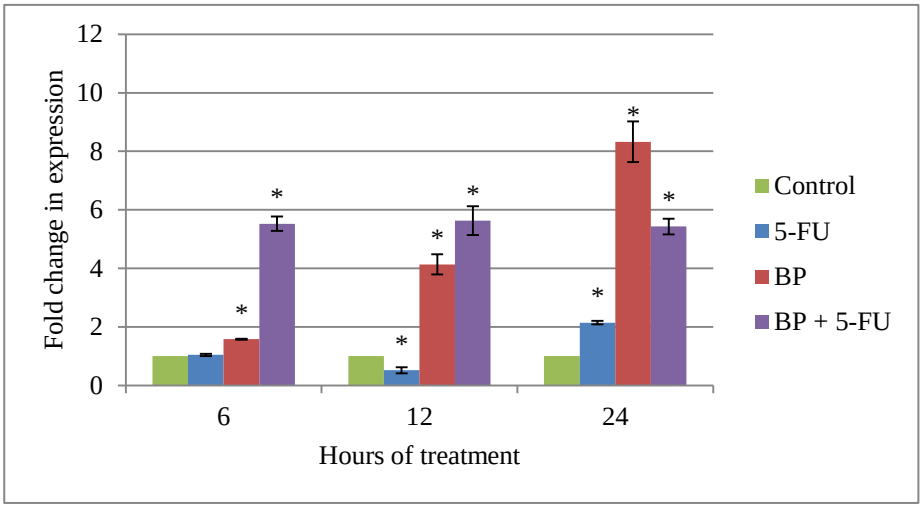


Figure 3.9 *CYP1A2* mRNA expression in response to 5-FU and/or BP treatment. Cells were treated with 5-FU, BP and 5-FU + BP in combination. Control cells were treated with 0.04% DMSO and all control mRNA expression levels are represented as one. The bar graph represents mRNA expression derived from qRT-PCR data and the fold changes are in reference to the control which has been normalised to one. The data are representative of three independent experiments. The * indicates significant differences ($p \leq 0.05$) compared to control cells.

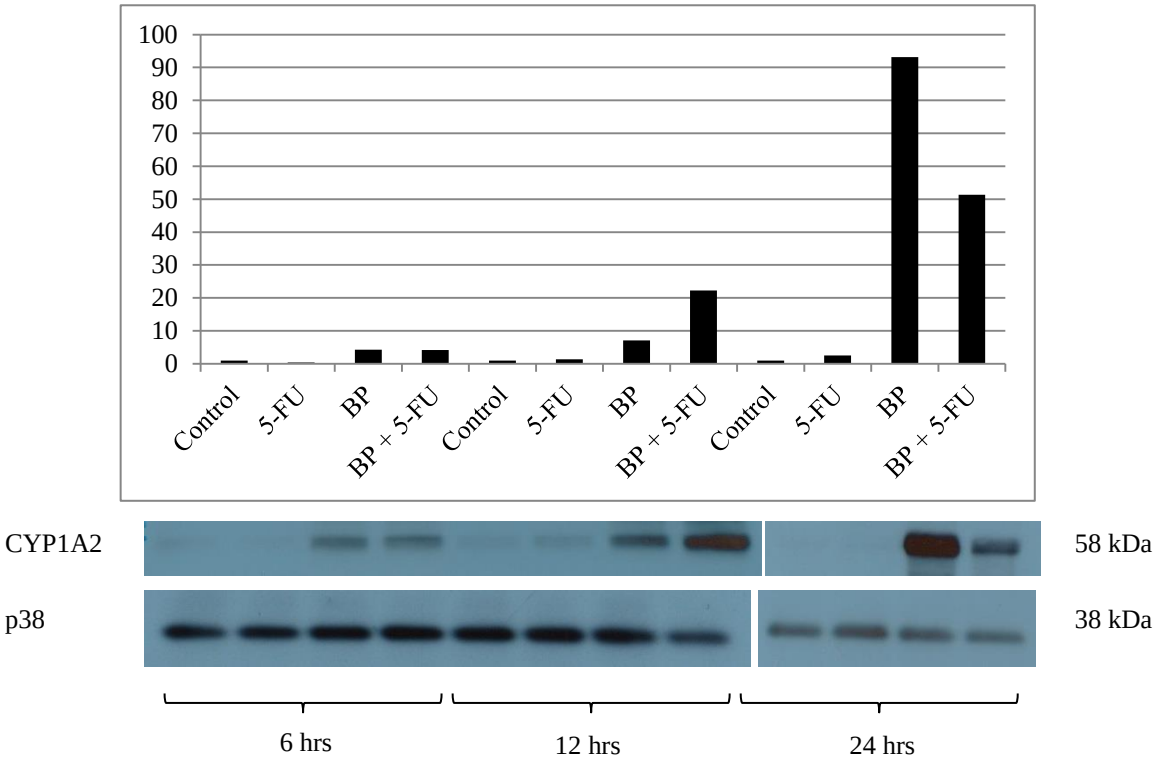


Figure 3.10 CYP1A2 protein expression in response to 5-FU and/or BP treatment. Cells were treated with 5-FU, BP and 5-FU + BP in combination. Control cells were treated with 0.04% DMSO. The bands shown (lower panel) were obtained by western blot analysis using the CYP1A2 antibody and the p38 antibody (loading control), and the corresponding bar graph derived from densitometric analyses of the western blots (upper panel) represents the expression level of CYP1A2 protein normalised to the internal loading control protein p38.

3.4.3 CYP1B1 mRNA was significantly induced by 5-FU

The expression of *CYP1B1* mRNA in WHCO1 cells treated with 5-FU was significantly increased, although at a low scale (Figure 3.11) which follows the trend observed for CYP1A1. A 1.4, 1.7 and 1.6-fold increase in mRNA expression was observed at 6, 12 and 24 hours respectively, while also similar to CYP1A1, no protein expression could be detected at these time points and under these treatment conditions, relative to the controls (Figure 3.12). Treatment with BP led to significant increases in both mRNA and protein expressions; an average increase of 8-fold in mRNA expression was observed at all three time points, while a ~40-fold, ~60-fold and ~70-fold increase in protein expression throughout the time points was observed. When the cells were co-treated with BP and 5-FU, *CYP1B1* mRNA expression steadily increased from ~1.3-fold at 6 hours, to ~5-fold at 12 hours, to ~9-fold at 24 hours, as shown in Figure 3.11. This pattern was reflected in the protein (Figure 3.12). However, it is interesting to note that at 6 and 12 hours, the extent of *CYP1B1* mRNA inductions were much reduced compared to induction with BP alone, which was also reflected at the protein level (at 6 hours). At 24 hours, both BP and BP + 5-FU induction was to the same extent in the mRNA (~8-fold) and the protein (~70-fold).

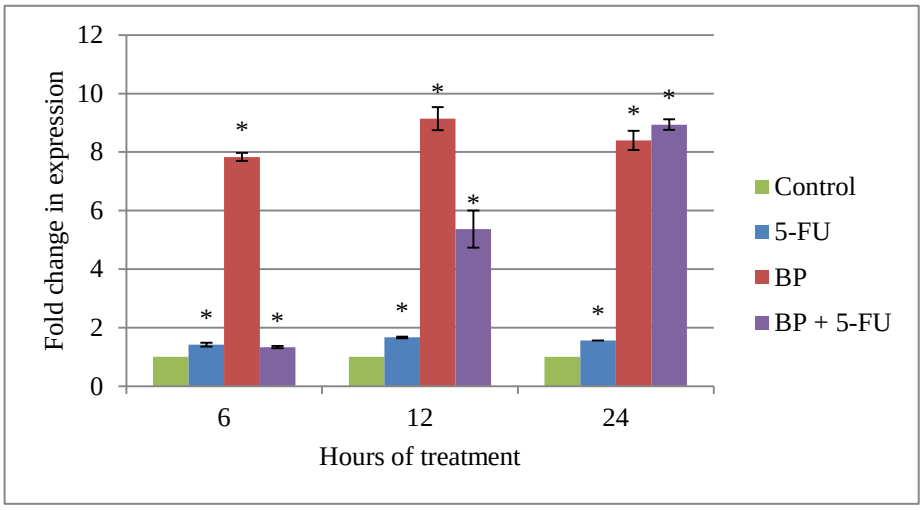


Figure 3.11 *CYP1B1* expression in response to 5-FU and/or BP treatment. Cells were treated with 5-FU, BP and 5-FU + BP in combination. Control cells were treated with 0.04% DMSO and all control mRNA expression levels are represented as one. The bar graph represents mRNA expression derived from qRT-PCR data and the fold changes are in reference to the control which has been normalised to one. The data are representative of three independent experiments. The * indicates significant differences ($p \leq 0.05$) compared to control cells.

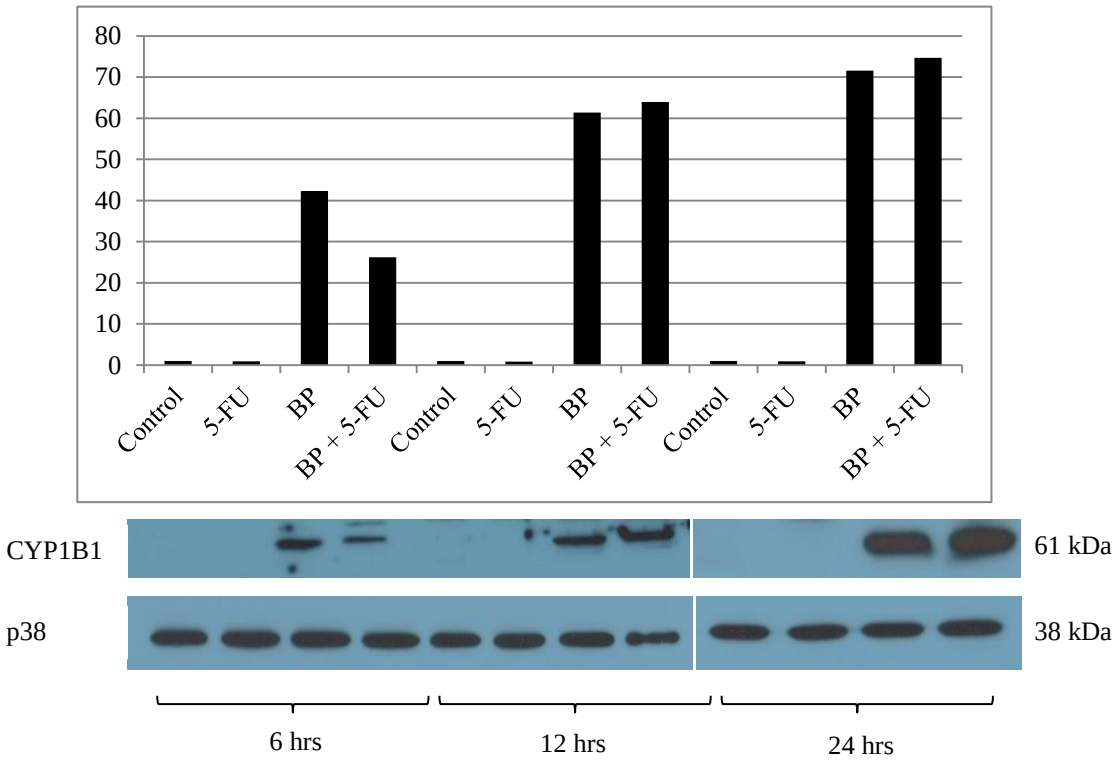


Figure 3.12 CYP1B1 protein expression in response to 5-FU and/or BP treatment. Cells were treated with 5-FU, BP and 5-FU + BP in combination. Control cells were treated with 0.04% DMSO. The bands shown (lower panel) were obtained by western blot analysis using the CYP1B1 antibody and the p38 antibody (loading control), and the corresponding bar graph derived from densitometric analyses of the western blots (upper panel) represents the expression level of CYP1B1 protein normalised to the internal loading control protein p38.

3.4.4 GSTP1 mRNA expression was significantly decreased by 5-FU

Exposure to 5-FU led to small but significant decreases in *GSTP1* mRNA expression, namely ~0.2-fold decreases throughout the time points (Figure 3.13). BP exposure did not lead to induction of *GSTP1* mRNA at 6 hours, but there was a significant increase at 12 hours (1.4-fold) and a significant decrease at 24 hours (~0.6-fold). Furthermore, treatment of the WHCO1 cells with 5-FU and BP combined did not cause any significant changes in mRNA expression at the early time points, although a slight but significant increase, 1.2-fold compared to the control, was observed at 24 hours. The pattern of GSTP1 protein expression generally did not follow that of the mRNA, and all the observed changes were below 1.6-fold (Figure 3.14).

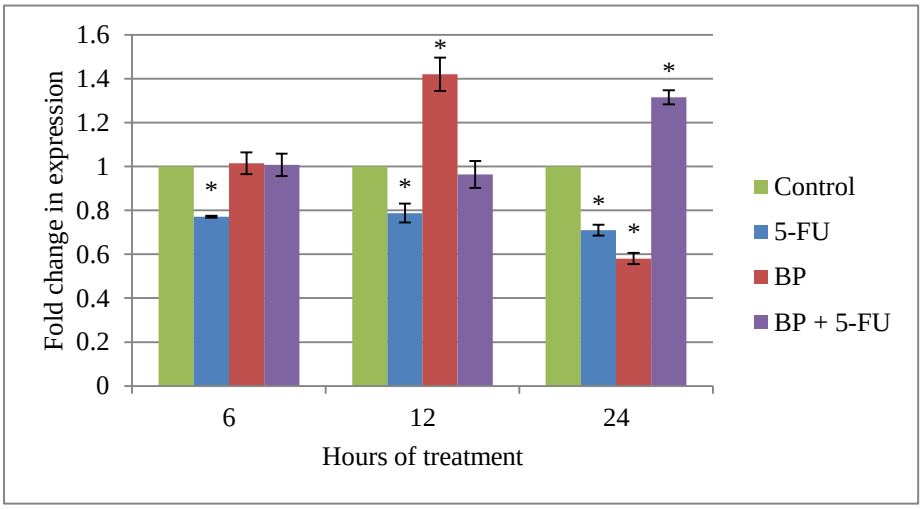


Figure 3.13 *GSTP1* gene expression in response to 5-FU and/or BP treatment. Cells were treated with 5-FU, BP and 5-FU + BP in combination. Control cells were treated with 0.04% DMSO and all control mRNA expression levels are represented as one. The bar graph represents mRNA expression derived from qRT-PCR data and the fold changes are in reference to the control which has been normalised to one. The data are representative of three independent experiments. The * indicates significant differences ($p \leq 0.05$) compared to control cells.

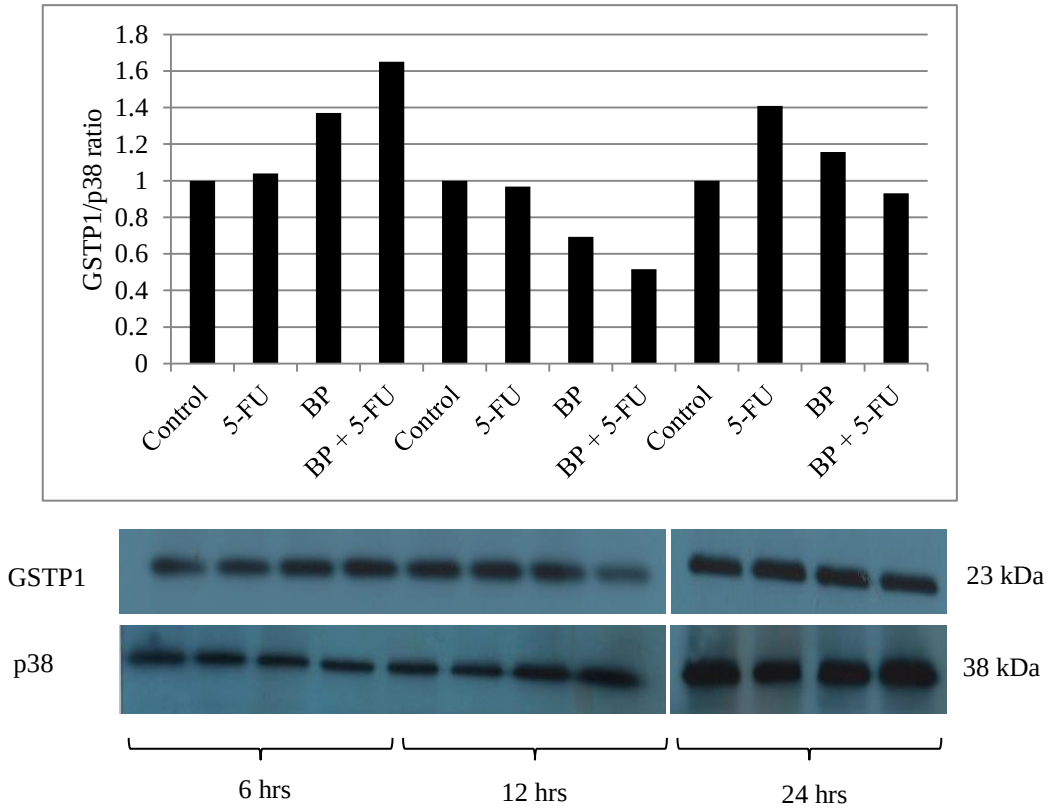


Figure 3.14 GSTP1 protein expression in response to 5-FU and/or BP treatment. Cells were treated with 5-FU, BP and 5-FU + BP in combination. Control cells were treated with 0.04% DMSO. The bands shown (lower panel) were obtained by western blot analysis using the GSTP1 antibody and the p38 antibody (loading control), and the corresponding bar graph derived from densitometric analyses of the western blots (upper panel) represents the expression level of GSTP1 protein normalised to the internal loading control protein p38.

The mRNA and protein expression changes of the four DMEs in response to the 5-FU and/or BP treatments, as well as the morphological changes observed in the WHCO1 cells, are summarised in Table 3.1.

Table 3.1 Summary of findings on the WHCO1 cells when treated with 5-FU, BP and 5-FU+BP. The fold changes of the significant increases and decreases in expression are given in parentheses.

	5-FU			BP			5-FU + BP		
	6 hrs	**12 hrs**	**24 hrs**	**6 hrs**	**12 hrs**	**24 hrs**	**6 hrs**	**12 hrs**	**24 hrs**
	Effects on morphology when compared to untreated control								
	None	None	None	None	None	None	None	None	Blebbing (< 1% of cells)
	Differences in RNA expression when compared to untreated control								
CYP1A1	Increase (1.4)	Increase (1.4)	Increase (1.8)	Increase (5.6)	Increase (8.6)	Increase (11.0)	Increase (6.1)	Increase (9.3)	Increase (4.5)
CYP1A2	None	Decrease (0.5)	Increase (2.1)	Increase (1.6)	Increase (4.1)	Increase (8.3)	Increase (5.5)	Increase (5.6)	Increase (5.4)
CYP1B1	Increase (1.4)	Increase (1.7)	Increase (1.6)	Increase (7.8)	Increase (9.1)	Increase (8.4)	Increase (1.3)	Increase (5.4)	Increase (8.9)
GSTP1	Decrease (0.8)	Decrease (0.8)	Decrease (0.7)	None	Increase (1.4)	Decrease (0.6)	None	None	Increase (1.3)
	Differences in protein expression when compared to untreated control								
CYP1A1	None	None	None	None	Increase (20.3)	Increase (69.8)	None	Increase (24.7)	Increase (40.7)
CYP1A2	None	None	None	Increase (4.2)	Increase (7.1)	Increase (93.2)	Increase (4.2)	Increase (22.2)	Increase (51.4)
CYP1B1	None	None	None	Increase (42.4)	Increase (61.3)	Increase (71.6)	Increase (26.2)	Increase (63.9)	Increase (74.7)
GSTP1	None	None	Increase (1.4)	Increase (1.4)	Decrease (0.7)	Increase (1.2)	Increase (1.7)	Decrease (0.5)	Increase (0.9)

3.5 The effects of CDDP and BP on the mRNA and protein expression of CYP1A1, CYP1A2, CYP1B1 and GSTP1

A second chemotherapeutic drug, CDDP, was also investigated in the same way as was done for 5-FU. Like 5-FU, CDDP is one of the most commonly used drugs for the treatment of various tumours. Below, observations on the work performed using CDDP, BP and the WHCO1 cells are presented.

3.5.1 CYP1A1 mRNA expression was differentially altered by CDDP

The exposure of the WHCO1 cells to CDDP led to minor but significant changes in *CYP1A1* mRNA expression, at 6 (1.3-fold increase) and 12 hours (0.6-fold decrease) but no significant difference at 24 hours, compared to the control (Figure 3.15). None of these changes were observed in the CYP1A1 protein (Figure 3.16). CDDP, when used in the presence of BP, caused an induction of *CYP1A1* at all the three time points, being ~4-fold at 6 hours and ~6-fold at both 12 and 24 hours (Figure 3.16), although these increases were less than what was observed for BP alone at 12 (~12-fold) and 24 hours (~16-fold). For the protein expression, the effect of co-administration of BP + CDDP was only observed at 12 hours (~40-fold) and expression was less detectable at 24 hours (below 10-fold), as shown in Figure 3.16. This effect was similar to that observed for 5-FU + BP and CYP1A1/1A2, compared to BP alone, at the 24 hour time point.

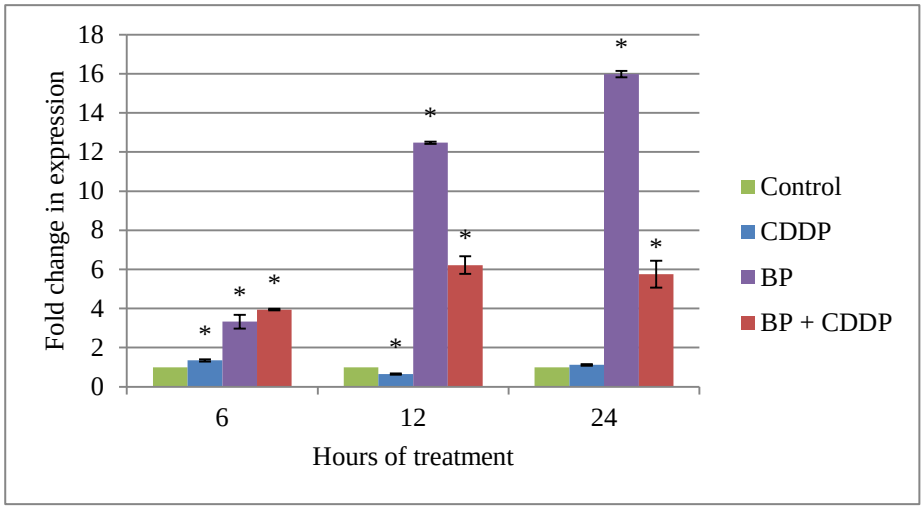


Figure 3.15 in *CYP1A1* gene expression in response to CDDP and/or BP treatment. Cells were treated with CDDP, BP and CDDP + BP in combination. Control cells were treated with 0.04% DMSO and all control mRNA expression levels are represented as one. The bar graph represents mRNA expression derived from qRT-PCR data and the fold changes are in reference to the control which has been normalised to one. The data are representative of three independent experiments. The * indicates significant differences ($p \leq 0.05$) compared to control cells.

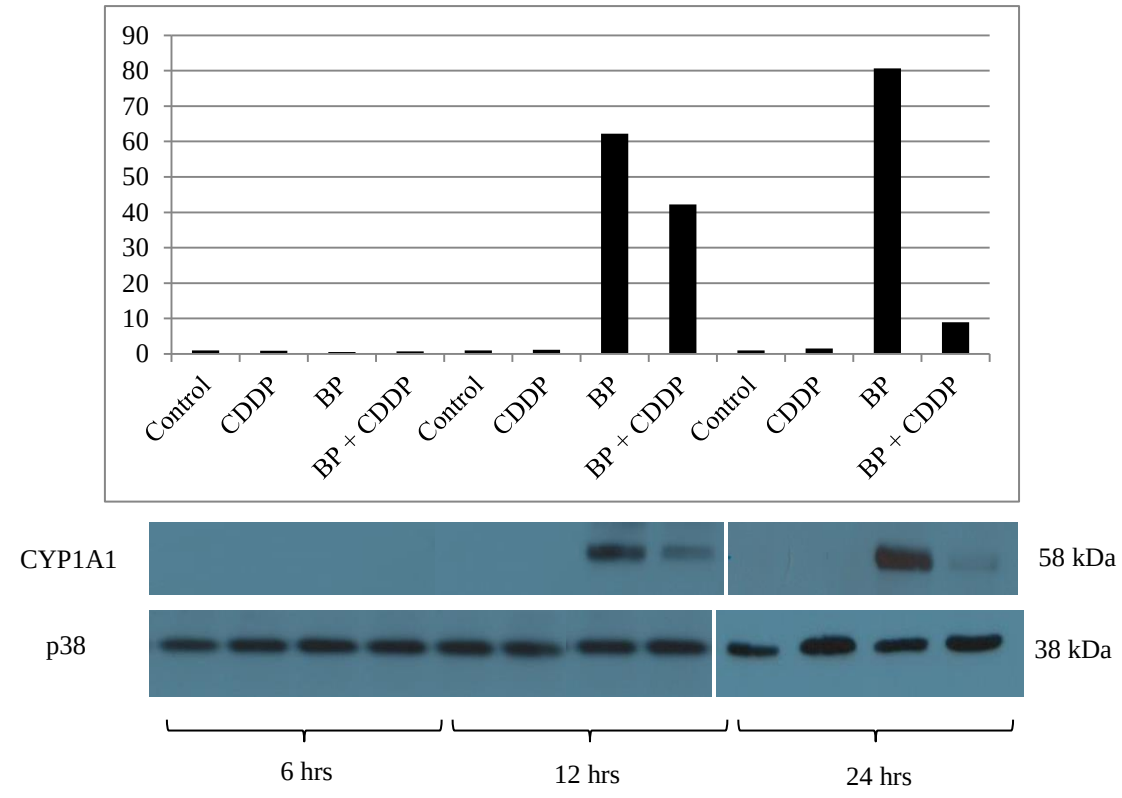


Figure 3.16 CYP1A1 protein expression in response to CDDP and/or BP treatment. Cells were treated with CDDP, BP and CDDP + BP in combination. Control cells were treated with 0.04% DMSO. The bands shown (lower panel) were obtained by western blot analysis using the CYP1A1 antibody and the p38 antibody (loading control), and the corresponding bar graph derived from densitometric analyses of the western blots (upper panel) represents the expression level of CYP1A1 protein normalised to the internal loading control protein p38.

3.5.2 CYP1A2 mRNA expression was significantly decreased by CDDP

CDDP treatment of WHCO1 cells led to a significant down-regulation of *CYP1A2* mRNA expression throughout the time points (Figure 3.17), with the level of mRNA expression almost undetectable at ~0.02 to 0.07-fold (Figure 3.18). The BP + CDDP treatment resulted in increases in *CYP1A2* mRNA expression at 6, 12 and 24 hours (~2.2-fold, 4-fold and 11-fold respectively, compared to the control), as shown in Figure 3.17. This pattern was also not reflected in the protein, but interestingly, followed the pattern of expression seen in response to BP treatment; i.e. the co-treatment led to a ~5-fold increase in expression at 6 hours, which increased to ~40-fold at 12 hours and subsequently decreased to ~7–fold at 24 hours (Figure 3.18). Unlike the ~90-fold induction of CYP1A2 protein by BP observed in Figure 3.10, treatment with BP in the CDDP experiment led to a protein induction level of only ~14-fold, as shown in Figure 3.18.Similarly to CYP1A1, the induction of CYP1A2 mRNA and protein in response to CDDP + BP was lower at 24 hours than the induction in response to BP alone.

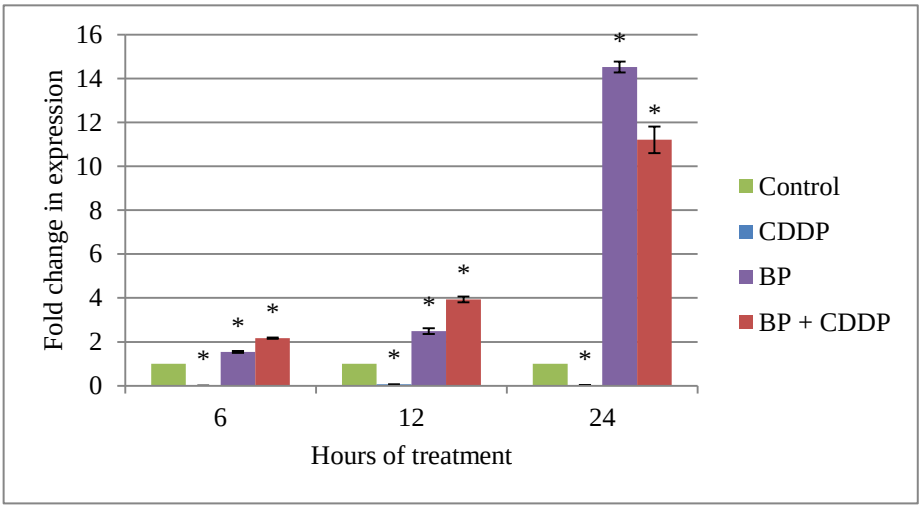


Figure 3.17 *CYP1A2* gene expression in response to CDDP and/or BP treatment. Cells were treated with CDDP, BP and CDDP + BP in combination. Control cells were treated with 0.04% DMSO and all control mRNA expression levels are represented as one. The bar graph represents mRNA expression derived from qRT-PCR data and the fold changes are in reference to the control which has been normalised to one. The data are representative of three independent experiments. The * indicates significant differences ($p \leq 0.05$) compared to control cells.

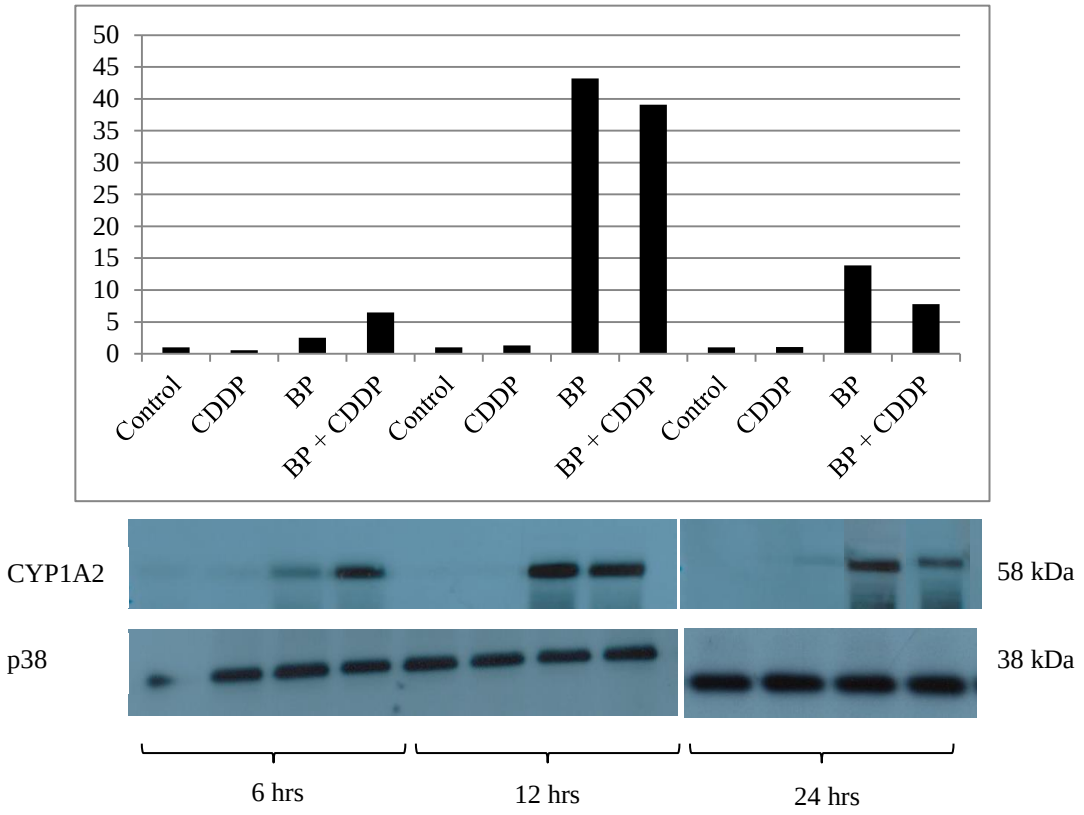


Figure 3.18 CYP1A2 protein expression in response to CDDP and/or BP treatment. Cells were treated with CDDP, BP and CDDP + BP in combination. Control cells were treated with 0.04% DMSO. The bands shown (lower panel) were obtained by western blot analysis using the CYP1A2 antibody and the p38 antibody (loading control), and the corresponding bar graph derived from densitometric analyses of the western blots (upper panel) represents the expression level of CYP1A2 protein normalised to the internal loading control protein p38.

3.5.3 CYP1B1 mRNA expression was significantly decreased by CDDP

In response to CDDP treatment, the expression of *CYP1B1* mRNA was significantly down-regulated at 6 (0.7-fold) and 12 hours (0.6-fold), but remained unaffected at 24 hours (Figure 3.19). Similarly to the response of CYP1B1 to 5-FU exposure, these changes were not observable at the protein level (Figure 3.20). Exposure of the WHCO1 cells to CDDP and BP in combination led to a steady increase in mRNA expression, from 8-fold at 6 hours to ~12-fold at 12 hours to ~16-fold at 24 hours, compared to the control, which mirrored the trend of induction caused by BP, but was higher than the level of induction by BP at 6 and 12 hours. Furthermore, this pattern was detected in the CYP1B1 protein, as shown in Figure 3.20, i.e. an 8-fold increase at 6 hours, followed by a ~50-fold increase at 12 hours and a ~60-fold increase at 24 hours, in response to BP + CDDP co-treatment.

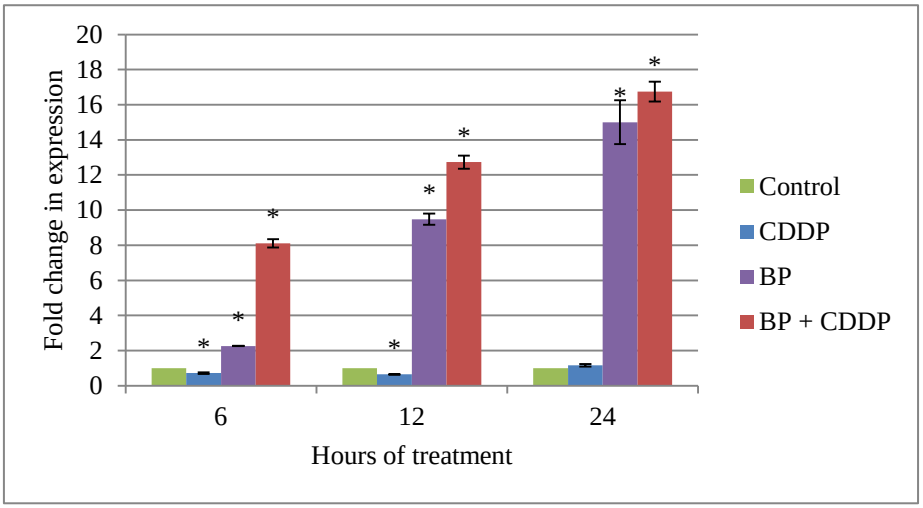


Figure 3.19 *CYP1B1* gene expression in response to CDDP and/or BP treatment. Cells were treated with CDDP, BP and CDDP + BP in combination. Control cells were treated with 0.04% DMSO and all control mRNA expression levels are represented as one. The bar graph represents mRNA expression derived from qRT-PCR data and the fold changes are in reference to the control which has been normalised to one. The data are representative of three independent experiments. The * indicates significant differences ($p \leq 0.05$) compared to control cells.

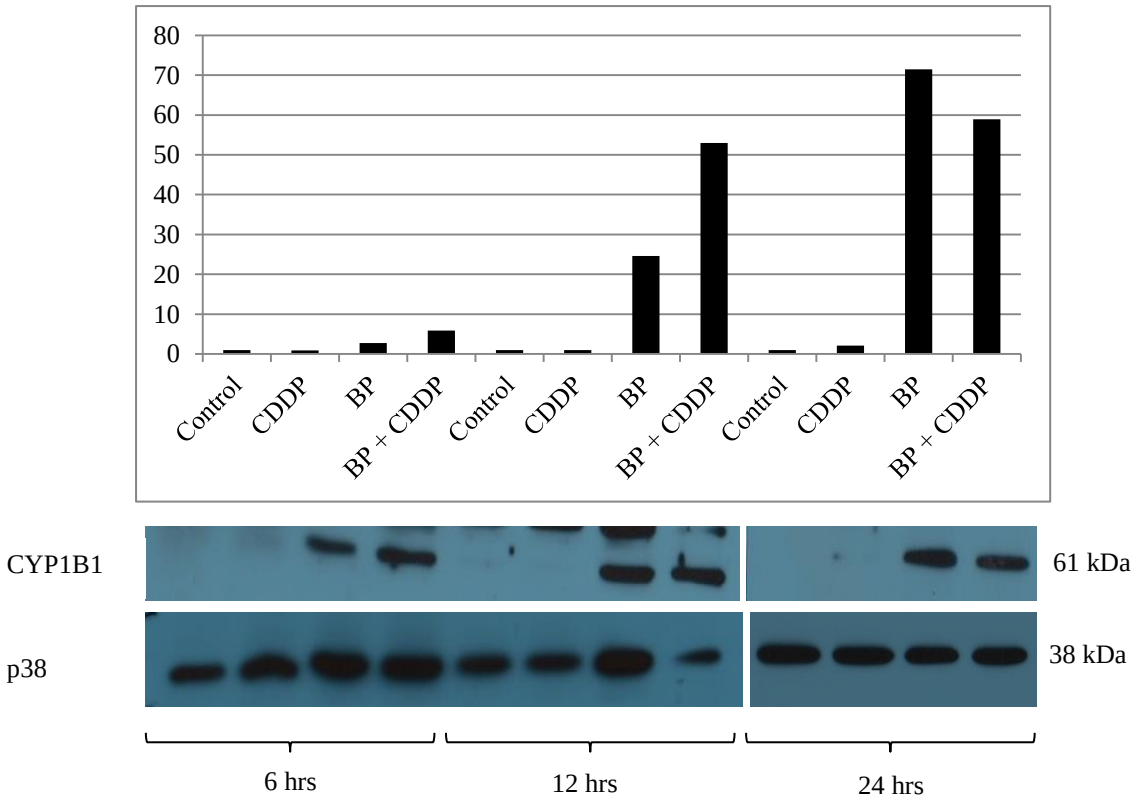


Figure 3.20 CYP1B1 protein expression in response to CDDP and/or BP treatment. Cells were treated with CDDP, BP and CDDP + BP in combination. Control cells were treated with 0.04% DMSO. The bands shown (lower panel) were obtained by western blot analysis using the CYP1B1 antibody and the p38 antibody (loading control), and the corresponding bar graph derived from densitometric analyses of the western blots (upper panel) represents the expression level of CYP1B1 protein normalised to the internal loading control protein p38.

3.5.4 GSTP1 expression was significantly increased by CDDP

In contrast to the response of *GSTP1* mRNA to 5-FU, there were small but significant increases in *GSTP1* mRNA expression in response to CDDP treatment (Figure 3.21), namely 1.3-fold at 6 hours, 1.2-fold at 12 hours and 1.8-fold at 24 hours, compared to the control. Exposure to BP also induced *GSTP1* mRNA expression to a minor but significant extent (~1.5-fold) throughout the time points. In the case of BP and CDDP combination treatment, there was a significant increase in mRNA expression only at 24 hours (1.2-fold) as shown in Figure 3.21, which is similar to the case of BP + 5-FU treatment at 24 hours. Also similar to the 5-FU and/or BP experiments, the protein expression pattern generally did not follow that of the mRNA expression in response to CDDP, where the protein levels exhibited no major or consistent changes compared to the control, and any changes that were observed were below ~1.4-fold, as shown in Figure 3.22.

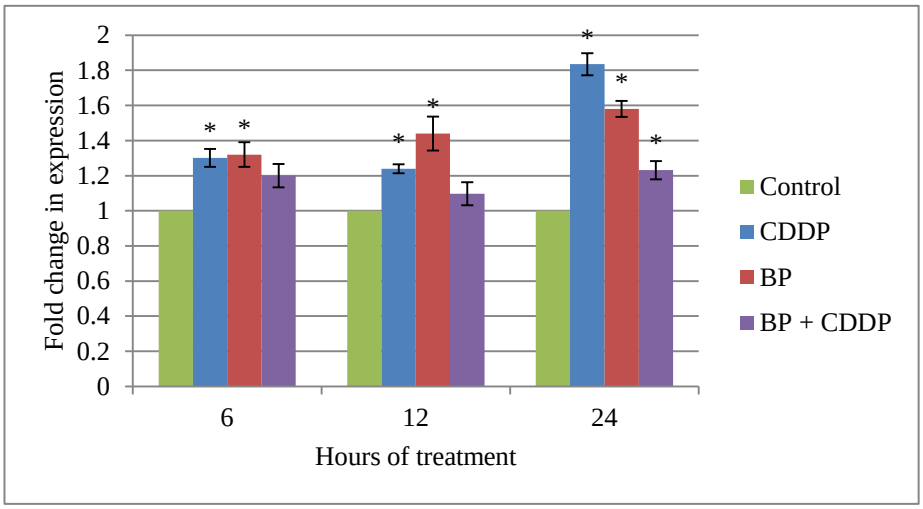


Figure 3.21 *GSTP1* gene expression in response to CDDP and/or BP treatment. Cells were treated with CDDP, BP and CDDP + BP in combination. Control cells were treated with 0.04% DMSO and all control mRNA expression levels are represented as one. The bar graph represents mRNA expression derived from qRT-PCR data and the fold changes are in reference to the control which has been normalised to one. The data are representative of three independent experiments. The * indicates significant differences ($p < 0.05$) compared to control cells.

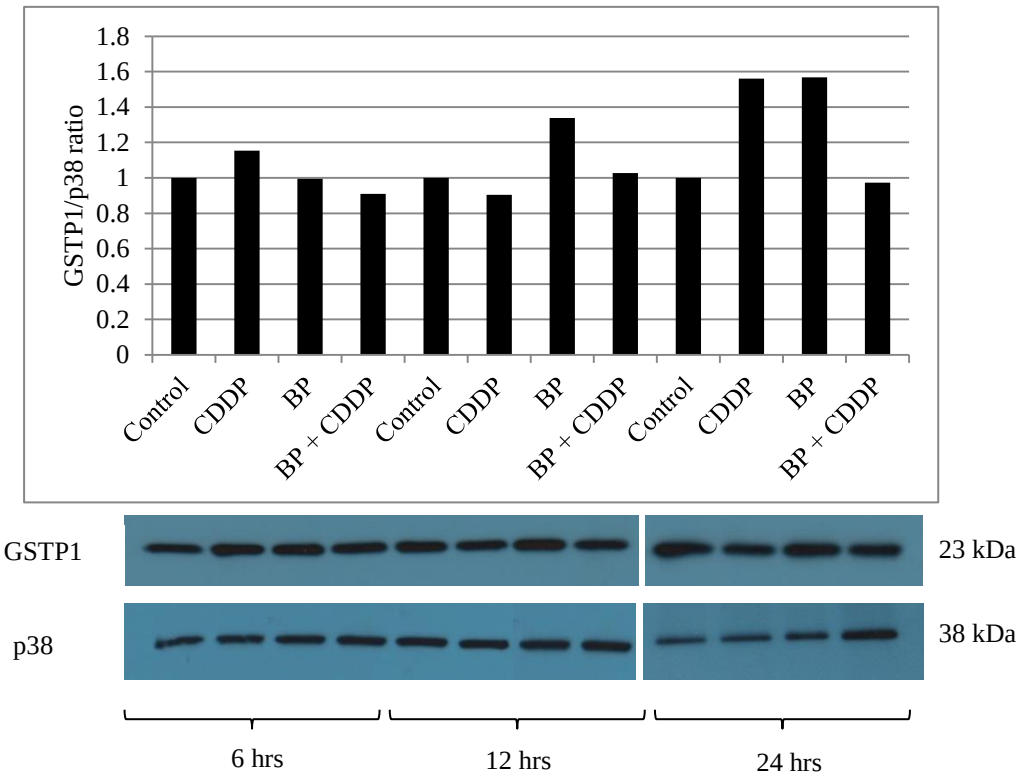


Figure 3.22 GSTP1 protein expression in response to CDDP and/or BP treatment. Cells were treated with CDDP, BP and CDDP + BP in combination. Control cells were treated with 0.04% DMSO. The bands shown (lower panel) were obtained by western blot analysis using the GSTP1 antibody and the p38 antibody (loading control), and the corresponding bar graph derived from densitometric analyses of the western blots (upper panel) represents the expression level of GSTP1 protein normalised to the internal loading control protein p38.

The mRNA and protein expression changes of the four DMEs in response to the CDDP and/or BP treatments, as well as the morphological changes observed in the WHCO1 cells, are summarised in Table 3.2.

Table 3.2 Summary of findings on the WHCO1 cells when treated with CDDP and BP. The fold changes of the significant increases and decreases in expression are given in parentheses.

	CDDP			**BP**			**CDDP + BP**		
	6 hrs	**12 hrs**	**24 hrs**	**6 hrs**	**12 hrs**	**24 hrs**	**6 hrs**	**12 hrs**	**24 hrs**
	Effects on morphology when compared to untreated control								
	None	None	None	None	None	None	None	None	Blebbing (< 1% of cells)
	Differences in RNA expression when compared to untreated control								
CYP1A1	Increase (1.3)	Decrease (0.7)	None	Increase (3.3)	Increase (12.5)	Increase (16.0)	Increase (3.9)	Increase (6.2)	Increase (5.8)
CYP1A2	Decrease (0.02)	Decrease (0.07)	Decrease (0.02)	Increase (1.5)	Increase (2.5)	Increase (14.5)	Increase (2.2)	Increase (3.9)	Increase (11.2)
CYP1B1	Decrease (0.7)	Decrease (0.6)	None	Increase (2.3)	Increase (9.5)	Increase (15.0)	Increase (8.1)	Increase (12.7)	Increase (16.7)
GSTP1	Increase (1.3)	Increase (1.2)	Increase (1.8)	Increase (1.3)	Increase (1.4)	Increase (1.6)	None	None	Increase (1.2)
	Differences in protein expression when compared to untreated control								
CYP1A1	None	None	None	None	Increase (62.2)	Increase (80.7)	None	Increase (42.2)	Increase (8.9)
CYP1A2	Decrease (0.5)	Increase (1.3)	None	Increase (2.5)	Increase (43.2)	Increase (13.9)	Increase (6.5)	Increase (39.1)	Increase (7.8)
CYP1B1	Decrease (0.8)	Decrease (0.9)	Increase (2.1)	Increase (2.8)	Increase (24.6)	Increase (71.4)	Increase (5.8)	Increase (53.0)	Increase (58.9)
GSTP1	Increase (1.1)	Decrease (0.9)	None	None	Increase (1.3)	Increase (1.6)	Decrease (0.9)	None	None

4. DISCUSSION

This study examined the effects of two chemotherapeutic drugs, 5-FU and CDDP, as well as the known *CYP*-inducing compound BP, on the expression of four DMEs, CYP1A1, 1A2, 1B1 and GSTP1. The results showed mRNA expression changes of various magnitudes i.e. fold changes (as summarised in Table 3.1 and Table 3.2). Many studies of this nature declare a certain fold change amount as a threshold, below which any changes in gene expression are not considered to be biologically meaningful. Early microarray publications considered a 2-fold change a reasonable cut-off (DeRisi *et al.,* 1996; Schena *et al.,* 1996), while more recent studies use combination criteria to accept genes as differentially expressed, for example only if they show a minimum fold change of 1.5 and also satisfy $p < 0.05$ (Peart *et al.*, 2005; Raouf *et al.*, 2008), or a fold change of at least 1.3 and $p < 0.2$ (Huggins *et al.*, 2008). Whilst the application of this method may appear to increase the strength of the findings, the combination criteria are determined *ad hoc*, thus the possibility remains that gene expression changes that are dismissed as not meaningful may in reality elicit important physiological changes (McCarthy and Smyth, 2009). In formal statistical terms, genes are considered to be differentially expressed when their expression levels change between two treatment conditions, irrespective of how small that change may be. Therefore in this project, no threshold was applied and any fold change in mRNA expression of the DMEs was regarded as potentially physiologically meaningful. The observed mRNA expression changes were reproducible amongst three independent cell treatment experiments and the data also had to satisfy $p < 0.05$ for statistical significance. This research serves as a preliminary study; future work may confirm this work by repeating it in additional cancer cell lines, and may investigate the biological effects of the resulting differential expressions and whether these changes may be extrapolated to potential clinical consequences. To the best of our knowledge, this is the first study to explore the effects of the two chemotherapeutic drugs on the *CYP* and *GST* DMEs in an oesophageal cancer cell line. The results will be discussed in detail below.

Treatment of the WHCO1 cells with 5-FU was associated with increased expression of *CYP1A1* and *CYP1B1,* which was generally enhanced in the presence of BP. The precise mechanism

responsible for the up-regulation caused by 5-FU remains to be elucidated, but given that the AhR pathway mediates *CYP1* transcription, it is likely that this pathway is involved. To the best of our knowledge, a direct link between 5-FU and the AhR pathway has not been established. However, previous studies have shown that 5-FU is capable of inducing the activity of cyclin A-cyclin-dependent kinase 2 (Cdk2), an important cell cycle regulatory protein, in four different human cancer cell lines, along with other cyclin proteins (Takeda *et al.*, 1999; Yoshikawa *et al.*, 2001). Interestingly, inhibition of the AhR has been found to be associated with down-regulation of cyclin and Cdk-2 expression. Thus it is possible that an up-regulation in Cdk2 activity, in this case caused by 5-FU, is linked to a corresponding up-regulation in AhR activity ultimately resulting in increased *CYP1* expression (Abdelrahim *et al.*, 2003; Korzeniewski1 *et al.*, 2010). Alternatively, 5-FU has also been shown to activate an important pathway known as the NF-E2 p45-regulated factor (Nrf2) signalling pathway. The regulatory region of the *Nrf2* gene encompasses several AhR-binding response elements (Miao *et al.*, 2005; Kohle and Bock, 2007; Hayes and McMahon, 2009); some studies have suggested that Nrf2 regulates *AhR* expression and hence modulates numerous downstream events of the AhR signalling cascade, including the transcriptional regulation of *CYP1* genes (Shin *et al.*, 2007). Therefore, it is also plausible that 5-FU increases Nrf2 expression and activity, ultimately leading to increased *CYP1* expression. Future work could begin to determine this by investigating the effects of 5-FU on AhR, Cdk2 and Nrf2 mRNA and protein expression levels in the WHCO1 cells.

The further increase in *CYP1A1* and *CYP1B1* expression in the presence of both 5-FU and BP may be explained by the additive inducing action of BP via the AhR pathway (Bao *et al.*, 2002). However, the potential roles of these pathway interactions need to be investigated further, because for *CYP1A2*, whilst the response to co-treatment was similar to the other *CYP*s i.e. a steady increase from 6 to 24 hours, the expression pattern in response to 5-FU appeared erratic, with a down-regulation at 12 hours followed by an up-regulation at 24 hours. Interestingly, the response of all three *CYP*s to the BP + CDDP co-treatment was similar to that of the BP + 5-FU co-treatment, in that the combined exposure generally led to increased *CYP1* expression, compared to

the control. It is well-known that the cytotoxic mechanisms of CDDP depend on Cdk2 activity, so it is possible that a similar mechanism to that of 5-FU, coupled with BP-mediated induction of AhR, is responsible for the increased expression observed throughout (Yu *et al.*, 2007).

In this study, the level of induction achieved by BP treatment was not always found to be consistent, as in the case of CYP1A2 protein induction levels between the 5-FU experiment (~90-fold, Figure 3.10) and the CDDP experiment (~14-fold, Figure 3.18). This could have been due to experimental error, but because the overall trends were reproducible, the difference in cell passage numbers between the 5-FU experiment and the CDDP experiment is more likely to have been responsible.

Whilst the possible mechanisms behind the results for the *CYP*s are noteworthy, this study has focused on the clinical relevance of the changes in DME expression observed in response to chemotherapy. The differential expression of the DMEs in response to 5-FU and CDDP, if shown to be physiologically significant by further research, is likely to affect the metabolism of other co-administered substances/drugs. In the example of 5-FU increasing *CYP1A1* expression, a cancer patient being administered 5-FU may also be taking acetaminophen, which is a widely-used analgesic agent that is present in over 600 over-the-counter and prescription drugs for treating pain, fever, cough, allergies and more. Acetaminophen is known to be metabolised by CYP1A1, and should there be a significant up-regulation of this enzyme by 5-FU, the patient may experience acetaminophen toxicity. This is because elevated CYP1A1 levels have been shown to result in accumulation of the acetaminophen metabolite N-acetyl-p-benzoquinone-imine, which is a potent cytotoxin (Casley *et al.*, 1997; Kim *et al.*, 2009; Laine *et al.*, 2009). In the case of the down-regulation of *CYP1A2* by CDDP, warfarin is one of the most commonly-used anticoagulant drugs worldwide and is metabolised by CYP1A2. Should the expression of CYP1A2 be repressed by CDDP in a cancer patient on chronic warfarin treatment, the metabolism of warfarin could be compromised, possibly leading to ADRs, as has been observed in the previously discussed example of 5-FU and CYP2C9 (section 1.3, Figure 1.9). These examples illustrate the importance of understanding the effects of the drugs administered to a patient on the DME genes, in order to

optimize the therapeutic efficiency of the medication without compromising the metabolism of other drugs.

Unlike in the case of 5-FU, CDDP treatment alone led to irregular changes in *CYP1A1* mRNA expression at the initial time points, which were later "normalised" to control levels after 24 hours. Similarly, *CYP1B1* mRNA expression levels were initially decreased by CDDP, but no difference from the control was observed after 24 hours. Kitamura *et al.* (2008) have demonstrated that subsequent to induction, CYP1A1 activity is gradually decreased to control levels after 24 hours This could explain the effect observed in this study, possibly for *CYP1B1* as well, given that both CYPs are controlled by the same pathway. In contrast to the results observed for *CYP1A1* and *CYP1B1*, CDDP down-regulated *CYP1A2* mRNA expression to levels that were almost undetectable. The work of Kitamura *et al.* (2008) further supports this, because in their study, *CYP1A2* mRNA levels only returned to control levels 48 hours after treatment. Thus, it is possible that the down-regulation of *CYP1A2* by CDDP in this study would be corrected at some point after 24 hours. Further work needs to be done to determine if this is the case, by treating the cells for a longer time period. Should the down-regulatory effect of CDDP on *CYP1A2* be shown to be sustained and to have physiological effects in the cell, there may be important clinical consequences for a patient who is being administered this drug. CYP1A2 is primarily detected in the liver, and as previously mentioned, this enzyme is responsible for the metabolism of a variety of clinically-prescribed drugs, both chemotherapeutic and otherwise, as well as many compounds found in the environment and diet. The repressive effect of CDDP on *CYP1A2* needs to be investigated further in liver cells, in order to ascertain whether this down-regulatory effect can in fact be detected at the protein level. This would suggest that patients who rely on the metabolic activity of CYP1A2 for the administered co-medications should be carefully evaluated when they are on CDDP-containing chemotherapeutic treatment, because the metabolism of co-medication may be compromised.

A further point to note is that when *CYP1A2* mRNA expression levels were down-regulated by CDDP exposure in this study, low levels of the corresponding protein were still detected by the

western blot analysis. Naturally, the synthesis of a protein cannot occur without the initial transcription of the relevant mRNA transcript. Given that the amplification specificity of the qRT-PCR primer sets was comprehensively validated, this phenomenon cannot be explained by inefficiencies in the qRT-PCR technique. Instead, there may have been a concurrent up-regulation of CYP1A2 expression through increase of the protein's half-life by stabilization. Alternatively, at the time points where total RNA and protein were isolated from the cells, there may have been accumulated CYP1A2 protein in sufficient amounts to allow detection by western blot analysis, despite the repression of *CYP1A2* mRNA expression by the presence of CDDP.

An additional interesting observation was that the response of the *CYP*s, particularly *CYP1A1* and *CYP1A2*, to both 5-FU and CDDP treatment in combination with BP was a lower expression level than what was observed for BP alone, at 24 hours. This was reflected at the protein level as well. As previously hypothesised, 5-FU or CDDP treatment may lead to increased *CYP1* expression via the Cdk2, Nrf2 and/or AhR signalling pathways. A further increase in expression could be expected in the presence of BP as well via AhR activation, yet the expression levels are lower in response to combined drug and BP treatments than in response to BP alone. This could be attributed to the generation of ROS due to increased cellular stress in the presence of two foreign compounds. Since CYP activity itself can also produce ROS in the cell, *CYP* expression is subject to a negative-feedback auto-regulatory loop, which ensures that the mRNA and protein levels for these enzymes are responsive to the ROS generated (Morel *et al.*, 1999). Thus, in the presence of the co-treatment, *CYP1A1* and *CYP1A2* may have been down-regulated to protect against excessive ROS production, and more so as time progressed and more ROS accumulated. This is further supported by the similarity in the pattern of findings for *CYP1A1* and *CYP1A2*, because these two genes share a bi-directional promoter containing at least 13 AhR response elements, some of which regulate both genes in co-ordination (Ueda *et al.*, 2006; Jorge-Nerbert *et al.*, 2010).

The formation of ROS may have also been responsible for the morphological changes observed in the WHCO1 cells. Interestingly, blebbing cells were observed in the presence of either 5-FU or CDDP in combination with BP, but not in the drug-treated cells, the BP-treated cells or the DMSO-

treated controls. The formation of blebs is a well-characterised sign of early apoptosis (Wickman *et al.,* 2013), and excessive ROS production may lead to apoptosis (Circu and Aw, 2010). Furthermore, in addition to their critical role in drug metabolism, the CYP1 enzymes also participate in various signalling pathways that control cell proliferation as well as apoptosis, and the AhR is known to play a role in regulating the cell cycle (Barhoover *et al.,* 2010). A recent study by Das *et al.* (2014) used *in silico* methods to determine that BP-CYP1B1 complexes show binding affinity towards certain caspases, which are the major proteins responsible for apoptosis, whilst CYP1B1 knockdown or inhibition suppressed BP-induced apoptosis. It would be of interest to replicate this study in CYP1B1-knockdown WHCO1 cells, to determine if the apoptosis was indeed caused by BP-CYP1B1 complexes. However in this study, the presence of apoptotic cells only in the cell culture dishes treated with 5-FU/CDDP and BP in combination suggests that there is some interaction between the drugs and BP that was responsible for apoptosis. Further studies comparing the ROS and active caspase levels between cells treated with the drug-BP combination vs. BP alone and drug alone, may begin to elucidate the mechanism behind apoptosis. Additionally, as previously explained, the potential effects of 5-FU and CDDP on the Cdk2 pathway may also be involved. It is especially important to determine whether the apoptosis in response to combination treatment is tumour-cell-specific because, if non-malignant cells remain unaffected, this provides a potential means for enhancing 5-FU/CDDP chemotherapy through the use of a CYP1-inducer. The manipulation of DME activity in order to optimize drug therapy is well-established. As an example, some current pharmacological approaches include the use of *GSTP1* inhibitors in cancer treatment, so as to protect the proficiency of chemotherapeutics that would otherwise be cleared by GSTP1 activity (Morales and Laborde, 2007).

The decreased level of induction of *CYP1A1/1A2* mRNA in response to 5-FU and BP in combination, compared to BP alone, was also observed for *CYP1B1* mRNA, although much earlier at 6 hours following treatment and then "corrected" by 24 hours. Conversely, exposure to CDDP enhanced the *CYP1B1*-inducing ability of BP initially, but this effect was also reversed by 24 hours. In general, *CYP1B1* is known to be less inducible than the other CYP1 genes (Abel *et al.,*

1996; Taylor *et al.*, 2009) and this might be why the DME was less susceptible to the effects of the chemotherapeutic drugs on BP-mediated induction than CYP1A1 and CYP1A2. Nevertheless, *CYP1B1* transcription is also controlled by the AhR pathway, and it would be interesting to investigate what mechanism differentiates the response of this gene from that of *CYP1A1* and *CYP1A2* to the chemotherapeutic drugs combined with BP. As mentioned in section 1.4.1, CYP1B1 is primarily expressed extra-hepatically and is detectable in a variety of tumours, but is usually not present in adjacent normal tissues. This means that there is therapeutic potential for the metabolism of chemotherapeutic agents by CYP1B1 specifically in cancer cells, which is why understanding the effects of these drugs on the expression of CYP1B1 has great value.

The purpose of including BP in this study was not only as a positive control of *CYP* expression, but also as a model of *CYP* induction. As discussed in section 1.3, there are various drugs, environmental substances and dietary compounds that are capable of inducing *CYP* expression. If the decrease in CYP1A1/1A2 mRNA and protein expression observed in response to 5-FU/CDDP + BP treatment, compared to BP treatment alone, cannot be explained by future studies (e.g. on ROS production), this decrease may have clinical consequences. For a cancer patient prescribed 5-FU/CDDP and also exposed to a *CYP1A1/1A2* inducer, there is the possibility of less available CYP1A1/1A2 enzyme and thus decreased metabolism of drugs and exogenous compounds. As an example of a *CYP*-inducing drug, omeprazole is one of the most widely-prescribed drugs worldwide and is available over-the-counter in many countries for the treatment of gastro-oesophageal reflux disease and other conditions caused by excess gastric acid. This drug is also a well-known inducer of *CYP1A1* and *CYP1A2* via the AhR pathway (Andersson, 1996; Frick *et al.*, 2003; Yoshinari *et al.*, 2008). Hypothetically, if a cancer patient prescribed 5-FU or CDDP is concurrently taking omeprazole, there may be reduced levels of these CYP enzymes. Consequently, the metabolism of any compounds that require these CYPs will be compromised. These compounds are many, including clozapine, caffeine, warfarin, fluvoxamine and paracetamol, to name a few for just CYP1A2.

It is important to bear in mind that DME expression, particularly *CYP* expression, is variable between individuals, tissues and the tumour type. Thus, whilst baseline mRNA expression levels of *CYP1A1*/*1A2* were low in the WHCO1 cell line and required induction to be more easily detectable, another patient/tissue/tumour cell type may exhibit mRNA expression levels of these *CYP*s equal to or higher than the induced levels observed in WHCO1, rendering them more susceptible to differential expression by 5-FU or CDDP. Assuming that this is the case, it may be disadvantageous to administer 5-FU or CDDP to a cancer patient if there is co-medication to be metabolised by the *CYP*s because, should the metabolism of the co-administered drug be decreased, accumulation of the drug may lead to toxicity and ultimately, ADRs for the patient. On the other hand, should the metabolism of the co-administered drug be increased, the efficiency of the drug might be compromised.

In this study, a slight but significant decrease in *GSTP1* mRNA expression was observed in response to 5-FU exposure. In terms of 5-FU treatment and GSTP1, little research has been performed to investigate the effect of this drug on the expression of detoxification enzymes, and many of the existing studies yield conflicting results. For example, a study by Nishiyama *et al.* (1999) has shown that when used alone, 5-FU increases *GSTP1* expression at certain concentrations. Another study has demonstrated that in human colon cancer cells, the effects of 5-FU on most of the antioxidant enzymes, including GSTP1, are low to non-existent (Akhdar *et al.,* 2009). Furthermore, this study shows that the cytoprotective enzymes that are affected by 5-FU, namely GSTS1 and GSTM3, increase in their mRNA levels only after 48 hours of incubation with 5-FU, due to the nuclear translocation and activation of Nrf2 by 5-FU. Fujishima *et al.* (1997) were unable to show a decrease in *GSTP1* expression in response to 5-FU exposure despite a marked decrease in the steady-state mRNA levels of two genes coding for rate-limiting enzymes for GSH synthesis, after 48 hours of 5-FU treatment. Thus, it is also possible that in this study, a greater effect of 5-FU on *GSTP1* expression might've been observable had the cells been treated for a longer period or at different drug concentrations. Nevertheless, the results of this study show that should the decreased expression of *GSTP1* have a significant physiological impact on the

cell, an oesophageal cancer patient taking 5-FU may have reduced levels of available GSTP1, which may compromise the detoxification of co-administered drugs as well as other exogenous compounds with harmful metabolites. However, the repressive effect of 5-FU may also increase the therapeutic efficiency of the drug when immediately followed by CDDP treatment. The sequential cytotoxic action of these two drugs has been shown by Scanlon *et al.* (1986) and is currently widely-used for treating tumours of the gastrointestinal tract.

In contrast to 5-FU, *GSTP1* mRNA expression was steadily increased over time in response to CDDP treatment. CDDP is known to be conjugated by the GSH enzymes including GSTP1, and exposure to CDDP has been shown to up-regulate the expression levels of *GSTP1* in other studies as well, along with several other detoxification enzymes (Goto *et al.*, 1999; Nishiyama *et al.*, 1999; Townsend *et al.*, 2009). For a patient taking CDDP, the increased levels of GSTP1 could lessen therapeutic efficacy by detoxifying the drugs too rapidly, leading to cellular resistance to chemotherapy, as mentioned in section 1.2.

It was interesting to note that unlike all of the CYP proteins in response to both chemotherapeutic drugs, GSTP1 protein was readily and generally equally detectable under all the treatment conditions, including in the control cells. One possible explanation for this is that GSTP1 protein might be up-regulated in the WHCO1 cell line, and therefore could not be further induced by CDDP, which showed an induction in *GSTP1* mRNA expression. This could be confirmed by measuring and comparing the basal levels of GSTP1 protein expression in a non-cancerous human oesophageal cell line. As mentioned in section 1.2, it has been shown that increased levels of this protein could have a protective effect against CDDP-induced toxicity, so patients may have an improved response to CDDP chemotherapy. On the other hand, elevated GSTP1 levels may also result in cellular resistance to CDDP, thus decreasing the efficacy of chemotherapy (Yellin *et al.*, 1994; Townsend *et al.*, 2009). Consequently it is important to first and foremost, understand the effects of the administered chemotherapeutic agents on the detoxifying enzymes such as GSTP1, before the treatment and/or dosages can be optimised for the patient.

Throughout the experimental findings of this study, there were varying differences between the gene expression level for a given DME and its corresponding level of protein expression. Experimental error cannot be completely ruled out; however the trends observed were reproducible and thus likely to be a true reflection of the results. Differences between gene and protein expression levels for a given gene can be explained by a number of known factors. Gene expression is regulated in numerous diverse ways and at different stages. Steady-state RNA levels are subject to transcriptional control (e.g. transcription factors, enhancers and silencers) and post-transcriptional control (e.g. RNA processing including alternative splicing, as well as RNA stability). In mammalian cells, an average of two mRNA transcripts is produced per hour, whereas dozens of proteins per mRNA per hour are transcribed. At the translational level protein stability is an important factor, as the half-life between different proteins can range from minutes to days, whilst the degradation rate of mammalian mRNA falls within a much tighter range of up to seven hours (Guo *et al.*, 2008). Therefore, although RNA expression levels may have predictive value in inferring potential protein expression levels, changes in gene expression are frequently not reflected at the protein level. In fact, the correlation between gene and protein expression can be as low as 40%, depending on the particular system (Vogel and Marcotte, 2012).

4.1 Limitations of this study

Although all the objectives of this project were met successfully, there were certain limitations that need to be acknowledged:

- Since DPD is the principal enzyme involved in 5-FU clearance, it would have been beneficial to measure the levels of DPD expression in WHCO1 cells. This would serve to confirm that the effects of 5-FU on the DMEs measured in this study were not affected by a high activity level of DPD and thus, rapid elimination of the drug from the cells.

- 5-FU and CDDP are frequently co-administered, especially for the treatment of oesophageal cancer. Due to the length of the cell culture experiments and other time

constraints, it was not possible to investigate the effect of these two chemotherapeutic drugs in combination on the expression of the DMEs, which may have yielded interesting potential implications for oesophageal cancer patients.

- Genetic variation in the four DMEs was not taken into account in this study, and variations such as SNPs can have an impact on the expression levels of the DME genes. It would have been useful to genotype the WHCO1 cell line for known SNPs affecting *CYP* and *GSTP1* expression, to support the expression data.

4.2 Future work

This research has yielded several interesting effects of the chemotherapeutic drugs 5-FU and CDDP, individually as well as in the presence of BP, on the mRNA and protein expression levels of four important DMEs. Firstly, this work serves as a preliminary study for the potential physiological significance of the differential expression of the DMEs to be determined. McCarthy and Smyth (2009) have described an *in silico* method to determine the biological relevance of gene expression changes, which may be used in future work based on the results of this study. To specifically elucidate the possible mechanisms behind the findings of this study, a good starting point would be to investigate the effects of 5-FU and CDDP on AhR, Cdk2 and Nrf2 expression levels in the WHCO1 cells. Furthermore, it would also be interesting to reproduce this study and increase the drug exposure time to determine if the changes observed in response to the various treatments are sustained beyond 24 hours and if so, for what length of time. This includes the decrease in *CYP1A1/1A2* mRNA induction levels by 5-FU/CDDP + BP exposure, compared to BP treatment alone, and it should also be determined if this is a result of the CYP negative regulatory feedback loop to reduce ROS. If this is not the case and there is some other mechanism behind the decrease in *CYP1A1/1A2* expression, the timing of a patient's administration of co-medication could be adjusted so as to maximise the required metabolic capacity of these important CYP enzymes. Also, as previously discussed, the down-regulatory effect of CDDP on *CYP1A2* mRNA

expression should be confirmed in future experiments involving liver cells, which have higher basal expression levels of *CYP1A2*.

Because the expression of the DMEs is highly tissue-specific and this study focused on one cell line, it would be of great value to replicate this work using other oesophageal cancer cell lines, as well as different tumour cell lines and especially liver cells, given that *CYP1A2* is expressed at high levels hepatically. This will serve to broaden the perspective on the possible impact of 5-FU, CDDP and/or *CYP*-inducers like BP, on patient response. As mentioned above (section 4.1), future work should also entail the use of these drugs in other tumour cell lines harbouring known genetic variations in the DME genes as this may affect the response to drug and/or BP treatment. Epigenetic factors such as DNA methylation and miRNA regulation also contribute to the response of DMEs to chemotherapeutics, and may also be incorporated into future studies of this nature.

5. CONCLUSION

The findings of this research demonstrated that 5-FU and CDDP significantly influence the expression of four critically important DME genes, *CYP1A1, 1A2, 1B1* and *GSTP1*. This may have important potential clinical consequences for patients with oesophageal cancer, which is a leading cause of death worldwide. These consequences include an impact on the metabolism of co-medication, dietary compounds, nutritional supplements and environmental compounds, leading to loss of drug efficacy or the accumulation of cytotoxic metabolites in the body. Furthermore the potential decrease of available CYP1A1/1A2 protein in the presence of BP, shown in this study, may result in poor metabolism of co-administered drugs, ultimately causing ADRs. This is especially important because BP is not only an omnipresent environmental pollutant (Perumal *et al.*, 2012), but it is also a model for other CYP-inducers present in both prescription and over-the-counter drugs and in various dietary substances. This study also showed that combining 5-FU and/or CDDP with BP resulted in early apoptosis of the oesophageal cancer cells, which depending on future work on non-cancerous cell lines, suggests that the use of CYP-inducers in conjunction with these chemotherapeutics may improve therapeutic response. Further studies are required to determine the mechanisms behind these effects.

The management of ADRs in cancer patients is a major challenge, because these reactions can be severe or even fatal. Understanding how chemotherapeutic drugs affect the enzymes that metabolise over 90% of medication as well as other exogenous and endogenous compounds, is crucial for improving patient response. The findings of this research contribute to the current knowledge on providing a means for predicting patient response to chemotherapy. Thus, this project has laid the foundation for future pharmacogenetics research, an important field which seeks to understand the influence of genetics on patient response to drugs, in order to maximise therapeutic efficiency whilst minimising adverse effects. The study of pharmacogenetics provides the key for the progression of the new era of personalised medicine, a field that holds promise as a means to ensure greater safety and efficacy in drug design and development.

References

Abel, J., Li, W., Dohr, O., Vogel, C., & Donat, S. (1996). Dose-response relationship of cytochrome P4501b1 mRNA induction by 2,3,7,8-tetrachlorodibenzo-p-dioxin in livers of C57BL/6J and DBA/2J mice. Archives of Toxicology, 70(8), 510-513.

Abdelrahim, M., Smith, R.,3rd, & Safe, S. (2003). Aryl hydrocarbon receptor gene silencing with small inhibitory RNA differentially modulates ah-responsiveness in MCF-7 and HepG2 cancer cells. Molecular Pharmacology, 63(6), 1373-1381.

Afsar, A., Lee, C., & Riddick, D. S. (1996). Modulation of the expression on constitutive rat hepatic cytochrome P450 isozymes by 5-fluorouracil. Canadian Journal of Physiology and Pharmacology, 74(2), 150-156.

Akhdar, H., Loyer, P., Rauch, C., Corlu, A., Guillouzo, A., & Morel, F. (2009). Involvement of Nrf2 activation in resistance to 5-fluorouracil in human colon cancer HT-29 cells. European Journal of Cancer (Oxford, England : 1990), 45(12), 2219-2227.

Alomirah, H., Al-Zenki, S., Husain, A., Sawaya, W., Ahmed, N., Gevao, B., *et al*. (2010). Benzo[a]pyrene and total polycyclic aromatic hydrocarbons (PAHs) levels in vegetable oils and fats do not reflect the occurrence of the eight genotoxic PAHs. Food Additives & Contaminants.Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment, 27(6), 869-878.

Alter, P., Herzum, M., Soufi, M., Schaefer, J. R., & Maisch, B. (2006). Cardiotoxicity of 5-fluorouracil. Cardiovascular & Hematological Agents in Medicinal Chemistry, 4(1), 1-5.

Andersson, T. (1996). Pharmacokinetics, metabolism and interactions of acid pump inhibitors. focus on omeprazole, lansoprazole and pantoprazole. Clinical Pharmacokinetics, 31(1), 9-28.

Arai, T., Miyoshi, Y., Kim, S. J., Akazawa, K., Maruyama, N., Taguchi, T., *et al*. (2008). Association of GSTP1 expression with resistance to docetaxel and paclitaxel in human breast cancers. European Journal of Surgical Oncology (EJSO), 34(7), 734-738.

Asteinza, J., Camacho-Carranza, R., Reyes-Reyes, R. E., Dorado-Gonzalez, V. V., & Espinosa-Aguirre, J. J. (2000). Induction of cytochrome P450 enzymes by albendazole treatment in the rat. Environmental Toxicology and Pharmacology, 9(1-2), 31-37.

Bao, H., Vepakomma, M., & Sarkar, M. A. (2002). Benzo(a)pyrene exposure induces CYP1A1 activity and expression in human endometrial cells. The Journal of Steroid Biochemistry and Molecular Biology, 81(1), 37-45.

Bapiro, T. E., Andersson, T. B., Otter, C., Hasler, J. A., & Masimirembwa, C. M. (2002). Cytochrome P450 1A1/2 induction by antiparasitic drugs: Dose-dependent increase in ethoxyresorufin O-deethylase activity and mRNA caused by quinine, primaquine and albendazole in HepG2 cells. European Journal of Clinical Pharmacology, 58(8), 537-542.

Barhoover, M. A., Hall, J. M., Greenlee, W. F., & Thomas, R. S. (2010). Aryl hydrocarbon receptor regulates cell cycle progression in human breast cancer cells via a functional interaction with cyclin-dependent kinase 4. Molecular Pharmacology, 77(2), 195-201.

Barhoumi, R., Mouneimne, Y., Ramos, K. S., Safe, S. H., Phillips, T. D., Centonze, V. E., *et al*. (2000). Analysis of benzo[a]pyrene partitioning and cellular homeostasis in a rat liver cell line. Toxicological Sciences : An Official Journal of the Society of Toxicology, 53(2), 264-270.

Battaglia, M., Pozzi, D., Grimaldi, S., & Parasassi, T. (1994). Hoechst 33258 staining for detecting mycoplasma contamination in cell cultures: A method for reducing fluorescence photobleaching.

Biotechnic & Histochemistry: Official Publication of the Biological Stain Commission, 69(3), 152-156.

Bompart, G. (1989). Cisplatin-induced changes in cytochrome P450, lipid peroxidation and drug metabolizing enzyme activities in rat kidney cortex. Toxicology Letters, 48(2), 193-199.

Bray, F., Jemal, A., Grey, N., Ferlay, J., & Forman, D. (2012). Global cancer transitions according to the human development index (2008–2030): A population-based study. The Lancet Oncology, 13(8), 790-801.

Candelaria, M., Garcia-Arias, A., Cetina, L., & Duenas-Gonzalez, A. (2006). Radiosensitizers in cervical cancer: cisplatin and beyond. Radiation Oncology (London, England), 1, 15.

Casley, W. L., Menzies, J. A., Mousseau, N., Girard, M., Moon, T. W., & Whitehouse, L. W. (1997). Increased basal expression of hepatic Cyp1a1 and Cyp1a2 genes in inbred mice selected for susceptibility to acetaminophen-induced hepatotoxicity. Pharmacogenetics, 7(4), 283-293.

Chen, A. M., Chen, L. M., Vaughan, A., Sreeraman, R., Farwell, D. G., Luu, Q., *et al*. (2011). Tobacco smoking during radiation therapy for head-and-neck cancer is associated with unfavorable outcome. International Journal of Radiation Oncology, Biology, Physics, 79(2), 414-419.

Circu, M. L., & Aw, T. Y. (2010). Reactive oxygen species, cellular redox systems, and apoptosis. Free Radical Biology & Medicine, 48(6), 749-762.

Code, E. L., Crespi, C. L., Penman, B. W., Gonzalez, F. J., Chang, T. K., & Waxman, D. J. (1997). Human cytochrome P4502B6: Interindividual hepatic expression, substrate specificity, and role in procarcinogen activation. Drug Metabolism and Disposition: The Biological Fate of Chemicals, 25(8), 985-993.

Cook, P. (1971). Cancer of the oesophagus in Africa. A summary and evaluation of the evidence for the frequency of occurrence, and a preliminary indication of the possible association with the consumption of alcoholic drinks made from maize. British Journal of Cancer, 25(4), 853-880.

Cunningham, D., Allum, W. H., Stenning, S. P., Thompson, J. N., Van de Velde, C. J., Nicolson, M., *et al*. (2006). Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. The New England Journal of Medicine, 355(1), 11-20.

Cunningham, D., Starling, N., Rao, S., Iveson, T., Nicolson, M., Coxon, F., *et al*. (2008). Capecitabine and oxaliplatin for advanced esophagogastric cancer. The New England Journal of Medicine, 358(1), 36-46.

Das, D. N., Panda, P. K., Mukhopadhyay, S., Sinha, N., Mallick, B., Behera, B., *et al*. (2014). Prediction and validation of apoptosis through cytochrome P450 activation by benzo[a]pyrene. Chemico-Biological Interactions, 208(0), 8-17.

de Bruin, W. C., Wagenmans, M. J., & Peters, W. H. (2000). Expression of glutathione S-transferase alpha, P1-1 and T1-1 in the human gastrointestinal tract. Japanese Journal of Cancer Research: Gann, 91(3), 310-316.

DeRisi, J., Penland, L., Brown, P. O., Bittner, M. L., Meltzer, P. S., Ray, M., *et al*. (1996). Use of a cDNA microarray to analyse gene expression patterns in human cancer. Nature Genetics, 14(4), 457-460.

Dhawan, D., Panchal, H., Shukla, S., & Padh, H. (2013). Genetic variability & chemotoxicity of 5-fluorouracil & cisplatin in head & neck cancer patients: A preliminary study. The Indian Journal of Medical Research, 137(1), 125-129.

Diasio, R. B., Schuetz, J. D., Wallace, H. J., & Sommadossi, J. P. (1985). Dihydrofluorouracil, a fluorouracil catabolite with antitumor activity in murine and human cells. Cancer Research, 45(10), 4900-4903.

Dos Santos, N. A., Carvalho Rodrigues, M. A., Martins, N. M., & dos Santos, A. C. (2012). Cisplatin-induced nephrotoxicity and targets of nephroprotection: An update. Archives of Toxicology, 86(8), 1233-1250.

Drexler, H. G., & Uphoff, C. C. (2002). Mycoplasma contamination of cell cultures: Incidence, sources, effects, detection, elimination, prevention. Cytotechnology, 39(2), 75-90.

Duffy, S. A., Ronis, D. L., Valenstein, M., Fowler, K. E., Lambert, M. T., Bishop, C., *et al*. (2007). Depressive symptoms, smoking, drinking, and quality of life among head and neck cancer patients. Psychosomatics, 48(2), 142-148.

Ekhart, C., Rodenhuis, S., Smits, P. H., Beijnen, J. H., & Huitema, A. D. (2009). An overview of the relations between polymorphisms in drug metabolising enzymes and drug transporters and survival after cancer drug treatment. Cancer Treatment Reviews, 35(1), 18-31.

Evans, A. M. (2000). Influence of dietary components on the gastrointestinal metabolism and transport of drugs. Therapeutic Drug Monitoring, 22(1), 131-136.

Ezzeldin, H., & Diasio, R. (2004). Dihydropyrimidine dehydrogenase deficiency, a pharmacogenetic syndrome associated with potentially life-threatening toxicity following 5-fluorouracil administration. Clinical Colorectal Cancer, 4(3), 181-189.

Fakih, M. G., Ross, M. E., & Starostik, P. (2007). Increased frequency of uridine diphosphate glucuronosyltransferase 1A1 7/7 in patients experiencing severe irinotecan-induced toxicities. Clinical Colorectal Cancer, 6(8), 583-587.

Frick, A., Kopitz, J., & Bergemann, N. (2003). Omeprazole reduces clozapine plasma concentrations. A case report. Pharmacopsychiatry, 36(3), 121-123.

Fujishima, H., Nakano, S., Masumoto, N., Esaki, T., Tatsumoto, T., Kondo, T., *et al*. (1997). Inhibition by 5-fluorouracil of ERCC1 and gamma-glutamylcysteine synthetase messenger RNA expression in a cisplatin-resistant HST-1 human squamous carcinoma cell line. Oncology Research, 9(4), 167-172.

Garner, C. M., Hubbold, L. M., & Chakraborti, P. R. (2000). Mycoplasma detection in cell cultures: A comparison of four methods. British Journal of Biomedical Science, 57(4), 295-301.

Gelderblom, W. C., Jaskiewicz, K., Marasas, W. F., Thiel, P. G., Horak, R. M., Vleggaar, R., *et al*. (1988). Fumonisins--novel mycotoxins with cancer-promoting activity produced by fusarium moniliforme. Applied and Environmental Microbiology, 54(7), 1806-1811.

Girennavar, B., Poulose, S. M., Jayaprakasha, G. K., Bhat, N. G., & Patil, B. S. (2006). Furocoumarins from grapefruit juice and their effect on human CYP 3A4 and CYP 1B1 isoenzymes. Bioorganic & Medicinal Chemistry, 14(8), 2606-2612.

Giunta, G. (2010). Adverse interaction between capecitabine and warfarin resulting in altered coagulation parameters: A review of the literature starting from a case report. Case Reports in Medicine, 426804.

Goekkurt, E., Hoehn, S., Wolschke, C., Wittmer, C., Stueber, C., Hossfeld, D. K., *et al*. (2006). Polymorphisms of glutathione S-transferases (GST) and thymidylate synthase (TS)--novel predictors for response and survival in gastric cancer patients. British Journal of Cancer, 94(2), 281-286.

Goto, S., Iida, T., Cho, S., Oka, M., Kohno, S., & Kondo, T. (1999). Overexpression of glutathione S-transferase pi enhances the adduct formation of cisplatin with glutathione in human cancer cells. Free Radical Research, 31(6), 549-558.

Guengerich, F. P. (2006). Cytochrome P450s and other enzymes in drug metabolism and toxicity. The AAPS Journal, 8(1), E101-11.

Guo, Y., Xiao, P., Lei, S., Deng, F., Xiao, G. G., Liu, Y., *et al*. (2008). How is mRNA expression predictive for protein expression? A correlation study on human circulating monocytes. Acta Biochimica Et Biophysica Sinica, 40(5), 426-436.

Hankinson, O. (1994). A genetic analysis of processes regulating cytochrome P4501A1 expression. Advances in Enzyme Regulation, 34, 159-171.

Hayes, J. D., & McMahon, M. (2009). NRF2 and KEAP1 mutations: Permanent activation of an adaptive response in cancer. Trends in Biochemical Sciences, 34(4), 176-188.

Heidelberger, C., Chaudhuri, N. K., Dannenberg, P., Mooren, D., Griesbach, L., Duschinsky, R. (1957). Fluorinated pyrimidines, a new class of tumour-inhibitory compounds. Nature, 179(4561), 663-666.

Hellum, B. H., Hu, Z., & Nilsen, O. G. (2007). The induction of CYP1A2, CYP2D6 and CYP3A4 by six trade herbal products in cultured primary human hepatocytes. Basic & Clinical Pharmacology & Toxicology, 100(1), 23-30.

Helm, C. W., & States, J. C. (2009). Enhancing the efficacy of cisplatin in ovarian cancer treatment - could arsenic have a role. Journal of Ovarian Research, 2, 2215-2222.

Hiyama, T., Yoshihara, M., Tanaka, S., & Chayama, K. (2007). Genetic polymorphisms and esophageal cancer risk. International Journal of Cancer. Journal International Du Cancer, 121(8), 1643-1658.

Huang, Z., Fasco, M. J., Figge, H. L., Keyomarsi, K., & Kaminsky, L. S. (1996). Expression of cytochromes P450 in human breast tissue and tumors. Drug Metabolism and Disposition: The Biological Fate of Chemicals, 24(8), 899-905.

Hundley, A. F., Yuan, L., & Visco, A. G. (2006). Skeletal muscle heavy-chain polypeptide 3 and myosin binding protein H in the pubococcygeus muscle in patients with and without pelvic organ prolapse. American Journal of Obstetrics and Gynecology, 194(5), 1404-1410.

Isohanni, M. H., Neuvonen, P. J., & Olkkola, K. T. (2006). Effect of fluvoxamine and erythromycin on the pharmacokinetics of oral lidocaine. Basic & Clinical Pharmacology & Toxicology, 99(2), 168-172.

Israel, D. I., & Whitlock, J. P., Jr. (1983). Induction of mRNA specific for cytochrome P1-450 in wild type and variant mouse hepatoma cells. The Journal of Biological Chemistry, 258(17), 10390-10394.

Iwao-Koizumi, K., Matoba, R., Ueno, N., Kim, S. J., Ando, A., Miyoshi, Y., *et al*. (2005). Prediction of docetaxel response in human breast cancer by gene expression profiling. Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology, 23(3), 422-431.

Jorge-Nebert, L. F., Jiang, Z., Chakraborty, R., Watson, J., Jin, L., McGarvey, S. T., *et al*. (2010). Analysis of human CYP1A1 and CYP1A2 genes and their shared bidirectional promoter in eight world populations. Human Mutation, 31(1), 27-40.

Joshi, M. B., Shirota, Y., Danenberg, K. D., Conlon, D. H., Salonga, D. S., Herndon, J. E.,2nd, *et al*. (2005). High gene expression of TS1, GSTP1, and ERCC1 are risk factors for survival in patients treated with trimodality therapy for esophageal cancer. Clinical Cancer Research : An Official Journal of the American Association for Cancer Research, 11(6), 2215-2221.

Kaschula, C. H., Hunter, R., Stellenboom, N., Caira, M. R., Winks, S., Ogunleye, T., *et al*. (2012). Structure-activity studies on the anti-proliferation activity of ajoene analogues in WHCO1 oesophageal cancer cells. European Journal of Medicinal Chemistry, 50, 236-254.

Kashigar, A., Habbous, S., Eng, L., Irish, B., Bissada, E., Irish, J., *et al*. (2013). Social environment, secondary smoking exposure, and smoking cessation among head and neck cancer patients. Cancer, 119(15), 2701-2709.

Kim, S. J., Lee, M. Y., Kwon do, Y., Kim, S. Y., & Kim, Y. C. (2009). Alteration in metabolism and toxicity of acetaminophen upon repeated administration in rats. Journal of Pharmacological Sciences, 111(2), 175-181.

Kimura, Y., & Okuda, H. (1999). Prevention by chitosan of myelotoxicity, gastrointestinal toxicity and immunocompetent organic toxicity induced by 5-fluorouracil without loss of antitumor activity in mice. Japanese Journal of Cancer Research, 90(7), 765-774.

Kitamura, R., Matsuoka, K., Nagayama, S., & Otagiri, M. (2008). Time-dependent induction of rat hepatic CYP1A1 and CYP1A2 expression after single-dose administration of the anti-angiogenic agent TSU-68. Drug Metabolism and Pharmacokinetics, 23(6), 421-427.

Kohle, C., & Bock, K. W. (2007). Coordinate regulation of phase I and II xenobiotic metabolisms by the ah receptor and Nrf2. Biochemical Pharmacology, 73(12), 1853-1862.

Korzeniewski, N., Wheeler, S., Chatterjee, P., Duensing, A., & Duensing, S. (2010). A novel role of the aryl hydrocarbon receptor (AhR) in centrosome amplification - implications for chemoprevention. Molecular Cancer, 9, 153-4598-9-153.

Lai, C. Y., Hsieh, L. L., Sung, F. C., Tang, R., Bai, C. H., Wu, F. Y., *et al*. (2013). Correction: Tumor site- and stage-specific associations between allelic variants of glutathione S-transferase and DNA-repair genes and overall survival in colorectal cancer patients receiving 5-fluorouracil-based chemotherapy. PloS One, 8(7), 10.1371

Laine, J. E., Auriola, S., Pasanen, M., & Juvonen, R. O. (2009). Acetaminophen bioactivation by human cytochrome P450 enzymes and animal microsomes. Xenobiotica; the Fate of Foreign Compounds in Biological Systems, 39(1), 11-21.

LaPensee, E. W., Schwemberger, S. J., LaPensee, C. R., Bahassi el, M., Afton, S. E., & Ben-Jonathan, N. (2009). Prolactin confers resistance against cisplatin in breast cancer cells by activating glutathione-S-transferase. Carcinogenesis, 30(8), 1298-1304.

Li, J., & Bluth, M. H. (2011). Pharmacogenomics of drug metabolizing enzymes and transporters: Implications for cancer therapy. Pharmacogenomics and Personalized Medicine, 4, 11-33.

Li, Y., Mizutani, Y., Shiraishi, T., Nakamura, T., Mikami, K., Takaha, N., *et al*. (2007). The significance of the expression of dihydropyrimidine dehydrogenase in prostate cancer. BJU International, 99(3), 663-668.

Liang, Z., Lippman, S. M., Kawabe, A., Shimada, Y., & Xu, X. C. (2003). Identification of benzo(a)pyrene diol epoxide-binding DNA fragments using DNA immunoprecipitation technique. Cancer Research, 63(7), 1470-1474.

Liu, H., Baliga, M., & Baliga, R. (2002). Effect of cytochrome P450 2E1 inhibitors on cisplatin-induced cytotoxicity to renal proximal tubular epithelial cells. Anticancer Research, 22(2A), 863-868.

Liu, H., & Baliga, R. (2003). Cytochrome P450 2E1 null mice provide novel protection against cisplatin-induced nephrotoxicity and apoptosis. Kidney International, 63(5), 1687-1696.

Long, J. R., Egan, K. M., Dunning, L., Shu, X. O., Cai, Q., Cai, H., *et al*. (2006). Population-based case-control study of AhR (aryl hydrocarbon receptor) and CYP1A2 polymorphisms and breast cancer risk. Pharmacogenetics and Genomics, 16(4), 237-243.

Longley, D. B., Harkin, D. P., & Johnston, P. G. (2003). 5-fluorouracil: Mechanisms of action and clinical strategies. Nature Reviews. Cancer, 3(5), 330-338.

Lovejoy, K. S., Todd, R. C., Zhang, S., McCormick, M. S., D'Aquino, J. A., Reardon, J. T., *et al*. (2008). Cis-diammine(pyridine)chloroplatinum(II), a monofunctional platinum(II) antitumor agent: Uptake, structure, function, and prospects. Proceedings of the National Academy of Sciences of the United States of America, 105(26), 8902-8907.

Lu, Y., & Cederbaum, A. (2007). The mode of cisplatin-induced cell death in CYP2E1-overexpressing HepG2 cells: Modulation by ERK, ROS, glutathione, and thioredoxin. Free Radical Biology & Medicine, 43(7), 1061-1075.

Marasas, W. F., Jaskiewicz, K., Venter, F. S., & Van Schalkwyk, D. J. (1988). Fusarium moniliforme contamination of maize in oesophageal cancer areas in transkei. South African Medical Journal, 74(3), 110-114.

Martins, N. M., Santos, N. A., Curti, C., Bianchi, M. L., & Santos, A. C. (2008). Cisplatin induces mitochondrial oxidative stress with resultant energetic metabolism impairment, membrane rigidification and apoptosis in rat liver. Journal of Applied Toxicology, 28(3), 337-344.

Masek, V., Anzenbacherova, E., Machova, M., Brabec, V., & Anzenbacher, P. (2009). Interaction of antitumor platinum complexes with human liver microsomal cytochromes P450. Anti-Cancer Drugs, 20(5), 305-311.

Matsuyama, T., Uetake, H., Aoyagi, H., Kobayashi, H., Ishikawa, T., Iida, S., *et al*. (2008). A case report of a patient with hand-foot syndrome induced by bolus 5-fluorouracil therapy. Gan to Kagaku Ryoho.Cancer & Chemotherapy, 35(12), 2147-2149.

McCarthy, D. J., & Smyth, G. K. (2009). Testing significance relative to a fold-change threshold is a TREAT. Bioinformatics (Oxford, England), 25(6), 765-771.

McIlwain, C. C., Townsend, D. M., & Tew, K. D. (2006). Glutathione S-transferase polymorphisms: Cancer incidence and therapy. Oncogene, 25(11), 1639-1648.

Miao, W., Hu, L., Scrivens, P. J., & Batist, G. (2005). Transcriptional regulation of NF-E2 p45-related factor (NRF2) expression by the aryl hydrocarbon receptor-xenobiotic response element signaling pathway: Direct cross-talk between phase I and II drug-metabolizing enzymes. The Journal of Biological Chemistry, 280(21), 20340-20348.

Morel, Y., Mermod, N., & Barouki, R. (1999). An autoregulatory loop controlling CYP1A1 gene expression: Role of H(2)O(2) and NFI. Molecular and Cellular Biology, 19(10), 6825-6832.

Motawi, T. K., Teleb, Z. A., El-Boghdady, N. A., & Ibrahim, S. A. (2013). Effect of simvastatin and naringenin coadministration on rat liver DNA fragmentation and cytochrome P450 activity: An in vivo and in vitro study. Journal of Physiology and Biochemistry, 70(1), 225-237.

Mulder, T. P., Verspaget, H. W., Sier, C. F., Roelofs, H. M., Ganesh, S., Griffioen, G., *et al*. (1995). Glutathione S-transferase pi in colorectal tumors is predictive for overall survival. Cancer Research, 55(12), 2696-2702.

Murray, G. I., Melvin, W. T., Greenlee, W. F., & Burke, M. D. (2001). Regulation, function, and tissue-specific expression of cytochrome P450 CYP1B1. Annual Review of Pharmacology and Toxicology, 41, 297-316.

Nebert, D. W., & Dalton, T. P. (2006). The role of cytochrome P450 enzymes in endogenous signalling pathways and environmental carcinogenesis. Nature Reviews. Cancer, 6(12), 947-960.

Nelson, D. R., Zeldin, D. C., Hoffman, S. M., Maltais, L. J., Wain, H. M., & Nebert, D. W. (2004). Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. Pharmacogenetics, 14(1), 1-18.

Nishiyama, M., Yamamoto, W., Park, J. S., Okamoto, R., Hanaoka, H., Takano, H., *et al*. (1999). Low-dose cisplatin and 5-fluorouracil in combination can repress increased gene expression of cellular resistance determinants to themselves. Clinical Cancer Research : An Official Journal of the American Association for Cancer Research, 5(9), 2620-2628.

Oeda, M., Yoshida, K., Sanada, Y., Wada, Y., Suzuki, T., Mizuiri, H., *et al*. (2006). The expression profiles of orotate phosphoribosyltransferase and dihydropyrimidine dehydrogenase in gastric cancer and their clinical significance. Oncology Reports, 16(6), 1165-1172.

Ofverholm, A., Arkblad, E., Skrtic, S., Albertsson, P., Shubbar, E., & Enerback, C. (2010). Two cases of 5-fluorouracil toxicity linked with gene variants in the DPYD gene. Clinical Biochemistry, 43(3), 331-334.

Oguri, T., Achiwa, H., Bessho, Y., Muramatsu, H., Maeda, H., Niimi, T., *et al*. (2005). The role of thymidylate synthase and dihydropyrimidine dehydrogenase in resistance to 5-fluorouracil in human lung cancer cells. Lung Cancer, 49(3), 345-351.

Ohta, T., Maruyama, T., Nagahashi, M., Miyamoto, Y., Hosoi, S., Kiuchi, F., *et al*. (2002). Paradisin C: A new CYP3A4 inhibitor from grapefruit juice. Tetrahedron, 58(33), 6631-6635.

Ozdemir, M., Aktan, Y., Boydag, B. S., Cingi, M. I., & Musmul, A. (1998). Interaction between grapefruit juice and diazepam in humans. European Journal of Drug Metabolism and Pharmacokinetics, 23(1), 55-59.

Pabla, N., & Dong, Z. (2008). Cisplatin nephrotoxicity: Mechanisms and renoprotective strategies. Kidney International, 73(9), 994-1007.

Pacella-Norman, R., Urban, M. I., Sitas, F., Carrara, H., Sur, R., Hale, M., *et al*. (2002). Risk factors for oesophageal, lung, oral and laryngeal cancers in black South Africans. British Journal of Cancer, 86(11), 1751-1756.

Park, J. Y., & Kim, K. A. (2003). Inhibitory effect of 5-fluorouracil on human cytochrome P450 isoforms in human liver microsomes. European Journal of Clinical Pharmacology, 59(5-6), 407-409.

Parker, A. C., Pritchard, P., Preston, T., & Choonara, I. (1998). Induction of CYP1A2 activity by carbamazepine in children using the caffeine breath test. British Journal of Clinical Pharmacology, 45(2), 176-178.

Peart, M. J., Smyth, G. K., van Laar, R. K., Bowtell, D. D., Richon, V. M., Marks, P. A., *et al*. (2005). Identification and functional significance of genes regulated by structurally different histone deacetylase inhibitors. Proceedings of the National Academy of Sciences of the United States of America, 102(10), 3697-3702.

Peer, C. J., McManus, T. J., Hurwitz, H. I., & Petros, W. P. (2012). Development and utilization of a combined LC-UV and LC-MS/MS method for the simultaneous analysis of tegafur and 5-fluorouracil in human plasma to support a phase I clinical study of oral UFT(R)/leucovorin. Journal of Chromatography, Analytical Technologies in the Biomedical and Life Sciences, 898, 32-37.

Peklak-Scott, C., Smitherman, P. K., Townsend, A. J., & Morrow, C. S. (2008). Role of glutathione S-transferase P1-1 in the cellular detoxification of cisplatin. Molecular Cancer Therapeutics, 7(10), 3247-3255.

Perumal Vijayaraman, K., Muruganantham, S., Subramanian, M., Shunmugiah, K. P., & Kasi, P. D. (2012). Silymarin attenuates benzo(a)pyrene induced toxicity by mitigating ROS production, DNA damage and calcium mediated apoptosis in peripheral blood mononuclear cells (PBMC). Ecotoxicology and Environmental Safety, 86, 79-85.

Plant, A. L., Benson, D. M., & Smith, L. C. (1985). Cellular uptake and intracellular localization of benzo(a)pyrene by digital fluorescence imaging microscopy. The Journal of Cell Biology, 100(4), 1295-1308.

Pollock RE, Morton DL. Principles of Surgical Oncology. In: Bast RC Jr, Kufe DW, Pollock RE, *et al*., editors. Holland-Frei Cancer Medicine. 5th edition. Hamilton (ON): BC Decker; 2000. Chapter 32.

Reedijk, J. (1987). Bioinorganic chemistry. inorganic chemistry in a perspective of biology, medicine and the environment. Die Naturwissenschaften, 74(2), 71-77.

Poorter, R. L., Bakker, P. J., Huizing, M. T., Taat, C. W., Rietbroek, R. C., Gouma, D. J., *et al*. (1995). Intermittent continuous infusion of ifosfamide and 5-fluorouracil in patients with advanced adenocarcinoma of the pancreas. Annals of Oncology : Official Journal of the European Society for Medical Oncology, 6(10), 1048-1049.

Raouf, A., Zhao, Y., To, K., Stingl, J., Delaney, A., Barbara, M., *et al*. (2008). Transcriptome analysis of the normal human mammary cell commitment and differentiation process. Cell Stem Cell, 3(1), 109-118.

Rochat, B., Morsman, J. M., Murray, G. I., Figg, W. D., & McLeod, H. L. (2001). Human CYP1B1 and anticancer agent metabolism: Mechanism for tumor-specific drug inactivation? The Journal of Pharmacology and Experimental Therapeutics, 296(2), 537-541.

Rodriguez-Antona, C., Donato, M. T., Pareja, E., Gomez-Lechon, M. J., & Castell, J. V. (2001). Cytochrome P-450 mRNA expression in human liver and its relationship with enzyme activity. Archives of Biochemistry and Biophysics, 393(2), 308-315.

Rodriguez-Antona, C., & Ingelman-Sundberg, M. (2006). Cytochrome P450 pharmacogenetics and cancer. Oncogene, 25(11), 1679-1691.

Rosenberg, B., Vancamp, L., & Krigas, T. (1965). Inhibition of cell division in escherichia coli by electrolysis products from a platinum electrode. Nature, 205, 698-699.

Rowe, J. D., Nieves, E., & Listowsky, I. (1997). Subunit diversity and tissue distribution of human glutathione S-transferases: Interpretations based on electrospray ionization-MS and peptide sequence-specific antisera. The Biochemical Journal, 325 (Pt 2), 481-486.

Sahu, B. D., Kuncha, M., Sindhura, G. J., & Sistla, R. (2013). Hesperidin attenuates cisplatin-induced acute renal injury by decreasing oxidative stress, inflammation and DNA damage. Phytomedicine : International Journal of Phytotherapy and Phytopharmacology, 20(5), 453-460.

Sakano, S., Wada, T., Matsumoto, H., Sugiyama, S., Inoue, R., Eguchi, S., *et al*. (2006). Single nucleotide polymorphisms in DNA repair genes might be prognostic factors in muscle-invasive bladder cancer patients treated with chemoradiotherapy. British Journal of Cancer, 95(5), 561-570.

Scanlon, K. J., Newman, E. M., Lu, Y., & Priest, D. G. (1986). Biochemical basis for cisplatin and 5-fluorouracil synergism in human ovarian carcinoma cells. Proceedings of the National Academy of Sciences of the United States of America, 83(23), 8923-8925.

Schena, M., Shalon, D., Heller, R., Chai, A., Brown, P. O., & Davis, R. W. (1996). Parallel human genome analysis: Microarray-based expression monitoring of 1000 genes. Proceedings of the National Academy of Sciences of the United States of America, 93(20), 10614-10619.

Schilsky, R. L. (1998). Biochemical and clinical pharmacology of 5-fluorouracil. Oncology (Williston Park, N.Y.), 12(10 Suppl 7), 13-18.

Scripture, C. D., Sparreboom, A., & Figg, W. D. (2005). Modulation of cytochrome P450 activity: Implications for cancer therapy. The Lancet Oncology, 6(10), 780-789.

Sharon, M., McLeod, H., Dolan, E., Shukla, S.J., Rabik, C. A, Gong, L., Hernandez-Boussard, T., Lou, X. J, Klein, T.E., Altman, R. B. Platinum pathway. (2009). Pharmacogenetics and genomics.

Sherratt, P. J., Pulford, D. J., Harrison, D. J., Green, T., & Hayes, J. D. (1997). Evidence that human class theta glutathione S-transferase T1-1 can catalyse the activation of dichloromethane, a liver and lung carcinogen in the mouse. comparison of the tissue distribution of GST T1-1 with that of classes alpha, mu and pi GST in human. The Biochemical Journal, 326 (Pt 3), 837-846.

Shin, S., Wakabayashi, N., Misra, V., Biswal, S., Lee, G. H., Agoston, E. S., *et al*. (2007). NRF2 modulates aryl hydrocarbon receptor signaling: Influence on adipogenesis. Molecular and Cellular Biology, 27(20), 7188-7197.

Somdyala, N. I., Bradshaw, D., Gelderblom, W. C., & Parkin, D. M. (2010). Cancer incidence in a rural population of south africa, 1998-2002. International Journal of Cancer.Journal International Du Cancer, 127(10), 2420-2429.

Spurr, N. K., Gough, A. C., Stevenson, K., & Wolf, C. R. (1987). Msp-1 polymorphism detected with a cDNA probe for the P-450 I family on chromosome 15. Nucleic Acids Research, 15(14), 5901.

Su, F., Hu, X., Jia, W., Gong, C., Song, E., & Hamar, P. (2003). Glutathion S transferase pi indicates chemotherapy resistance in breast cancer. The Journal of Surgical Research, 113(1), 102-108.

Sulem, P., Gudbjartsson, D. F., Geller, F., Prokopenko, I., Feenstra, B., Aben, K. K., *et al*. (2011). Sequence variants at CYP1A1-CYP1A2 and AHR associate with coffee consumption. Human Molecular Genetics, 20(10), 2071-7.

Sutoh, I., Kohno, H., Nakashima, Y., Hishikawa, Y., Tabara, H., Tachibana, M., *et al*. (2000). Concurrent expressions of metallothionein, glutathione S-transferase-pi, and P-glycoprotein in colorectal cancers. Diseases of the Colon and Rectum, 43(2), 221-232.

Takechi, T., Fujioka, A., Matsushima, E., & Fukushima, M. (2002). Enhancement of the antitumour activity of 5-fluorouracil (5-FU) by inhibiting dihydropyrimidine dehydrogenase activity (DPD) using 5-chloro-2,4-dihydroxypyridine (CDHP) in human tumour cells. European Journal of Cancer (Oxford, England: 1990), 38(9), 1271-1277.

Takeda, H., Haisa, M., Naomoto, Y., Kawashima, R., Satomoto, K., Yamatuji, T., *et al*. (1999). Effect of 5-fluorouracil on cell cycle regulatory proteins in human colon cancer cell line. Japanese Journal of Cancer Research : Gann, 90(6), 677-684.

Taylor, R. T., Wang, F., Hsu, E. L., & Hankinson, O. (2009). Roles of coactivator proteins in dioxin induction of CYP1A1 and CYP1B1 in human breast cancer cells. Toxicological Sciences: An Official Journal of the Society of Toxicology, 107(1), 1-8.

Townsend, D. M., Deng, M., Zhang, L., Lapus, M. G., & Hanigan, M. H. (2003). Metabolism of cisplatin to a nephrotoxin in proximal tubule cells. Journal of the American Society of Nephrology, 14(1), 1-10.

Townsend, D. M., Tew, K. D., He, L., King, J. B., & Hanigan, M. H. (2009). Role of glutathione S-transferase pi in cisplatin-induced nephrotoxicity. Biomedicine & Pharmacotherapy, 63(2), 79-85.

Ueda, R., Iketaki, H., Nagata, K., Kimura, S., Gonzalez, F. J., Kusano, K., *et al*. (2006). A common regulatory region functions bidirectionally in transcriptional activation of the human CYP1A1 and CYP1A2 genes. Molecular Pharmacology, 69(6), 1924-1930.

Urruticoechea, A., Alemany, R., Balart, J., Villanueva, A., Vinals, F., & Capella, G. (2010). Recent advances in cancer therapy: An overview. Current Pharmaceutical Design, 16(1), 3-10.

Van Cutsem, E., Moiseyenko, V. M., Tjulandin, S., Majlis, A., Constenla, M., Boni, C., *et al*. (2006). Phase III study of docetaxel and cisplatin plus fluorouracil compared with cisplatin and fluorouracil as first-line therapy for advanced gastric cancer: A report of the V325 study group. Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology, 24(31), 4991-4997.

van der Straaten, T., Kweekel, D., Tiller, M., Bogaartz, J., & Guchelaar, H. J. (2006). Multiplex pyrosequencing of two polymorphisms in DNA repair gene XRCC1. The Journal of Molecular Diagnostics : JMD, 8(4), 444-448.

van Kuilenburg, A. B., Meinsma, R., & van Gennip, A. H. (2004). Pyrimidine degradation defects and severe 5-fluorouracil toxicity. Nucleosides, Nucleotides & Nucleic Acids, 23(8-9), 1371-1375.

van Rooyen, B. A., Schafer, G., Leaner, V. D., & Parker, M. I. (2013). Tumour cells down-regulate CCN2 gene expression in co-cultured fibroblasts in a Smad7- and ERK-dependent manner. Cell Communication and Signaling, 11, 75-811X-11-75.

Veale, RB, Thornley, AL. Atypical cytokeratins synthesized by human oesophageal carcinoma cells in culture. South African Journal of Science 1984; 80; 260-267.

Vogel, C., & Marcotte, E. M. (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nature Reviews: Genetics, 13(4), 227-232.

Wang, D., & Lippard, S. J. (2005). Cellular processing of platinum anticancer drugs. Nature Reviews.Drug Discovery, 4(4), 307-320.

Wickman, G. R., Julian, L., Mardilovich, K., Schumacher, S., Munro, J., Rath, N., *et al*. (2013). Blebs produced by actin-myosin contraction during apoptosis release damage-associated molecular

pattern proteins before secondary necrosis occurs. Cell Death and Differentiation, 20(10), 1293-1305.

Wigmore, P. M., Mustafa, S., El-Beltagy, M., Lyons, L., Umka, J., & Bennett, G. (2010). Effects of 5-FU. Advances in Experimental Medicine and Biology, 678, 157-164.

Wogan, G. N., Hecht, S. S., Felton, J. S., Conney, A. H., & Loeb, L. A. (2004). Environmental and chemical carcinogenesis. Seminars in Cancer Biology, 14(6), 473-486.

Wrighton, S. A., & Stevens, J. C. (1992). The human hepatic cytochromes P450 involved in drug metabolism. Critical Reviews in Toxicology, 22(1), 1-21.

Yellin, S. A., Davidson, B. J., Pinto, J. T., Sacks, P. G., Qiao, C., & Schantz, S. P. (1994). Relationship of glutathione and glutathione-S-transferase to cisplatin sensitivity in human head and neck squamous carcinoma cell lines. Cancer Letters, 85(2), 223-232.

Yoshikawa, R., Kusunoki, M., Yanagi, H., Noda, M., Furuyama, J. I., Yamamura, T., *et al*. (2001). Dual antitumor effects of 5-fluorouracil on the cell cycle in colorectal carcinoma cells: A novel target mechanism concept for pharmacokinetic modulating chemotherapy. Cancer Research, 61(3), 1029-1037.

Yoshinari, K., Ueda, R., Kusano, K., Yoshimura, T., Nagata, K., & Yamazoe, Y. (2008). Omeprazole transactivates human CYP1A1 and CYP1A2 expression through the common regulatory region containing multiple xenobiotic-responsive elements. Biochemical Pharmacology, 76(1), 139-145.

Yoshisue, K., Nagayama, S., Shindo, T., & Kawaguchi, Y. (2001). Effects of 5-fluorouracil on the drug-metabolizing enzymes of the small intestine and the consequent drug interaction with nifedipine in rats. The Journal of Pharmacology and Experimental Therapeutics, 297(3), 1166-1175.

Yu, F., Megyesi, J., Safirstein, R. L., & Price, P. M. (2007). Involvement of the CDK2-E2F1 pathway in cisplatin cytotoxicity in vitro and in vivo. American Journal of Physiology.Renal Physiology, 293(1), F52-9.

Zanger, U. M., & Schwab, M. (2013). Cytochrome P450 enzymes in drug metabolism: Regulation of gene expression, enzyme activities, and impact of genetic variation. Pharmacology & Therapeutics, 138(1), 103-141.

APPENDIX A

Solutions

All solutions stored at room temperature unless stated otherwise.

1% Agarose gel with ethidium bromide

1 g Agarose
100 mL 1X TBE (in DEPC-treated water)
Dissolve by pulse-heating in microwave with gentle agitation in between. Allow to cool on shaker and add 4 µL ethidium bromide before pouring.

30% Acryl-bisacrylamide mix

29 g Acrylamide
1 g N,N'-methylenebisacrylamide
Dissolve in 60 mL dH_2O and heat to 37 ºC. Adjust volume to 100 mL with dH_2O and store at 4 ºC protected from light.

10% Ammonium persulphate

1 g ammonium persulphate
8 mL dH_2O
Adjust volume to 10 mL with H_2O. Store at 4 ºC protected from light and use with 2 weeks.

0.5 M EDTA (pH 8.0)

37.22 g $Na_2EDTA.2H_2O$
140 mL dH_2O
Adjust pH to 8.0 with 10 M NaOH and volume to 200 mL with dH_2O, and autoclave.

70% Ethanol

70 mL Absolute ethanol
Make up to 100 mL with dH_2O

DEPC-treated sterile dH_2O

Add 100 µL DEPC to 100 mL dH_2O, stir, leave to stand overnight and autoclave.

5% Fat-free blocking solution

5 g Fat-free milk powder
Make up to100 mL with PBST. Store at 4 ºC and use within 1 week.

Fixing solution

500 mL Methanol
100 mL Acetic acid
400 mL dH_2O
Store at room temperature and use within 1 month.

Freezing medium

1 mL DMSO
9 mL FCS
Store at -20 ºC.

1 M HCl

Add 6.13 µL concentrated HCl to 200 mL H_2O.

MTT reagent

100 g Thiazolyl blue tetrazolium powder
20 mL 1X PBS
Incubate at 37 ºC for 15 minutes and store at 4 ºC protected from light for up to 1 month.

10X PBS

2 g KCl
80 g NaCl
2 g KH_2PO_4
11.5 g Na_2HPO_4
Adjust pH to 7.4 using 1 M HCl or 1 M NaOH if necessary; make up to 1 L with dH_2O and autoclave.

1X PBS

100 mL 10X PBS
900 mL dH_2O
Store at 4 ºC.

1X PBST

100 mL 1X PBS
1 mL Tween-20
Place on magnetic stirrer for 30 minutes and store at 4 ºC.

8% Resolving gel

4.6 mL dH_2O

2.7 mL 30% Acryl-bisacrylamide mix
2.5 mL 1.5 M Tris (pH 8.8)
100 µL 10% SDS
100 µL 10% Ammonium persulphate
6 µL TEMED

12% Resolving gel

3.3 mL dH_2O
4.0 mL 30% Acryl-bisacrylamide mix
2.5 mL 1.5 M Tris (pH 8.8)
100 µL 10% SDS
100 µL 10% Ammonium persulphate
4 µL TEMED

1X RIPA buffer

1 mL 10X RIPA buffer
9 mL dH_2O
Store at 4 ºC.

1X RIPA buffer with protease inhibitor cocktail

10X protease inhibitor cocktail tablet
2.5 mL 1X PBS
Dissolve tablet in PBS and add 20 µL of this solution to 180 µL 1X RIPA buffer just before use.

2X RNA loading buffer

900 µL Formamide
99 µL Sucrose
0.5 µL Bromophenol blue
0.5 µL Xylene cyanol

10% SDS

10 g SDS
90 mL dH_2O
Heat to ~70 ºC to dissolve and adjust volume to 100 mL with dH_2O.

5X SDS loading buffer

12.5 mL 2 M Tris-HCl (pH 6.8)
10 g SDS
30 mL Glycerol
5 mL 14.3 M β-Mercaptoethanol
52 mL Bromophenol blue

Adjust volume to 100 mL with dH_2O.

10X SDS running buffer

10 g SDS
30.3 g Tris
144.1 g Glycine
800 mL dH_2O
Adjust volume to 1 L with dH_2O.

1X SDS running buffer

100 mL 10X SDS running buffer
900 mL dH_2O

10X SDS transfer buffer

144 g Glycine
38 g Tris
Adjust volume to 1 L with dH_2O.

1X SDS transfer buffer

100 mL 10X SDS transfer buffer
200 mL Isopropanol
700 mL dH_2O
Store at 4 ºC.

Solubilisation reagent

50 g SLS
5 mL 1M HCl
Adjust volume to 500 mL with dH_2O.

5% Stacking gel

2.1 mL dH_2O
500 µL 30% Acryl-bisacrylamide mix
380 µL 1.5 M Tris (pH 6.8)
30 µL 10% SDS
30 µL 10% Ammonium Persulphate
3 µL TEMED

Stripping buffer

690 µL 14.3 M β-Mercaptoethanol
20 mL 10% SDS

6.25 mL 1 M Tris-HCl (pH 6.7)
73.06 mL dH_2O
Store and use in fume-hood.

10X TBE (DEPC-treated)

108 g Tris
55 g Boric acid
40 mL 0.5 M EDTA (pH 8.0)
Adjust volume to 1 L with DEPC-treated dH_2O and autoclave.

1X TBE

100 mL 10X TBE
900 mL DEPC-treated dH_2O

1.5 M Tris pH 6.8

60.5 g Tris base
300 mL dH_2O
Adjust pH to 6.8 with concentrated HCl and volume to 500 mL with dH_2O. Store at 4 ºC.

1.5 M Tris pH 8.8

60.5 g Tris base
300 mL dH_2O
Adjust pH to 8.8 with concentrated HCl and volume to 500 mL with dH_2O. Store at 4 ºC.

1 M Tris-HCl (pH 6.7)

24.22 g Tris base
160 mL dH_2O
Adjust pH to 6.7 with concentrated HCl and volume to 200 mL with dH_2O. Store at 4 ºC.

1X Trypsin-EDTA

1 mL 10X Trypsin-EDTA
9 mL dH_2O
Store at -20 ºC.

APPENDIX B

Calculating IC_{50} values from a sigmoidal-dose response curve using GraphPad PRISM® v 5.0 software (continued from section 3.2.1)

After entering the required raw data, which are the concentration gradient values and corresponding absorbance readings obtained from the MTT assay (section 2.2.2), the GraphPad PRISM® software generates a sigmoidal dose-response curve and uses the following equation to calculate an IC_{50} value:

$$Y = Bottom + (Top - Bottom) / 1 + 10^{(LogIC50 - X) \cdot Hillslope}$$

The equation is known as a four-parameter logistic equation, where Bottom refers to the Y value at the bottom plateau and Top refers to the Y value at the top plateau of the curve (Figure B1). $LogIC^{50}$ is the X value when the response if halfway between the Top and the Bottom. The Hillslope, also known as the slope factor or Hill coefficient, describes the steepness of the curve. When the Hillslope is positive, the curve increases as X increases as shown in the three examples of Hillslope values in Figure A (0.5, 1.0 and 1.5), and vice versa.

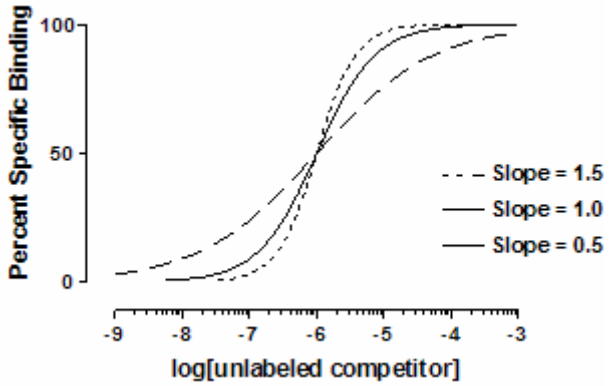


Figure B1 A classic sigmoidal-dose response curve. (Obtained from http://www.graphpad.com/guides/prism/6/curve-fitting/index.htm?reg_classic_dr_variable.htm).

When the curve goes in an uphill direction, as shown in the example in Figure B1, the variable is called the ED^{50} (half maximal effective dose), whereas when the curve goes downhill, as in Figure 3.2 of this study, the variable is called the IC^{50} (half maximal inhibitory concentration).

APPENDIX C

Melt curves for qRT-PCR products (continued from section 3.3.2)

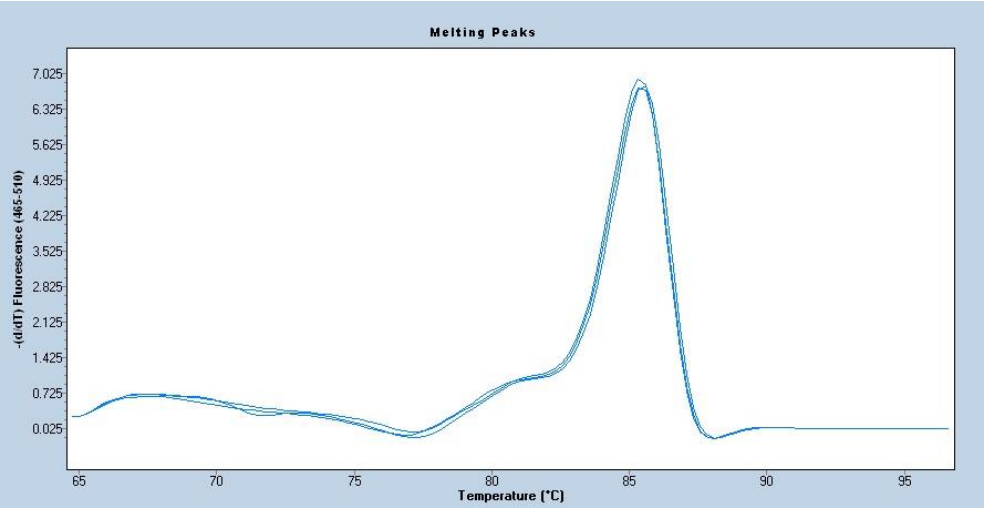


Figure C1 Melt curve for CYP1A1 product after qRT-PCR showing primer specificity. Three matched peaks can be observed, indicating that the primers amplified a single product (in triplicate), in this case, CYP1A1, and confirming that no primer dimers were formed.

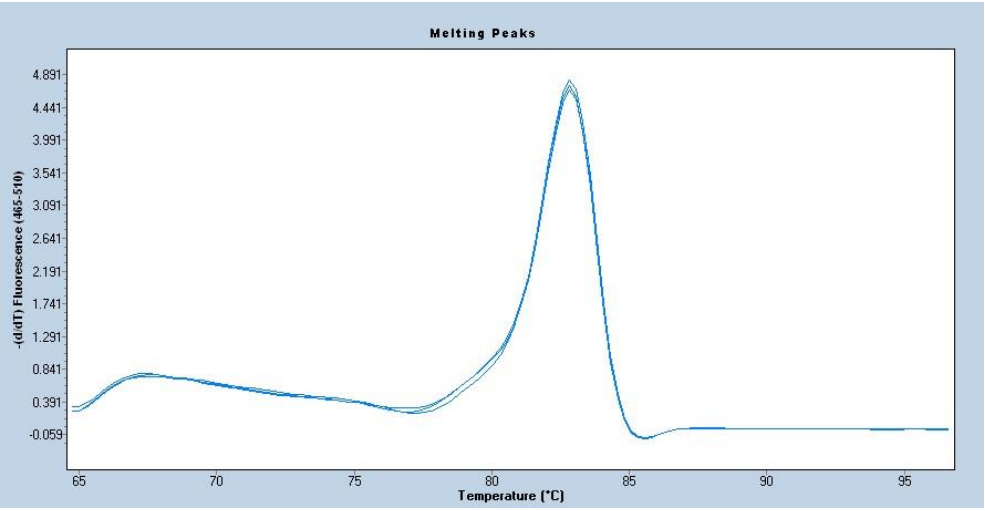


Figure C2 Melt curve for CYP1A2 product after qRT-PCR showing primer specificity. Three matched peaks can be observed, indicating that the primers amplified a single product (in triplicate), in this case, CYP1A2, and confirming that no primer dimers were formed.

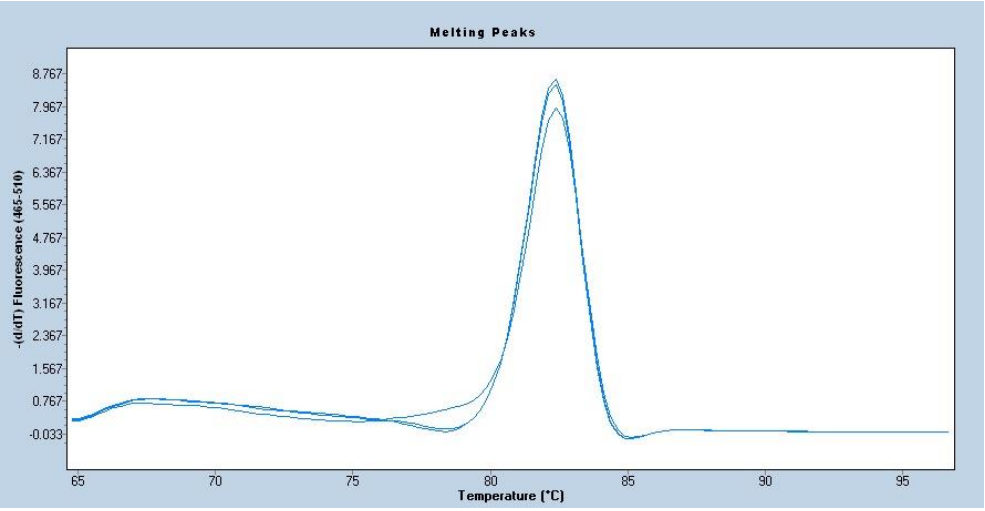


Figure C3 Melt curve for GSTP1 product after qRT-PCR showing primer specificity. Three matched peaks can be observed, indicating that the primers amplified a single product (in triplicate), in this case, GSTP1, and confirming that no primer dimers were formed.

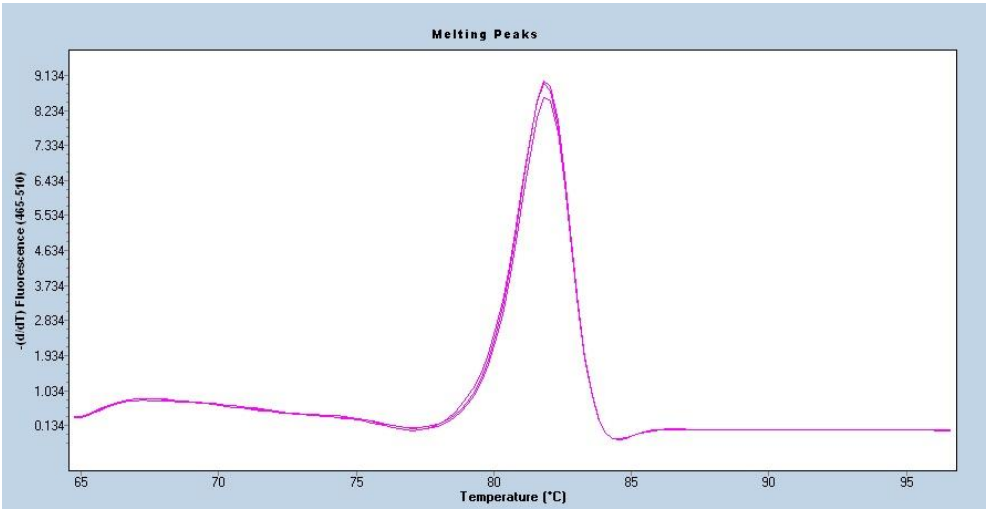


Figure C4 Melt curve for GAPDH product after qRT-PCR showing primer specificity. Three matched peaks can be observed, indicating that the primers amplified a single product (in triplicate), in this case, GAPDH, and confirming that no primer dimers were formed.

APPENDIX D

Amplification of qRT-PCR products (continued from section 3.3.2)

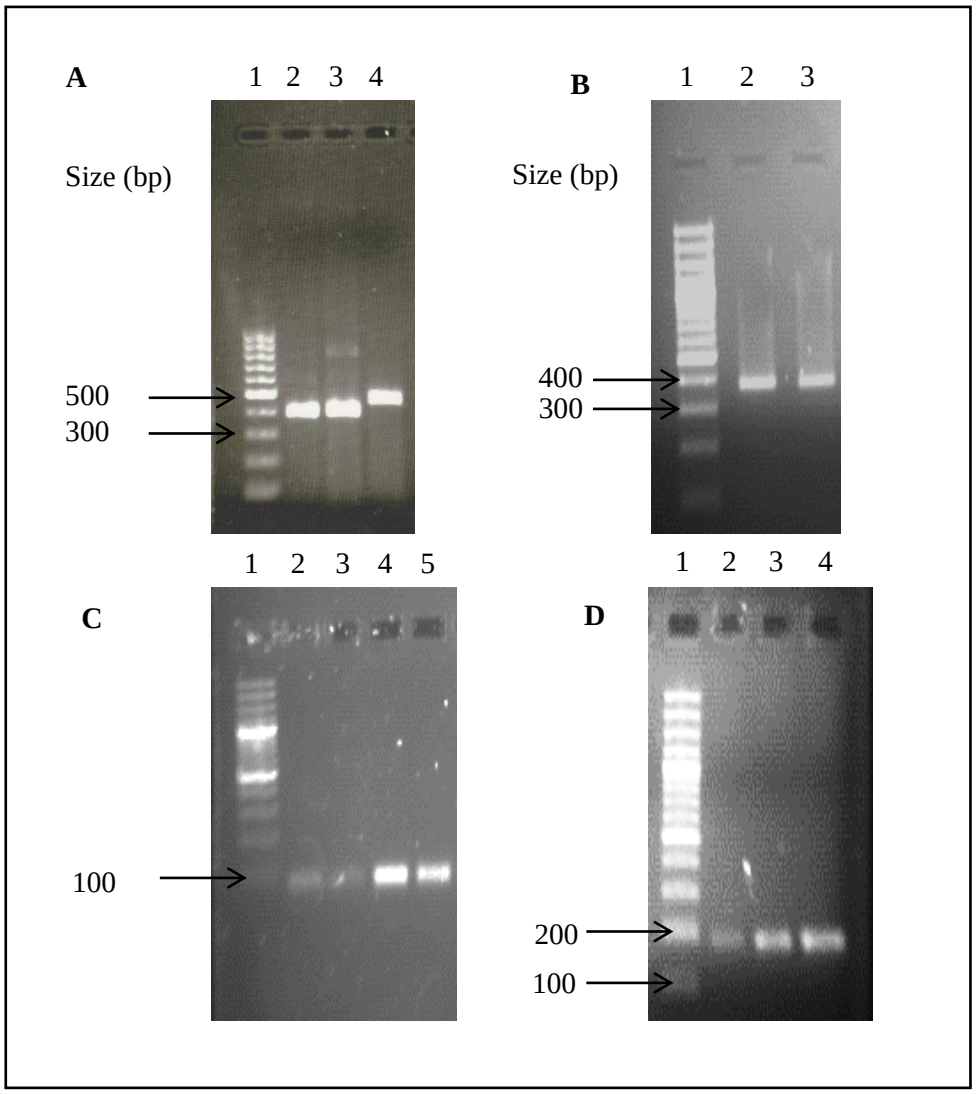


Figure D1 Confirmation of qRT-PCR products by agarose gel electrophoresis. The products of the qRT-PCR were electrophoresed on a 1% agarose gel alongside a 100 bp molecular weight maker (shown in lane 1 of each gel image). The gel images show the correct fragment sizes for A. CYP1A1 (lanes 2 and 3), CYP1A2 (lane 4), B. CYP1B1 (lanes 2 and 3), C. GSTP1 (lanes 2-5) and D. GAPDH (lanes 2-4).