STUDIES TO UNDERSTAND THE EFFECT OF CANCER ON HEPATIC CYP2C19 ACTIVITY

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Abstract

Inter-patient variation in effectiveness and toxicity of cancer chemotherapy may be due to differences in pharmacokinetics, influenced by genetic and environmental factors controlling the activity of hepatic drug metabolising enzymes. One such enzyme, CYP2C19, displays genetic variation; homozygous variant individuals have a poor metaboliser (PM) phenotype. Whilst this relationship is valid in healthy populations, genotype-phenotype discordance has been reported in cancer patients. The aim of this thesis was to determine if discordance occurs in a wider range of cancer patients and to elucidate mechanisms responsible for decreased CYP2C19 enzyme activity.

Two independent clinical studies were undertaken. Of 33 patients with terminal cancer, 37% were PM, significantly ($P < 0.0005$) higher than predicted from genotype. For 29 patients with colorectal carcinoma, 27% in stage IV and 14% of resected patients were PM. Although RECIST analysis of stage IV patients did not demonstrate a significant relationship between CYP2C19 activity and tumour burden, the one patient tested both before and after tumour resection, changed from a poor to an extensive metaboliser.

In patients with terminal cancer, no correlation between CYP2C19 status and inflammatory markers was observed. In contrast, PM phenotype in stage IV and resected patients was associated with elevated CRP ($P < 0.05$) and decreased serum TGF-β ($R_S = -0.5331$, $P < 0.005$). Interestingly, six patients changed phenotype categories over three test occasions reflected by changes in TGF-β. There was also an association between BMI and CYP2C19 activity ($R_S = 0.4953$, $P = 0.0063$).

NO-donor compounds reversibly inhibited CYP2C19 activity in human liver microsomes and cells over-expressing CYP2C19. In addition, 24h exposure of cells to NO-donor compounds irreversibly decreased CYP2C19 activity ($P < 0.0005$), which was blocked by MG132, an inhibitor of proteasomal degradation. However, there was no relationship between plasma nitrate/nitrite concentrations and CYP2C19 activity in the patients.

Total plasma protein and unbound drug fraction were determined for individual patients. It was demonstrated that the high drug/metabolite ratio in the PM subjects was not due to altered drug-protein binding and could only be accounted for by decreased enzyme activity (intrinsic clearance, CL_{int}).

In conclusion, some cancer patients have compromised CYP2C19 activity that may be due to factors including inflammation, obesity and nitrosative damage. Non-inherited variation in CYP2C19 activity may account for variable pharmacokinetics of some anticancer drugs, thus identification of phenotypic PM prior to treatment may reduce the wide variation in both toxicity and response to these agents.

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Table of Contents

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

List of Abbreviations and Symbols

°C	Degree Celsius
5’OH OMP	5’hydroxy omeprazole
α-MEM	Alpha-minimal essential media
A	Adenine
AAG	$Alpha_1$-acid glycoprotein
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AST	Aspartate transaminase
AUC	Area under the time-concentration curve
bid	bis in die, twice a day
BMI	Body mass index
bp	Base pair
C	Cytosine
CG	Cycloguanil
CL	Clearance
CL_H	Hepatic clearance
CL_{int}	Intrinsic clearance
CRP	C-reactive protein
CT scan	X-ray computed tomography scan
CYP450	Cytochrome P450
dL	Decilitre
DNA	Deoxyribonucleic acid
E	Hepatic extraction ratio
EDTA	Ethylenediaminetetraacetic acid
EM	Extensive metaboliser
f_U	Fraction unbound
g	Gravitational acceleration
G	Guanine
g	Gram
HDL	High-density lipoprotein
HI	Hydroxylation index
HPLC	High performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
IFN-γ	Interferon-gamma
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6

IQR	Interquartile range
IS	Internal standard
iv	Intravenous
kg	Kilogram
K_m	Michaelis constant
L	Litre
LDL	Low-density lipoprotein
M	Molar
m	Metre
mg	Milligram
min	Minutes
mL	Millilitre
mM	Millimolar
mm	Millimetre
mm	Millimetre
mRNA	Micro RNA
n	Number
NADPH	β-Nicotinamide adenine dinucleotide phosphate (reduced form)
NED	No-evaluable disease
ng	Nanogram
nm	Nanometre
NO	Nitric oxide
NOC-18	2,2'-(hydroxynitrosohydrazino)bis-ethanamine
OD	Optical density
OMP	Omeprazole
P	*P*-value
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pg	Picogram
PG	Proguanil
PM	Poor metaboliser
po	*Per os*, oral administration
PVDF	Polyvinylidene difluoride
Q	Liver blood-flow
qPCR	Real-time polymerase chain reaction
R_B	Blood-to-plasma ratio
RECIST	Response evaluation criteria in solid tumours
RFLP	Restriction fragment length polymorphism
R_S	Spearman rank correlation
SD	Standard deviation
SE	Standard error

SNAP	S-nitroso *N*-acetylpenicillamine
SNP	Single nucleotide polymorphism
SP	Sodium nitroprusside
T	Thymine
$t_{1/2}$	Half-life
TBS	Tris-buffered saline
TGF-β	Transforming growth factor-beta
TNF-α	Tumour necrosis factor-alpha
U	Units
UV	Ultraviolet
v/v	Volume to volume
var	Variant allele
Vd	Volume of distribution
V_{max}	Maximum catalytic rate
w/v	Weight to volume
wt	Wildtype allele
µg	Microgram
µL	Microlitre
µM	Micromolar

CHAPTER 1

General introduction

1.1 Therapeutic variability

The outcomes of drug therapy can vary significantly between individuals. Achieving optimal efficacy and safety of drugs in all patients is an important focus of clinical practice and drug development. Therapeutic failure and/or drug toxicity in subpopulations of patients contributes to the quality of health care and economic burden worldwide.

Adverse drug reactions (ADR) have been reported to account for between 1.7 and 6.7% of hospital admissions in various countries (Lazarou et al. 1998; Pirmohamed et al. 2004; Bond et al. 2006; Carrasco-Garrido et al. 2010). These ADR can be very expensive, with the annual cost of the 6.5% of hospital admissions due to ADR in the United Kingdom projected to be £466 million. Importantly, much of this burden could be avoidable (Pirmohamed et al. 2004).

There is also large inter-individual variation in the therapeutic response. The therapeutic response to many major drugs varies between 25% to 60% (Spear et al. 2001; Wilkinson 2005). For example, the highest percentage of patients responding to treatment was for COX-2 inhibitors (80%) and lowest was 25% for anticancer therapies (Spear et al. 2001). The relatively poor efficacy of cancer therapies is possibly due to the effect of genetic variation in the somatic genome of tumours. Somatic mutations result in altered expression of drug targets and the development of resistance.

The narrow therapeutic index associated with anticancer therapy is a well recognised limitation in the use of these cytotoxic agents. The small margins of safety compared with other drugs mean that confounding factors, such as co-morbidity, polypharmacy and altered physiological status in patients that change drug pharmacokinetics and pharmacodynamics, can have a large impact on therapeutic effectiveness. Changes in pharmacokinetics, in particular, can result in relatively unpredictable inter-individual variation in both therapeutic response and also the incidence of adverse toxicity with these drugs (Slaviero et al. 2003).

1.1.1 Pharmacokinetic variability in cancer chemotherapy

The majority of cancer chemotherapy drugs are administered based on body surface area (mg/m^2) (Pinkel 1958). The DuBois formula was the first widely used formula to estimate body surface area from a patient's weight and height (Du Bois et al. 1916). Further modifications to the DuBois formula have also been proposed to estimate body surface area (Gehan et al. 1970; Haycock et al. 1978; Mosteller 1987). Despite this dose normalisation, the pharmacokinetic parameters of cancer chemotherapeutic drugs remain highly variable and unpredictable between patients. Some examples of the wide inter-patient variability in pharmacokinetic parameters of anticancer drugs that are dosed based on body surface area are illustrated in Table 1.1 (Gurney 1996).

Drug	Inter-patient variation in pharmacokinetic parameters	References
Doxorubicin	Vd = 62%, CL = 65%, AUC = 65%	(Piscitelli et al. 1993)
Busulfan	AUC = 4 fold	(Vassal et al. 1992)
Teniposide	C_{ss} = 3-6 fold	(Rodman et al. 1987)
Cisplatin	AUC of free platinum = 19%	(Sumiyoshi et al. 1995)
Carboplatin	AUC = 2-3 fold	(Madden et al. 1992)
Etoposide	$t_{1/2}$ = 43%, Vd = 23%, CL = 10%, AUC = 12%	(Lowis et al. 1998)
Ifosfamide	Isophosphoramide AUC = 10 fold	(Boddy et al. 1993)
Cyclophosphamide, methotrexate, 5-fluorouracil	AUC = 3-4 fold for each drug	(Moore et al. 1994)
Vincristine	AUC = 11 fold	(Van den Berg et al. 1982)
5-fluorouracil	AUC = 5 fold	(Milano et al. 1988)
Irinotecan	AUC = 20%	(Sumiyoshi et al. 1995)
Paclitaxel	CL = 5 fold	(Sonnichsen et al. 1994)

Table 1.1. ***Inter-patient variability in the pharmacokinetic parameters of various anticancer drugs.*** The percentages represent the coefficient of variation and others reflect the fold range. Vd = volume of distribution, CL = clearance, AUC = area under the time-concentration curve, C_{ss} = steady-state concentration, $t_{1/2}$ = half-life. Adapted from Gurney (1996).

For example, the AUC of vincristine varied 11 fold and ranged from 16 to 182 ng/mL/h per mg/kg in 39 cancer patients receiving 0.4-1.54 mg/m^2 as part of conventional treatment protocols (Van den Berg et al. 1982).

The pharmacokinetics of docetaxel also displays large inter-individual variability in cancer patients with various solid tumours (Clarke et al. 1999; Goh et al. 2002). The reported clearance of docetaxel was 15.9 ± 4.0 L/h/m^2 in the 31 patients receiving 75 or 100 mg/m^2 dose (Goh et al. 2002)

Another example is the considerable inter-individual pharmacokinetic variability of cyclophosphamide in cancer patients (Moore 1991; Moore et al. 1994; Zhang et al. 2006). The clearance of cyclophosphamide was reported to be 78 ± 20 mL/min in 23 breast cancer patients (Moore et al. 1994). These patients were also given 5-fluorouracil and methotrexate as part of the schedule. The AUC of all of these drugs was also highly variable, 51,955 ± 11,315, 1008 ± 199 and 8689 ± 3582 μM.min for cyclophosphamide, methotrexate and 5-fluorouracil respectively (Moore et al. 1994).

One reason for this pharmacokinetic variability is altered drug clearance due to changes in hepatic drug metabolism. This can be influenced by intrinsic (genetic) and/or extrinsic (environmental) factors. The intrinsic factors include variation in the patient's germline genome which may affect both the pharmacokinetics and pharmacodynamics of a drug. Extrinsic factors include polypharmacy, age and morbidity.

Some of the factors that may influence the pharmacokinetic variability in cancer chemotherapy are listed in Table 1.2 (Masson et al. 1997).

Parameter	**Source of variability**
Dose selection	Physician's preference Patient's condition
Dose administration	Patient compliance Medication error
Systemic exposure	Drug-drug interactions Altered renal and/or hepatic function Protein binding

Table 1.2. ***The influential factors on the pharmacokinetic variability in chemotherapy.*** Adapted from Masson et al. (1997).

Polypharmacy refers to multiple medications and is common in elderly patients (Bjerrum et al. 1998; Delafuente 2003; Hajjar et al. 2007). The concurrent use of multiple drugs increases the risk of unwanted drug-drug interactions, adverse drug reactions as well as non-compliance (Colley et al. 1993; Delafuente 2003). In addition, polypharmacy is also influenced by large variations in the practice patterns of different practitioners (Bjerrum et al. 1999). The mechanisms involved can be pharmacodynamic, with additive, synergistic or antagonistic effects; or pharmacokinetic, with changes in drug metabolism/transport or protein binding.

Differences in protein binding due to variable concentrations of drug-binding proteins in cancer patients may also contribute to the variations in pharmacokinetics of anticancer drugs. In particular, the concentrations of the major drug-binding proteins, albumin and alpha$_1$-acid glycoprotein are known to be altered in cancer (Fearon et al. 1998; Uslu et al. 2003), and may affect the level of drug-protein binding. Variation in drug-protein binding has been reported for anticancer drugs such as etoposide (Stewart et al. 1989) and docetaxel (Urien et al. 1996).

Altered renal and/or hepatic function also contributes to the variation in the pharmacokinetic parameters of anticancer drugs due to changes in hepatic and/or renal clearance (Koren et al. 1992; Kintzel et al. 1995). For example, altered renal function due to age or malignancy can change the pharmacokinetics of drugs that are associated with significant renal clearance, such as carboplatin (Kintzel et al. 1995). In addition, inter-individual differences in the activity of hepatic drug metabolising enzymes can also account for variation in hepatic clearance of drugs within populations.

1.1.2 Drug metabolising enzymes

Drug biotransformation is catalysed by drug metabolising enzymes which are mainly located in the liver (Williams 1972). Drug metabolism involves reactions such as, oxidation, hydrolysis, reduction and dehydrogenation/hydrogenation as well as conjugation (Meyer 1996). Following catalysis of the drug substrates, the metabolites are either excreted or undergo further biotransformation by conjugation with endogenous hydrophilic moieties such as glutathione, acetate, glucuronate, sulfate or glycine which make the drug more water soluble and readily excreted. Enzymes involved in this conjugation include uridine diphosphate glucuronosyltransferase (UGT) and glutathione

S-transferase (GST). The main enzymes involved in oxidation, reduction and hydroxylation reactions are a family of proteins called cytochrome P450 (CYP).

1.1.2.1 Cytochrome P450 enzymes

The cytochrome P450 (CYP) drug metabolising enzymes are responsible for approximately 75% of phase I metabolism of exogenous compounds (Bertz et al. 1997; Evans et al. 1999). These enzymes are haem-containing proteins that are anchored in the membranes of the endoplasmic reticulum of hepatocytes.

There are multiple families and subfamilies of cytochrome P450 enzymes. Based on the amino acid sequence identity, enzymes that share ≥ 40% identity are assigned to a family designated by an Arabic numeral, and those with ≥ 55% are assigned to a subfamily designated by a letter (Nebert et al. 1987). Humans have a total of 57 *CYP* genes and 33 pseudogenes which is arranged into 18 families and 42 subfamilies (Nelson et al. 2004).

The major CYP isoforms present in human liver are CYP2C and CYP3A (Shimada et al. 1994; Ingelman-Sundberg 2004a). CYP2 is the largest CYP family in humans with 13 subfamilies and 16 genes (Nebert et al. 2002). Members of the CYP2C subfamily, CYP2C8, CYP2C9, CYP2C18 and CYP2C19, together metabolise more than half of all frequently prescribed drugs, as well as arachidonic acid and some steroids (Bertz et al. 1997; Evans et al. 1999; Ingelman-Sundberg 2004b).

It has previously been reported that 51% of drugs are metabolised by CYP3A and 19% of drugs by CYP2C (Shimada et al. 1994) and hence CYP3A4 catalysis is a relatively more common route than CYP2C. However, substrate specificity for certain CYP isoforms exists and a list of commonly used anticancer agents and the CYP drug metabolising enzymes that are responsible at least in part of the metabolic pathways is shown in Table 1.3 (Rendic 2002; Fujita 2006; Scripture et al. 2006).

Drug metabolising enzyme	Anticancer drug substrate
CYP1A2	Dacarbazine, etoposide [a], flutamide,
CYP2A6	Tegafur, tamoxifen [a]
CYP2B6	Cyclophosphamide, ifosfamide
CYP2C8	Paclitaxel, all-*trans*-retinoic acid
CYP2C9	Tauromustine, tamoxifen [a]
CYP2C19	Bortezomib [b], cyclophosphamide, thalidomide, indisulam, nilutamide
CYP2D6	Tamoxifen
CYP3A4/5	Docetaxel, doxorubicin, erlotinib, etoposide, gefitinib, irinotecan, vinblastine, vincristine, vinorelbine

Table 1.3. ***Examples of major contributing CYP450 drug metabolising enzymes responsible for the metabolism of anticancer drugs.*** Adapted from Fujita (2006), Rendic (2002) and Scripture et al. (2006). [a] minor contribution by the enzyme and [b] other enzymes involved.

It is of note that the two major drug metabolising enzyme families (CYP2C and CYP3A) are involved in the metabolism of at least 18 chemotherapeutic drugs. Differences in the activity of these CYP enzymes may play an important role in the pharmacokinetic variability observed with many of these agents. For example, variation in CYP3A4 activity may be one explanation for the inter-individual pharmacokinetic differences observed following docetaxel treatment (Hirth et al. 2000; Yamamoto et al. 2005). The erythromycin breath test and the urinary clearance of endogenous 6-β-hydroxycortisol are validated phenotypic tests for CYP3A4 (Rivory et al. 2000; Yamamoto et al. 2000). A significant positive correlation between docetaxel clearance and measured CYP3A4 activity was observed (Hirth et al. 2000; Yamamoto et al. 2000). Furthermore, the clearance of docetaxel is a strong predictor of neutropenia and fluid retention (Bruno et al. 1998) and low CYP3A4 activity is associated with increased toxicity to vinorelbine and docetaxel in cancer patients (Slaviero et al. 2003).

Variable activity of CYP enzymes can be influenced by numerous factors including drug-drug interactions (inhibition and induction of activity), as well as changes in regulation or expression due to gender/age and morbidity. Importantly, variation in drug metabolism can also be influenced by germline genetic variation. This inherited variation in CYP activity is termed pharmacogenetics and is particularly important for CYP enzymes such as CYP2C19 and CYP2D6.

1.1.2.2 Other drug metabolising enzymes

It is important to note that, as well as the CYP isoforms involved in the metabolism of cytotoxic anticancer agents, a number of these drugs and metabolites are then substrates for further metabolism by enzymes such as UDP-glucuronosyl transferase. Examples of additional enzymes important in the pharmacokinetic disposition of anticancer drugs are shown in Table 1.4 (Rendic 2002; Fujita 2006; Scripture et al. 2006). Many of these enzymes are also subject to drug-drug interactions (inhibition and induction of activity), as well as changes in regulation or expression due to gender/age and morbidity and germline pharmacogenetic variation.

Drug metabolising enzyme	Anticancer drug substrate
Dihydropyrimidine dehydrogenase	5-Fluorouracil
Glutathione *S*-transferases	Busulfan, carmustine, chlorambucil, cisplatin, doxorubicin, melphalan, oxaliplatin
N-acetyltransferases	Amonafide
Thiopurine methyltransferase	6-Mercaptopurine, 6-thioguanine
UDP-glucuronosyl transferases	Irinotecan

Table 1.4. ***Examples of other drug metabolising enzymes responsible for the metabolism of anticancer drugs.*** Adapted from Fujita (2006), Rendic (2002) and Scripture et al. (2006).

1.2 Pharmacogenetics

The genetic variability that influences a drug disposition or toxic response has been termed “pharmacogenetics” (Vogel 1959; Evans et al. 2004; Meyer 2004). There is abundant evidence of genetic polymorphisms in drug metabolising enzymes such as CYP which account for inter-individual differences in drug disposition. The application of information from the human genome to predict drug response in an individual is an additional way to minimise the currently unpredictable inter-patient variability in the pharmacokinetic parameters of cancer chemotherapeutic drugs.

Four potential phenotypes can arise from mutations/variants in drug metabolising genes, a) ultra-rapid metabolisers: individuals who have more than two functional (normal/wildtype) copies of the gene or have gene mutations which result in increased function, b) extensive metabolisers: individuals who have two functional (normal/wild-

type) genes, c) intermediate metabolisers: individuals who either carry one non-functional gene or two decreased functional genes and d) poor metabolisers who have two non-functional copies of the gene or gene deletion (Ingelman-Sundberg et al. 2007).

One example which demonstrates the influence of pharmacogenetics on anticancer therapy is thiopurine methyltransferase (TMPT). This enzyme is involved in the methylation and hence the inactivation of azathioprine, mercaptopurine and thioguanine (Table 1.4), drugs which are used in the treatment of leukemia, inflammatory bowel disease and rheumatoid arthritis (Burchenal et al. 1953; Lennard 1992; Ansari et al. 2002). TPMT activity is highly variable in the Caucasian population with 86.6% having high activity, 11.1% intermediate activity and 0.3% have null activity (Corominas et al. 2000; McLeod et al. 2002). Twenty variant *TPMT* alleles have been identified and of these, *TPMT*2*, *TPMT*3A-D* accounts for more than 95% of the patients with defective TPMT activity (Gardiner et al. 2000; McLeod et al. 2000; Schaeffeler et al. 2004). Due to the gene-dose effect of TPMT on 6-thioguanines (McLeod et al. 2000; McLeod et al. 2002; Schaeffeler et al. 2004), dose reduction has been advocated. Approximately 0.3% of the population are homozygous poor metabolisers and 10% are heterozygotes, hence these patients require a dose reduction of 10% and 30-50% respectively (Kaskas et al. 2003; Gardiner et al. 2008).

Other drug metabolising enzymes that have pharmacogenetic variants include UGT1A1 (uridine diphosphate glucuronosyltransferase) and DYPD (dihydropyrimidine dehydrogenase). These two enzymes are responsible for the metabolism of the common anticancer agents, irinotecan and 5-fluorouracil (Table 1.4) respectively (Harris et al. 1990; Fleming et al. 1992; Iyer et al. 1998). Mutations in these genes can lead to decreased enzyme activity and cause severe toxicity due to decreased clearance of each of these drugs (Fleming et al. 1992; Takimoto et al. 1996; Ando et al. 2000).

CYP2D6 is responsible for the conversion of tamoxifen to the more potent 4-hydroxy-tamoxifen and endoxifen metabolites (Desta et al. 2004). This drug is used in the treatment of breast cancer (Fisher et al. 1989; Fisher et al. 1998). Over 70 polymorphisms in *CYP2D6* have been identified and the common alleles which account for 97% of null CYP2D6 activity in Caucasian population are *CYP2D6*3*, *CYP2D6*4*, *CYP2D6*5* and *CYP2D6*6* (Gaedigk et al. 1999). The plasma concentration of endoxifen was significantly lower in patients who are homozygous variant for *CYP2D6* compared with wildtype individuals (Jin et al. 2005). In tamoxifen-treated breast cancer

patients, those that were homozygous variant for the *CYP2D6*4* allele appeared to be predisposed to a higher risk of disease relapse and a lower incidence of hot flashes (Goetz et al. 2005).

As mentioned in section 1.1.2.1 above, CYP2C and CYP3A isoforms are involved in the metabolism of at least 18 chemotherapeutic drugs. The CYP2C family consists of CYP2C8, 2C9, 2C18 and 2C19 and genetic variants of these enzymes exists. Of major interest is the genetic polymorphism observed in *CYP2C19*.

1.2.1 *CYP2C19* genetic polymorphism

CYP2C19 metabolises a number of cancer chemotherapeutic drugs (Table 1.3) and also numerous other pharmacological agents (Table 1.5) (Desta et al. 2002).

Class	Drug	References
Antineoplastic	Bortezomib	(Uttamsingh et al. 2005)
	Cyclophosphamide	(Griskevicius et al. 2003; Timm et al. 2005)
	Thalidomide	(Ando et al. 2002a; Li et al. 2007)
	Tamoxifen	(Coller et al. 2002; Schroth et al. 2007)
	Indisulam	(Zandvliet et al. 2007)
	Nilutamide	(Horsmans et al. 1991)
Proton pump inhibitors	Omeprazole	(Chiba et al. 1993)
	Lansoprazole	(Sohn et al. 1997)
	Pantoprazole	(Andersson 1996)
	Rabeprazole	(Yasuda et al. 1995)
Anticonvulsants, hypnosedatives, muscle relaxants	Phenytoin	(Ieiri et al. 1997)
	S-Mephenytoin	(Wilkinson et al. 1989)
	Methylphenytoin	(Schellens et al. 1990)
	Diazepam	(Andersson et al. 1994)
	Desmethyldiazepam	(Bertilsson et al. 1989)
	Flunitrazepam	(Coller et al. 1999b)
	Phenobarbital	(Mamiya et al. 2000)
	Hexobarbital	(Yasumori et al. 1990)
	Mephobarbibal	(Küpfer et al. 1985)
	Carisoprodol	(Dalén et al. 1996)
Anti-infectives	Proguanil	(Helsby et al. 1990c)
	Chlorproguanil	(Wright et al. 1995)
	Nelfinavir	(Burger et al. 2006)
	Voriconazole	(Murayama et al. 2007)
Antidepressants	Citalopram	(Sindrup et al. 1993)
	Fluoxetine	(Liu et al. 2001)
	Sertraline	(Wang et al. 2010)
	Imipramine	(Skjelbo et al. 1993)
	Clomipramine	(Nielsen et al. 1994)
	Trimipramine	(Eap et al. 2000)
	Amitriptyline	(Breyer-Pfaff et al. 1992)
	Nortriptyline	(Olesen et al. 1997)
	Moclobemide	(Gram et al. 1995)
Cardiovascular agents	Clopidogrel	(Kim et al. 2008)
	Propanolol	(Ward et al. 1989a)

Table 1.5. ***Known CYP2C19 substrates.*** Adapted from Desta et al (2002).

Considerable inter-individual variability in the activity of CYP2C19 is observed. On the basis of an individual's ability to metabolise CYP2C19 substrates such as *S*-mephenytoin or omeprazole, individuals can be categorised as extensive or poor metabolisers. The first study to report the discovery of a poor metaboliser of *S*-mephenytoin was published in 1979 (Küpfer et al. 1979; Küpfer et al. 1982). This *S*-mephenytoin phenotypic test was validated and used extensively to describe inter-individual variability (Küpfer et al. 1984). A genetic component to this "poor metabolism" of *S*-mephenytoin was demonstrated using classical pedigree studies and it was demonstrated to be inherited as an autosomal recessive trait (Ward et al. 1987; Brøsen et al. 1995). Further *in vitro* studies using human liver identified CYP2C19 as the protein that was responsible for the metabolism of *S*-mephenytoin (Wrighton et al. 1993). Subsequent studies identified genetic variants of this enzyme that are responsible for this differential metabolism of CYP2C19 drug substrates (de Morais et al. 1994a; de Morais et al. 1994b).

The *CYP2C19* gene is located on chromosome 10 (10q24.1-q24.3) and comprises of nine exons (Desta et al. 2002). *CYP2C19* is highly polymorphic and more than 20 genetic variants have been identified to date as shown in Table 1.6 (Sim 2008).

Allele	Nucleotide change	Gene change	Effect	Protein change	Enzyme activity	References
**1*	None		Wildtype		Normal	(Romkes et al. 1991; Richardson et al. 1995)
**2*	681 G>A	19154 G>A	Splicing defect at exon 5	Premature termination of protein synthesis	Inactive	(de Morais et al. 1994b; Ibeanu et al. 1998b)
**3*	636 G>A	17948 G>A	Premature stop codon	Truncated protein	Inactive	(de Morais et al. 1994a)
**4*	1 A>G	1 A>G	Mutation of initiation codon	Inhibits translation	Inactive	(Ferguson et al. 1998)
**5*	1297 C>T	90033 C>T	Arg433→Trp		Inactive	(Ibeanu et al. 1998a)
**6*	395 G>A	12748 G>A	Arg 132→Gln		Inactive	(Ibeanu et al. 1998b)
**7*		19294 T>A	Splicing defect at intron 5		Inactive	(Ibeanu et al. 1999)
**8*	358 T>C	12711 T>C	Trp120→Arg		Decrease	(Ibeanu et al. 1999)
**17*		-806 C>T			Increase	(Sim et al. 2006)

Table 1.6. ***Common genetic variants of CYP2C19 allele identified to date and the major SNPs/alterations responsible for the phenotype of the corresponding allele.*** Adapted from Sim (2008).

The majority of non-functional CYP2C19 “poor metaboliser” subjects can be attributed to two of these genetic defects: *CYP2C19*2* and *CYP2C19*3*. Both of these variants are null alleles which arise from a single base change, G>A, at exon 5 and exon 4 respectively. The *CYP2C19*2* (681 G>A) variant results in a splice site mutation and the *CYP2C19*3* (636 G>A) variant results in a premature stop codon (de Morais et al. 1994a; de Morais et al. 1994b). Both of these variants result in a loss of functional enzyme due to a truncated protein or lack of binding to the haem moiety (de Morais et al. 1994a; de Morais et al. 1994b).

The availability of RFLP-PCR tests (de Morais et al. 1994a; de Morais et al. 1994b; Goldstein et al. 1996) for the *CYP2C19*2* and *CYP2C19*3* variants led to the confirmation that poor metabolisers (PM) of *S*-mephenytoin (Masimirembwa et al. 1995), proguanil (Hoskins et al. 1998), omeprazole (Chang et al. 1995b) and diazepam (Kosuge et al. 2001) are homozygous variant for *CYP2C19* whereas extensive metabolisers (EM) are homozygous or heterozygous wild type for this gene.

There are also additional loss-of-function variants such as *CYP2C19*4, CYP2C19*5, CYP2C19*6, CYP2C19*7* and *CYP2C19*8*, however these are relatively rare in comparison to *CYP2C19*2* and *CYP2C19**3 (Desta et al. 2002; Rosemary et al. 2007).

Recent reports of the *CYP2C19*17* variant indicates ultrarapid metabolism of CYP2C19 drugs (Sim et al. 2006; Rudberg et al. 2008; Sibbing et al. 2010). Mutation in the 5’ regulatory region (-806 C>T and -3402 C>T) of *CYP2C19* results in the recruitment of a transcription factor to the mutated site which increases the transcription of the *CYP2C19* gene (Sim et al. 2006).

Other less well-characterised variant alleles have been identified (*CYP2C19*9-*20*) (Sim 2008), however, little is known about the CYP2C19 activity of these alleles (Blaisdell et al. 2002; Morita et al. 2004; Fukushima-Uesaka et al. 2005; Rosemary et al. 2007; Sim 2008; Lee et al. 2009b; Zhou et al. 2009; Drögemöller et al. 2010).

1.2.1.1 Inter-ethnic variability

There is considerable inter-ethnic variability in the incidence of poor metaboliser *CYP2C19*2* and *CYP2C19*3* variants. The frequency of the *CYP2C19*2* allele is different between different ethnic populations: 15% in Caucasian, 17% in African-Americans and 30% in Chinese populations (Xie et al. 1999b; Desta et al. 2002). Although the *CYP2C19*3* allele is rare in Caucasian and African populations, it makes up the remainder of the defective alleles in Asian populations (Goldstein et al. 1997). The inter-ethnic variability in the distribution of the *CYP2C19*2* and *CYP2C19*3* alleles are shown in Table 1.7 (Rosemary et al. 2007).

Ethnicity	**n**	***CYP2C19*2***	***CYP2C19*3***	**Reference**
Caucasian				
Belgian	121	9.1	0	(Allabi et al. 2003)
Dutch	765	13.3	0.2	(Tamminga et al. 2001)
Russian	290	11.4	0.3	(Gaikovitch et al. 2003)
Croatian	200	15.0	0	(Bozina et al. 2003)
Italian	360	11.1	0	(Scordo et al. 2004)
African				
African	922	17.3	0.4	(Xie et al. 1999a)
Tanzanian	216	10	0	(Bathum et al. 1999)
African-American	108	25	0	(Goldstein et al. 1997)
Asian				
Chinese Han	101	36.6	7.5	(Xiao et al. 1997)
Chinese Bai	202	25.7	5.5	(Xiao et al. 1997)
Japanese	53	23	10.4	(Goldstein et al. 1997)
Korean	103	21	12	(Roh et al. 1996)
Filipino	52	39	8	(Goldstein et al. 1997)
Pacific				
Vanuatu	100	57	25	(Kaneko et al. 1999b)
Vanuatu	5538	63.3	14.4	(Kaneko et al. 1999b)
PNG [a]	47	37	34	(Hsu et al. 2008)
PNG [a] Sepik	401	45	15.8	(Masta et al. 2003)

Table 1.7. ***The inter-ethnic variability in the incidence of CYP2C19*2 and CYP2C19*3 variant alleles.*** Adapted from Rosemary et al (2007). [a] PNG = Papua New Guinea.

The incidence of genotypic poor metabolisers (homozygous variant) in a Caucasian population was reported to be 1-6% (Xie et al. 1999b). A similar incidence of 1-7.5% was reported in the African population (Xie et al. 1999a). In contrast, a much higher incidence of 12-23% was reported in the Asian population (Bertilsson 1995; Goldstein et al. 1997). The incidence of genotypic poor metabolisers is also extremely high in Indonesia with 34% who are homozygous variant (Yusuf et al. 2003). Moreover, more than 70% CYP2C19 poor metabolisers have been reported in the Melanesian island of Vanuatu (Kaneko et al. 1997; Kaneko et al. 1999a). Homozygous variant for *CYP2C19*2* and *CYP2C19**3 (poor metaboliser) occurs in 48.9% of the Iruna population of Papua New Guinea (Hsu et al. 2008). It was suggested that the high incidence of poor metabolism in Vanuatu may be due to a genetic drift originating from Papua New Guinea as the *CYP2C19*2* allele is predominant in the Vanuatu population with a lower contribution from the *CYP2C19*3* variant (Helsby 2008; Hsu et al. 2008). The Maori population in New Zealand was thought to be originated from Southeast Asia, spreading through Philippines, Indonesia, Melanesia and the Pacific Islands including Fiji, Samoa, Tonga and the Cook Islands (Moodley et al. 2009; Wilmshurst et al. 2010). Consistent with this hypothesis, the allele frequency of *CYP2C19*2* and *CYP2C19*3* variants were significantly higher in Maori compared with Caucasians (Lea et al. 2008; Helsby et al. 2010a).

Relatively little has been reported about the inter-ethnic differences in the frequency of the *CYP2C19*17* allele. This allele was identified in 18% of Swedes and Ethiopians with a lower incidence of 4% observed in Chinese individuals (Sim et al. 2006). Another study has reported a low incidence of 0.65% in 20 healthy Chinese male volunteers (Wang et al. 2009). Similarly, a low incidence of 1.3% has been reported in healthy Japanese volunteers (Sugimoto et al. 2008). In addition, a high frequency of 19.2% occurs in a Tamil Indian population (Anichavezhi et al. 2011).

1.2.1.2 CYP2C19 phenotype probe/tests

S-Mephenytoin has been used previously as the probe substrate for measuring CYP2C19 activity (Wrighton et al. 1993). However it is not an ideal probe drug as it has many side effects including sedation, which limit its use (Wilkinson et al. 1989; Setiabudy et al. 1992; Balian et al. 1995).

Another CYP2C19 substrate, omeprazole, is a widely used phenotypic probe for CYP2C19 (Desta et al. 2002). In order to measure the phenotypic activity, a 2 hour post dose (20 mg po omeprazole) blood sample is analysed by HPLC to measure the levels of omeprazole and the 5'hydroxy omeprazole metabolite. The omeprazole log hydroxylation index (log HI) is calculated as log [OMP]/[5'OH OMP]. Individuals with no detectable 5'hydroxy omeprazole metabolite are given an arbitrary value of 2. Several studies have demonstrated that the log HI is bimodally distributed in a healthy population and that the antimode value to categorise poor metabolisers is a log HI index ≥ 1 (Balian et al. 1995; Kortunay et al. 1997a). Therefore individuals with a log HI index ≥ 1 are classified as a poor metabolisers and < 1 as extensive metabolisers. Moreover, the omeprazole hydroxylation index consistently correlate with *S*-mephenytoin metabolism ($n = 40$, $r^2 = 0.681$, $P < 0.001$) (Balian et al. 1995).

It is important however, to note that chronic administration of omeprazole can lead to auto-induction of metabolic clearance due to increased activity of CYP1A2 (Diaz et al. 1990; Rost et al. 1992). In such situations, *CYP2C19* genotype will not predict omeprazole metabolism and omeprazole is not a suitable probe for measuring CYP2C19 activity in patients receiving this commonly prescribed drug.

An additional phenotypic probe drug for CYP2C19 is proguanil (Helsby et al. 1990c; Wright et al. 1995). Proguanil is an anti-malarial prodrug that is converted by CYP2C19 to its major metabolite, cycloguanil (Armstrong 1973). The metabolic route of proguanil is shown in Figure 1.1.

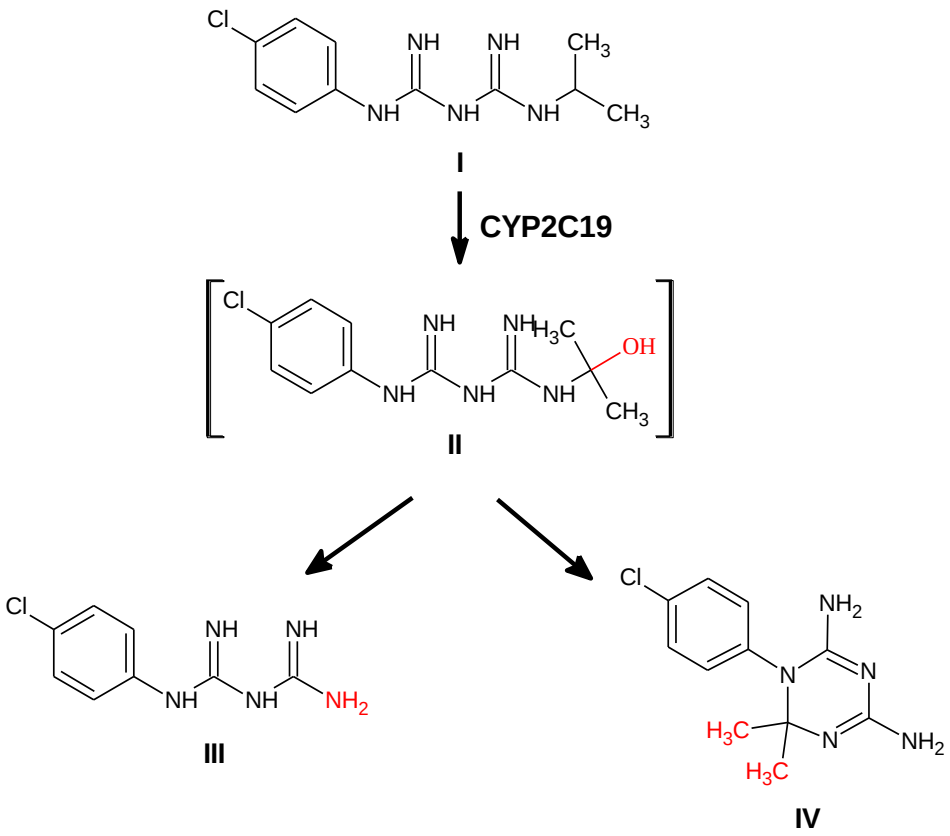

Figure 1.1. ***The proposed metabolic route of proguanil in humans.*** Structure I = proguanil, II = unstable intermediate, III = phenylbiguanide, IV = cycloguanil. Adapted from Herrlin et al (2000).

Following an oral dose of proguanil (200 mg), a 3 hour post-dose blood sample is analysed by HPLC to measure the concentration of proguanil and cycloguanil. The CYP2C19 phenotypic activity is reported as the metabolic ratio of proguanil/cycloguanil and a ratio greater than 10 is categorised as a poor metaboliser (Ward et al. 1989b; Helsby et al. 1990a).

An excellent linear correlation between the plasma proguanil metabolic ratio and omeprazole index was reported in healthy individuals that were phenotyped at two different occasions (Spearman R = 0.87, $P < 0.01$) (Herrlin et al. 2000). Similarly, proguanil metabolism correlates with *S*-mephenytoin metabolism (Ward et al. 1991).

Many studies (n = 22) have shown the correlation between genotypic poor metabolisers and phenotypic poor metabolisers as reviewed by Xie (Xie et al. 1999b) and some examples are shown in Table 1.8. It is important to note that these studies have been undertaken young healthy subjects.

Genotype		Phenotype			Reference
	%	Probe drug		%	
Brazilian, n = 38					
EM	89	omeprazole	EM	89	(Linden et al. 2009)
PM	11		PM	11	
Korean, n = 103					
EM	88	omeprazole	EM	87.4	(Roh et al. 1996)
PM	12		PM	12.6	
Chinese, n = 193					
EM	90.7	*S*-mephenytoin	EM	89.6	(He et al. 2002)
PM	9.3		PM	10.4	
Columbian, n = 44					
EM	95.5	omeprazole	EM	95.5	(Isaza et al. 2007)
PM	4.5		PM	4.5	
Swedish, n = 160					
EM	71	omeprazole	EM	71	(Chang et al. 1995a)
PM	29		PM	29	

Table 1.8. ***Concordance between CYP2C19 genotype and phenotype in healthy populations.***

1.2.1.3 Non-genetic factors affecting CYP2C19 activity and expression

The concordance between *CYP2C19* genotype and phenotype is clearly valid in healthy populations of any ethnicity (Table 1.8). The application of such genotypic test to predict drug response in an individual is one possible way to personalise medicine and decrease variability in drug response and drug toxicity (Johnson 2003; Eichelbaum et al. 2006; Blakey et al. 2011; Sim et al. 2011). However, for the *CYP2C19* pharmacogenetic test to be clinically useful, it must also be robust and accurate in the clinical situation. However, there is little information about genotype-phenotype relationship in subjects with disease. Other environmental factors that may affect CYP2C19 activity include age, gender and disease.

It has been reported that omeprazole disposition is altered with advanced age. A decreased elimination rate and prolonged plasma half-life of omeprazole is observed in the elderly compared with young adults (Landahl et al. 1992). A significant negative relationship between omeprazole hydroxylation index (CYP2C19 activity) and increasing age was reported in a study of 28 elderly (66-85 years) and 23 young (21-36 years)

Japanese healthy volunteers (Ishizawa et al. 2005). Moreover, there was a discordance between CYP2C19 genotype and phenotype in the elderly, where elderly *wt/wt* or *wt/var* were found to be poor metaboliser phenocopies (Kimura et al. 1999). A similar discordance was recently observed in a cohort of very elderly patients who had previously experienced an adverse drug reaction to omeprazole (Helsby et al. 2010c).

Severe liver diseases such as cirrhosis and hepatitis may also have a negative effect on CYP2C19 activity (Andersson et al. 1993b; Rost et al. 1995; Kimura et al. 1999; Ohnishi et al. 2005). The mean bioavailability of omeprazole increased from 56% in healthy volunteers compared with 98% in patients with liver cirrhosis, which indicates a decrease in first-pass metabolism (Andersson et al. 1993b). Similarly, the hepatic clearance of omeprazole was significantly decreased in the patients with cirrhosis (Andersson et al. 1993b). There were also reports of poor metaboliser phenocopies in patients with liver disease who were genotypic extensive (*wt/wt* or *wt/var*) metabolisers (Rost et al. 1995; Kimura et al. 1999).

A number of medications are CYP2C19 inhibitors including cimetidine, oral contraceptives, fluoxetine and loratadine (Jeppesen et al. 1996; Somogyi et al. 1996; Laine et al. 2000; Barecki et al. 2001; Desta et al. 2002). Hence these co-medications may interfere with drug clearance and result in apparent CYP2C19 poor metaboliser phenocopies.

In addition, induction of CYP2C19 activity by co-medications may result in an underestimation of CYP2C19 metabolic activity when based on genotype information alone. Clinical evidence has shown that at least two drugs, artemisinin (Svensson et al. 1998; Mihara et al. 1999) and rifampicin (Zhou et al. 1990; Smith et al. 1991) are able to induce CYP2C19 activity. However, the molecular mechanisms behind this induction has not been clarified (Desta et al. 2002). It has been suggested that rifampicin may activate CAR and PXR (Raucy et al. 2002; Chen et al. 2003; Chen et al. 2005; Sahi et al. 2009), which are hypothesised to be involved in the regulation of CYP enzymes. This inducing effect appears to be gene-dose related (Zhou et al. 1990). There is also some evidence that the ability of rifampicin to induce CYP2C19-dependent hexobarbital clearance is decreased in elderly compared with young healthy volunteers (Smith et al. 1991).

Interestingly, CYP2C19 activity has been shown to be influenced by pregnancy and usage of oral contraceptives. It has been reported that the peak cycloguanil concentrations were lower and the proguanil metabolic ratio were higher in pregnant women compared with women two months post-partum (Wangboonskul et al. 1993; McGready et al. 2003). Importantly, this effect was only observed in phenotypic extensive metabolisers (McGready et al. 2003). Oral contraceptives and hormone replacement therapy were also found to decrease CYP2C19 activity significantly in healthy females (Hoskins et al. 1998; Laine et al. 2000; Hägg et al. 2001; Palovaara et al. 2003). Other *in vitro* studies have also demonstrated the inhibition of CYP2C19 activity by synthetic estrogens, 17α-ethinylestradiol and estradiol (Jurima et al. 1985; Laine et al. 2003; Rodrigues 2004). The decreased activity may be due to the activation of the estrogen receptor-α, which can bind to the *CYP2C19* promoter and thus inhibit transcription of the *CYP2C19* gene (Mwinyi et al. 2010a).

Some studies have also suggested that there may be an intrinsic gender difference in CYP2C19 activity (Hooper et al. 1990; Tamminga et al. 1999; Tanaka 1999), although conflicting evidence exists as to whether there is gender-related difference (Laine et al. 2000; Kim et al. 2002; Bebia et al. 2004).

1.2.1.4 Genotype-phenotype discordance in disease

Although studies have demonstrated that CYP2C19 activity in individuals who express functional protein (*wt/wt* or *wt/var*) can vary due to extrinsic (environmental) factors. There is also some limited evidence that this environmental regulation of the *CYP2C19* gene expression can in some cases results in “phenocopies”. For example, the decline in CYP2C19 activity associated with advanced age resulted in some elderly individuals with *wt/wt* or *wt/var* genotype having a log omeprazole hydroxylation index > 1 and a poor metaboliser phenotypic status (Kimura et al. 1999).

Importantly, a limited number of studies have demonstrated that a discordance between genotype and phenotype exists in patients with diseases such as cancer and congestive heart failure. In a small study of patients (n = 16) with advanced cancer (*wt/wt* and *wt/var*), the overall omeprazole hydroxylation index (CYP2C19 activity) of these cancer patients were significantly decreased ($P < 0.0001$) compared with a healthy reference

population (Williams et al. 2000). More importantly, 25% of these patients were CYP2C19 poor metaboliser phenocopies (Williams et al. 2000).

Such genotype-phenotype discordance was also seen in a study of patients with congestive heart failure (Frye et al. 2002). This study was conducted in a group of sixteen congestive heart failure patients who were all genotypic extensive metabolisers. However, seven of these patients were classified as phenotypic poor metabolisers based on the low recovery levels of the 4'-hydroxymephenytoin metabolite (Frye et al. 2002).

Importantly, genotype-phenotype discordance in patients with disease was not only observed for the CYP2C19 enzyme, but has also been reported for NAT2 and CYP2D6. Patients with HIV and AIDS have been reported to have compromised NAT2 and CYP2D6 activity (O'Neil et al. 1997; O'Neil et al. 2000). The bimodal distribution of NAT2 and CYP2D6 phenotype that is usually observed in a healthy population was not observed in the HIV and AIDS population. Other drug metabolising enzyme activities, including CYP3A4 and XO were also reported to be altered in HIV-infected patients (Jones et al. 2010).

CYP3A4 activity has also been reported to decrease in cancer patients. There was a significant decrease in CYP3A4 activity measured using the erythromycin breath test in patients with advanced cancer (n = 29) compared with healthy volunteers (n = 32) (Slaviero et al. 2003). This may be due to down-regulation of the gene. Animal models (*CYP3A4* transgenic mice model inoculated with Engelbreth-Holm-Swarm, EHS, sarcoma), have demonstrated that the hepatic expression of *CYP3A4* was decreased in the presence of an extrahepatic tumour compared with saline-treated controls (Charles et al. 2006). Furthermore, murine CYP3A protein and activity was also significantly decreased in these tumour-bearing mice (Charles et al. 2006). The mechanism may be due to the impairment of the CAR and PXR activation on the gene regulation of CYP3A (Kacevska et al. 2011). This EHS model was associated with a tumour-mediated inflammatory response due to an increase in acute phase response proteins and inflammatory cytokines (Charles et al. 2006; Kacevska et al. 2008; Sharma et al. 2008).

It has been demonstrated previously that patients with sepsis have decreased CYP450 drug metabolism (Carcillo et al. 2003). Those sepsis patients with elevated plasma IL-6 concentrations had increased plasma nitrite/nitrate concentrations as well as increased organ failure score (Doughty et al. 1996). Sepsis is characterised by a "cytokine

cascade" which involves IL-1, IL-6 and TNF-α (Blackwell et al. 1996; Gogos et al. 2000; Ulloa et al. 2005). Furthermore in healthy volunteers, the administration of endotoxin, which elicits an inflammatory response, decreases CYP450 metabolism (Shedlofsky et al. 1994; Shedlofsky et al. 1997).

The environmental/extrinsic factors associated with this compromised activity of certain drug metabolising enzymes in diseases such as cancer is not well characterised but may involve inflammation.

1.3 Inflammation as a contributing factor to compromised drug metabolism

It has long been recognised that the inflammatory response is associated with decreased CYP activity. Inflammation causes the down-regulation of gene expression or enzyme activity of CYP involved in hepatic drug metabolism. Cytokines have been shown to down-regulate P450 expression in human hepatocyte cultures (Abdel-Razzak et al. 1993). In addition, turpentine-induced inflammation in IL-6 gene knockout mice failed to down-regulate CYP expression (Siewert et al. 2000; Ashino et al. 2004). The down-regulation of CYP enzymes may lead to decreased drug clearance and elevated drug plasma concentrations which may cause toxicity. A general scheme illustrating the inflammation-mediated downregulation of CYP expression or activity is shown in Figure 1.2 (Renton 2004).

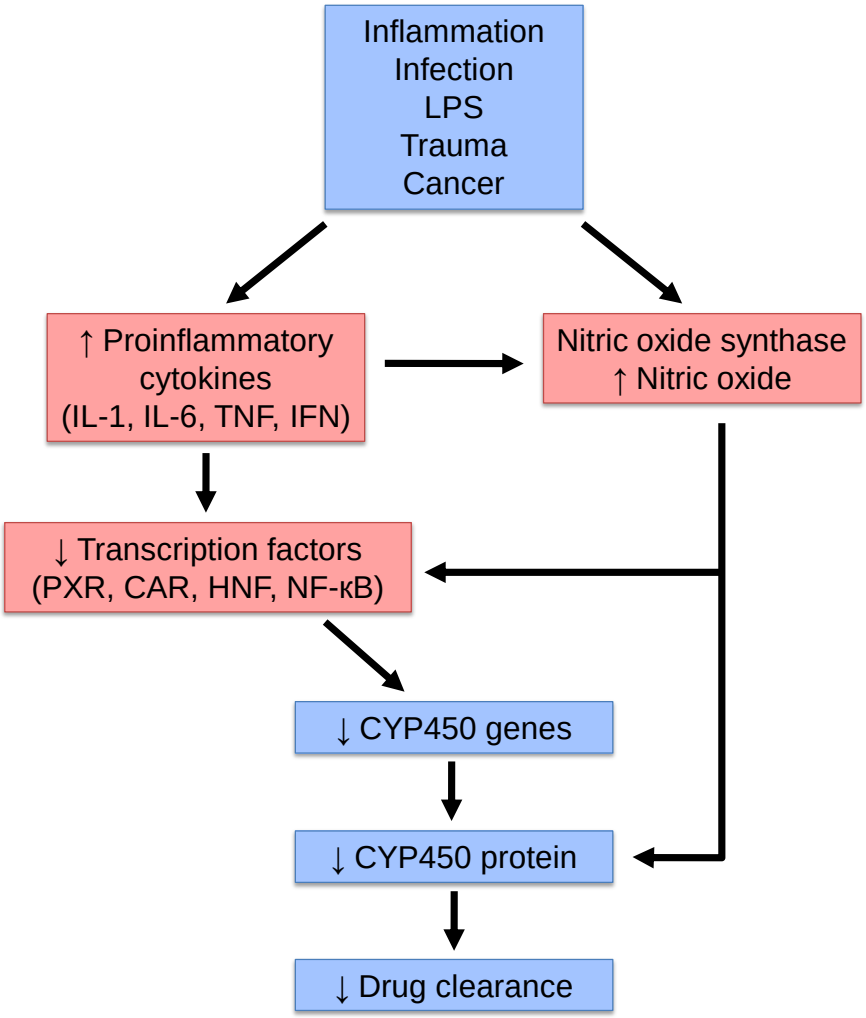

Figure 1.2. ***The proposed downregulation of CYP450 enzymes during inflammation and infection.*** Modified from Renton (2004). ↑ = increased ↓ = decreased.

A recent study carried out in human hepatocyte culture *in vitro* has reported that cytokines were able to down-regulate drug metabolising enzyme mRNA expression (Aitken et al. 2007). The major inflammatory cytokines that regulate hepatic CYP expression are IL-1, IL-6, TNF-α and IFN-γ, and they have been previously reviewed extensively (Morgan 1997; Renton 2004; Morgan et al. 2008; Lee et al. 2010). It has been suggested that cytokines down-regulate CYP gene expression through the decrease in expression of transcription factors such as CAR (Pascussi et al. 2000; Chen et al. 2009). Another possible pathway is due to the inflammatory induction of the inducible nitric oxide synthase in hepatocytes which then generates nitric oxide (Nussler et al. 1992). Nitric oxide can down-regulate CYP at both the gene and protein level (Morgan et al. 2002).

A summary of the clinical studies that have investigated the effect of inflammation on drug metabolism is shown in Table 1.9 (Vet et al. 2011).

Inflammation mechanism	Drug	CYP450 involved	Effect [a]	Correlate [b]	Reference
Healthy					
LPS	Antipyrine, theophylline hexobarbital	Overall	↓ 22-35%	TNF-α, IL-6	(Shedlofsky et al. 1994; Shedlofsky et al. 1997)
	Chlorzoxazone	2E1	↔	ND	(Poloyac et al. 1999)
IL-10	Midazolam	3A	↓ 12%	IL-10	(Gorski et al. 2000)
	Tolbutamide	2C9	↔	ND	
	Caffeine	1A2	↔	ND	
	Dextromethorphan	2D6	↔	ND	
Influenza vaccine	Erythromycin breath test	3A4	↓ 4%	IFN-γ	(Hayney et al. 2003)
Inflammatory disease					
Elective surgery	Erythromycin breath test	3A4	↓ 20-60%	IL-6	(Haas et al. 2003)
Allogeneic bone marrow transplantation	Cyclosporine	3A4	↓	IL-6, CRP	(Chen et al. 1994)
HIV	Midazolam	3A	↓ 18%	TNF-α	(Jones et al. 2010)
	Dextromethorphan	2D6	↓ 90%	ND	
	Midazolam	3A	↓ 50%	ND	(Jetter et al. 2010)
	Dextromethorphan	2D6	↔	ND	
Hepatitis C	Lidocaine	3A4	↓ 60-70%	ND	(Giannini et al. 2003)
Rheumatoid arthritis	Verapamil	3A4, 1A2	↓	IL-6	(Mayo et al. 2000)
Infection	Clozapine	1A2	↓	ND	(Haack et al. 2003)
Congestive heart failure	Caffeine	1A2	↓	IL-6, TNF-α	(Frye et al. 2002)
	S-Mephenytoin	2C19	↓	IL-6, TNF-α	
	Chlorzoxazone	2E1	↔	ND	
Cancer [c]	Erythromycin breath test	3A	↓ 30%	CRP, IL-6	(Rivory et al. 2002)
	Erythromycin breath test	3A	↓	AAG	(Baker et al. 2004)
	Midazolam	3A	↓	Ferritin	(Alexandre et al. 2007)
	Tolbutamide	2C9	↔	ND	(Shord et al. 2008)
	Omeprazole	2C19	↓	ND	(Williams et al. 2000)

Table 1.9. ***A summary of the clinical studies demonstrating the effect of inflammation on CYP450 drug metabolism in adults.*** Adapted from Vet et al (2011). [a] ↓ = decrease in CYP450 activity, ↔ = no change in CYP450 activity. [b] the relationship between inflammatory markers and CYP450. [c] solid tumour of multiple type. ND = not determined in the reported study.

The inflammatory response in disease is associated with changes in activity of a number of CYP enzymes, in particular CYP3A4. It was observed that higher plasma concentrations of TNF-α were significantly associated with lower CYP3A4 activity, and to a lesser extent, decreased CYP2D6 activity in HIV-infected patients (Jones et al. 2010). In addition elevated CRP has been associated with low CYP3A4 activity in cancer patients (Rivory et al. 2002).

However, relatively little is known about whether inflammation is associated with altered CYP2C19 activity, although Frye *et al* demonstrated that there was an inverse relationship between CYP2C19 activity and plasma concentrations of TNF-α (R_S = -0.61, $P < 0.05$) and IL-6 (R_S = -0.63, $P < 0.01$) in patients with congestive heart disease (Frye et al. 2002).

Relatively little is known about the transcription factors that control *CYP2C19* expression. However, recent reports suggest that the promoter region of the gene contains putative sites for a number of transcription factor binding sites including the nuclear receptor, constitutive androstane receptor (CAR) and GATA-4 (Chen et al. 2003; Mwinyi et al. 2010b). CAR is constitutively active and activation will increase its cytosol translocation into the nucleus (Waxman 1999). The presence of a CAR binding site in the promoter region suggests that these receptors may constitutively up-regulate *CYP2C19* and possibly response to drugs (Gerbal-Chaloin et al. 2001; Raucy et al. 2002; Urquhart et al. 2007). Interestingly, the proinflammatory cytokine IL-6, has been shown to negatively regulate *CAR* mRNA expression in human hepatocyte culture (Pascussi et al. 2000). Therefore it may be possible that cytokines such as IL-6 are able to decrease the basal expression of *CYP2C19* via CAR.

1.3.1 Cancer-associated inflammation

It has long been recognised that there is a high level of inflammation in cancer. The first association was made following the discovery of leucocytes present in neoplastic tissues by Rudolf Virchow in 1863 (Balkwill et al. 2001). It is estimated that 15-20% of all cancer deaths are due to underlying infection and inflammation (Wingo et al. 1999; Aggarwal et al. 2009; Balkwill et al. 2010). The relationship between inflammation and cancer is very complex and involves both an intrinsic and extrinsic pathway (Mantovani et al. 2008). Oncogenes can be intrinsically activated by genetic mutations such as point mutation,

chromosomal rearrangement or amplification, or inactivation of tumour-suppressor genes (Hanahan et al. 2000). Conversely, the extrinsic pathway includes infection and inflammatory events which may augment the risk of developing cancer. These two pathways both activate certain transcription factors in the tumour cells which in turn produce chemokines/cytokines and promoting further inflammation and development of cancer as illustrated in Figure 1.3 (Mantovani et al. 2008).

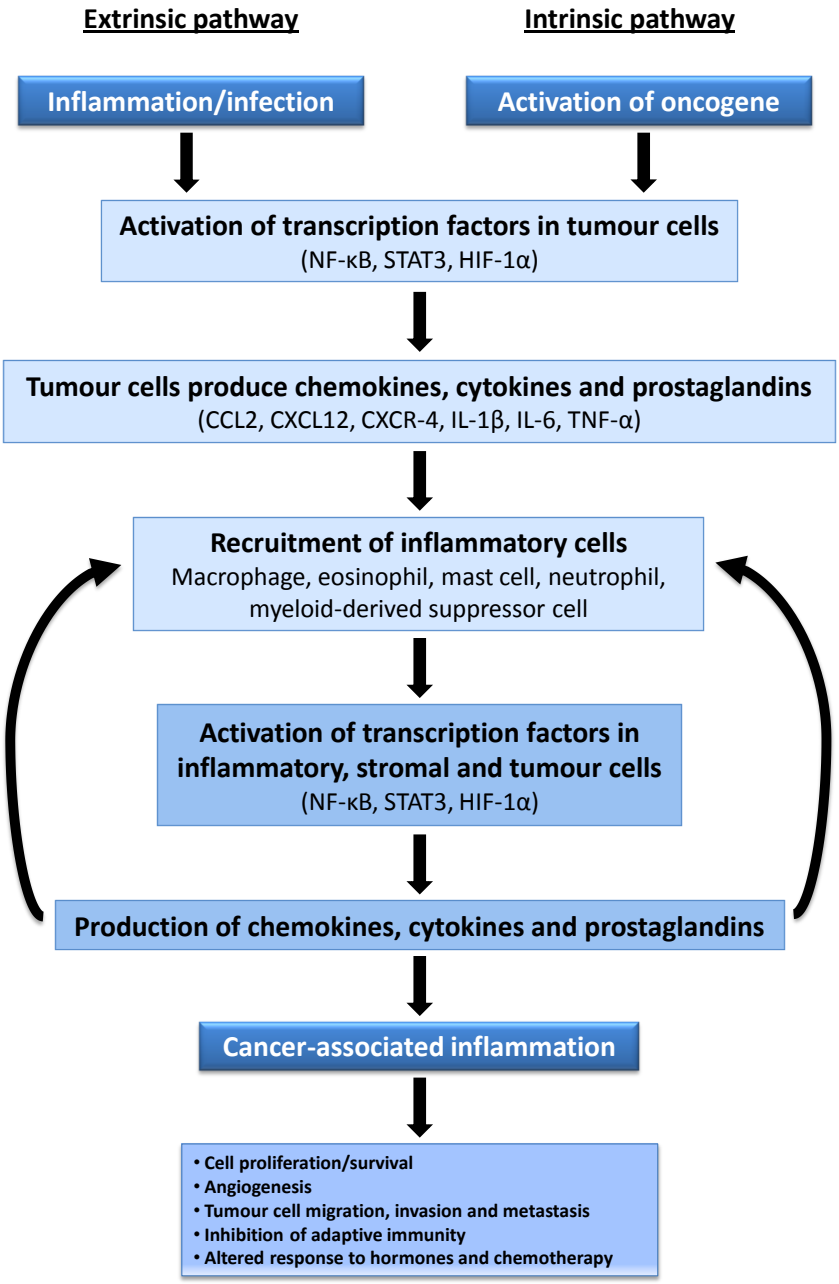

Figure 1.3. ***The complex relationship between cancer and inflammation.*** Adapted from Manotovani et al. (2008). The intrinsic and extrinsic pathways of cancer lead to activation of transcription factors which causes the tumour cells to produce an inflammatory response that becomes a feedback loop to elicit further inflammation.

Inflammatory markers such as cytokines and C-reactive protein (CRP) are known to be elevated in cancer patients (Table 1.10).

Type of cancer	Healthy	Cancer	*P-value*	Reference
Gastrointestinal[c]	*n = 15*	*n = 20*		
IL-1β	0.98 ± 0.22	7.12 ± 1.30		
IL-6	6.98 ± 1.96	17.47 ± 2.37	< 0.01	(Dülger et al. 2004)[a]
TNF-α	7.31 ± 0.94	17.12 ± 3.14		
CRP	8.75 ± 0.84	36.63 ± 2.06		
Oral cavity	*n = 15*	*n = 42*		
IL-1β	30.5 ± 4.7	123.3 ± 93.2		
IL-6	10.3 ± 5.1	79.6 ± 42.1	< 0.01	(Jablonska et al. 1997)[b]
TNF-α	12.7 ± 4.9	45.8 ± 37.0		
CRP	0.31 ± 0.26	3.2 ± 2.1		
Multiple myeloma	*n = 15*	*n = 14*		
IL-1β	4.3 ± 0.2	10.1 ± 2.8		
IL-6	4.4 ± 0.2	11.4 ± 3.2	< 0.0001	(Kuku et al. 2005)[b]
TNF-α	6.3 ± 1.3	18.6 ± 3.7		
CRP	0.41 ± 0.15	5.0 ± 1.9		
Ovarian	*n = 30*	*n = 20*		
IL-1α	28 ± 6	274 ± 27		
IL-1β	318 ± 40	779 ± 51	< 0.001	(Macciò et al. 1998)[a]
IL-6	11 ± 2	125 ± 10		
TNF-α	0	29 ± 15		
CRP	0.4 ± 0.2	70 ± 5		
Melanoma	*n = 378*	*n = 179*		
IL-1α	13.1 ± 3.2	174.4 ± 92.0		
IL-1β	40.2 ± 8.9	405.7 ± 97.0	< 0.01	(Yurkovetsky et al. 2007)[a]
IL-6	22.8 ± 7.0	1045 ± 287.6		
TNF-α	34.3 ± 11.5	1041 ± 308.6		
Head and neck	*n = 29*	*n = 24*		
IL-1β	31.5 ± 13.2	41.2 ± 26.4		
IL-6	20.0 ± 11.6	43.6 ± 17.8	NS	(Hathaway et al. 2005)[a]
TNF-α	8.4 ± 2.6	9.7 ± 5.3		
IFN-γ	2.1 ± 0.7	2.2 ± 0.5		
Prostate[c]	*n = 19*	*n = 9*		
IL-6	1.53 ± 0.30	93.15 ± 48.05	< 0.05	(Adler et al. 1999)[a]
TNF-α	3.36 ± 0.19	4.34 ± 0.46		
TGF-β	8.83 ± 1.18	15.25 ± 3.23		

Table 1.10. ***The inflammatory cytokine concentrations in healthy individuals versus cancer patients with various types of cancer.*** All units are pg/mL except for TGF-β (ng/mL) and CRP (mg/L). [a] reported as mean ± SE and [b] reported as mean ± SD. [c] The inflammatory cytokine concentrations were reported for gastrointestinal cancer patients who were non-cachectic and prostate cancer patients with bone metastases. NS = not significant due to small sample size.

Hence, elevated levels of cytokines and/or CRP in cancer may cause the discordance seen between *CYP2C19* genotype and phenotype (Williams et al. 2000) and the low activity of CYP3A4 (Rivory et al. 2002). This could be a potential extrinsic disease-associated factor that influences the large variability observed in the pharmacokinetics of many anticancer drugs. Due to the narrow therapeutic index of many anticancer drugs, disease itself may influence both therapeutic activity and toxicity of these important agents.

1.4 Objectives of this thesis

The objectives of this thesis were to investigate the reported discordance observed between CYP2C19 genotype and phenotype in a cancer population and to elucidate the potential mechanisms in this discordance, including the inflammatory status of the patients.

1.5 Thesis organisation

Chapter two describes the general methods and the data analysis used in the thesis.

Chapter three reports an investigation into the reported discordance observed between the CYP2C19 genotype and phenotype in a cancer population. This clinical study is the first to examine the relationship between the inflammatory status and CYP2C19 metabolic activity of cancer patients with advanced incurable (terminal) cancer.

Chapter four describes further examination into the CYP2C19 genotype-phenotype discordance in cancer patients at earlier stages of disease progression who are on active first-line chemotherapy. This clinical study includes patients with stage IV or resected (no evaluable disease) colorectal cancer. Potential factors that may be associated with this discordance, such as inflammatory markers, nutritional status, tumour burden and chemotherapy were also investigated.

Chapter five reports experiments to explore the possible mechanism of nitric oxide as an inflammatory mediator on CYP2C19 activity and expression in an *in vitro* system.

Chapter six describes experiments to determine whether variable drug-protein binding may affect the apparent drug metabolic ratio of each patient.

Chapter seven presents the final discussion and conclusion of the current study, as well as proposing potential future studies arising from the work in this thesis.

CHAPTER 2

Methods

2.1 Introduction

The methods and data analysis used in this thesis are described in this chapter.

2.2 Determination of *CYP2C19* genotype

2.2.1 DNA extraction from whole blood

In order to determine the *CYP2C19* genotype of the individuals, DNA was initially obtained from whole blood.

Blood (8.5 mL) was collected in PAXgene Blood DNA tubes (Qiagen, Hilden, Germany) and was stored at -20°C prior to the DNA extraction process. DNA was extracted from the blood sample using the PAXgene Blood DNA kit (Qiagen, Hilden, Germany). All the contents from the PAXgene blood tube were poured into the processing tube containing 25 mL Buffer BG1 for blood lysis and were mixed by inverting 5 times. The tube was centrifuged (2500 *g*, 5 minutes) and the supernatant was discarded. Buffer BG2 (5 mL) was added to wash the pellet by vortexing vigorously for 5 seconds, with subsequent centrifugation (2500 *g*, 3 minutes) and removal of supernatant. The pellet was resuspended in Buffer BG3 (5 mL) and PreAnalytiX protease (1% v/v) for protein digestion by vortexing for 20 seconds at high speed until the pellet was completely redissolved and then heated at 65°C for 10 minutes. The mixture was vortexed again for 5 seconds at high speed prior to the addition of isopropanol (100%; 5 mL) to precipitate DNA by inverting at least 20 times followed by centrifugation (2500 *g*, 3 minutes). The supernatant was discarded and DNA was dried by inversion for 1 minute. The DNA pellet was washed in ethanol (70% v/v; 5 mL), vortexed briefly and centrifuged (2500 *g*, 3 minutes) and the supernatant discarded. To prevent ethanol contamination in the downstream analysis, the tube was inverted and air-dried for at least 10 minutes. The DNA pellet was finally dissolved in Buffer BG4 (1 mL) and incubated at 65°C for 1 hour followed by incubation overnight at room temperature to allow complete resuspension.

The yield of genomic DNA extracted from the blood samples were determined by UV spectrophotometry using a NanoDrop spectrophotometer (ND 1000, Nanodrop Technology Inc.) according to the manufacturer's instructions. To initiate the spectrophotometer, sterile water (2 µL) was applied to the measurement pedestal followed by Buffer BG4 (2 µL) as a blank. Subsequently, genomic DNA samples (2 µL) were placed on the measurement pedestal and absorbance readings were measured at 260 and 280 nm. Pure DNA has an absorbance ratio (260/280 nm) of 1.7 to 1.9. The concentration of DNA was calculated from the absorbance reading at 260 nm when one optical density (OD) unit equates to 50 ng/µL of double-stranded DNA (Stephenson 2003) and is summarised as follows.

$$\text{DNA concentration (ng/µL)} = OD_{260} \times 50 \text{ ng/µL}$$

DNA samples were stored at -20°C until further analysis.

2.2.2 Polymerase chain reaction (PCR)

The *CYP2C19*2, CYP2C19*3* and *CYP2C19*17* status in the DNA samples was determined by PCR-RFLP to classify individuals as extensive (at least one or more *CYP2C19*1* or *CYP2C19*17* allele) or poor metabolisers (two of *CYP2C19*2* and/or *CYP2C19*3* alleles).

2.2.2.1 *CYP2C19* primers

The *CYP2C19* forward and reverse primers are listed in Table 2.1 and were obtained from Invitrogen Life Technologies, New Zealand. All primer sequences were as quoted from reported literature (Goldstein et al. 1996; Sim et al. 2006; Baldwin et al. 2008).

Gene		Primer sequence
*CYP2C19*2*	forward	5'-CAGAGCTTGGCATATTGTATC
	reverse	5'-GTAAACACAAAACTAGTCAATG
*CYP2C19*3*	forward	5'-AAATTGTTTCCAATCATTTAGCT
	reverse	5'-ACTTCAGGGCTTGGTCAATA
*CYP2C19*17*		
part 1	forward	5'-GCCCTTAGCACCAAATTCTC
	reverse	5'-ATTTAACCCCCTAAAAAAACACG
part 2	forward	5'-AAATTTGTGTCTTCTGTTCTCAATG
	reverse	5'-AGACCCTGGGAGAACAGGAC

Table 2.1. ***The primer sequence of the CYP2C19 alleles studied in this thesis (CYP2C19*2, CYP2C19*3 and CYP2C19*17).***

All primers were reconstituted in sterile nuclease-free water to a stock solution concentration of 100 µM and were further diluted with sterile nuclease-free water to a working solution of 10 µM. All primers and PCR reagents were stored at -20°C prior to use.

2.2.2.2 *CYP2C19*2* and *CYP2C19*3* amplification

All PCR preparation steps were carried out in a sterile area (CleanSpot PCR/UV work station, Coy Laboratory Products, MI, USA). The PCR master mix (Qiagen, New Zealand) comprised 2.5 units of *Taq* DNA polymerase, 10x Qiagen PCR buffer containing 1.5 mM $MgCl_2$ and 200 µM of each dNTP's. For each reaction, PCR master mix (25 µL), forward primer (10 µM; 2 µL), reverse primer (10 µM; 2 µL), 18 µL nuclease-free water and DNA template (100 ng/µL; 3 µL) were added to a sterile PCR microcentrifuge tube. All amplification reactions were carried out in a thermal cycler (Techne Cambridge Ltd, Cambridge, UK).

The amplification conditions, as described by Goldstein and Blasidell (1996) were: one 5 minute cycle at 94°C for initial denaturation, followed by 37 cycles of 20 seconds duration at 94°C for denaturation, 53°C for 10 seconds for annealing and 72°C for 10 seconds for extension and lastly 1 cycle of 72°C for 5 minutes for final extension.

*2.2.2.3 CYP2C19*17* amplification

The amplification process for *CYP2C19*17* consists of two parts as previously reported (Sim et al. 2006; Baldwin et al. 2008). In the initial amplification process, PCR master mix (25 µL), forward primer (10 µM; 2 µL), reverse primer (10 µM, 2 µL), nuclease-free water (19 µL) and DNA template (100 ng/µL; 2 µL) were added to a sterile PCR microcentrifuge tube. The amplification conditions comprised 35 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 53°C for 30 seconds and extension at 72°C for 30 seconds. An initial denaturation step at 95°C for 1 minute and a final extension step at 72°C for 7 minutes were also performed.

In the second part of the amplification process, PCR master mix (25 µL), forward primer (10 µM; 1.25 µL), reverse primer (10 µM, 1.25 µL), nuclease-free water (20.5 µL) and PCR amplicon from above (100 ng/µL; 2 µL) were added to a sterile PCR microcentrifuge tube. The initial denaturation was allowed to proceed at 95°C for 1 minute, then 25 cycles are performed with denaturation at 95°C for 30 seconds, annealing at 51°C for 30 seconds and extension at 72°C for 30 seconds. The products were then extended for an additional 7 minutes at 72°C.

2.2.3 Restriction fragment length polymorphism (RFLP)

Following *CYP2C19* gene amplification as described in section 2.2.2, the amplicon was subjected to RFLP analysis to determine the *CYP2C19* genotype of the samples. All restriction enzymes and associated NEB buffer were supplied by New England BioLabs Inc, Massachusetts, USA. Agarose was obtained from BDH Chemicals, VWR International Limited, Lutterworth, United Kingdom. Other reagents and chemicals used were of analytical grade. A heterozygote control, which has been previously sequenced to confirm its heterozygosity, was included. Other controls included ones with or without DNA, and with or without restriction enzyme to ensure that there was no contamination of DNA or incomplete digestion by the restriction enzyme.

*2.2.3.1 CYP2C19*2*

The *CYP2C19*2* RFLP analysis was as described previously (Goldstein et al. 1996). The PCR product (20 µL) was incubated with *Sma*I (10,000 U/mL; 1 µL), NEBuffer4 (1x v/v; 2.5 µL) and nuclease-free water (1.5 µL) overnight at room temperature.

*2.2.3.2 CYP2C19*3*

The *CYP2C19*3* RFLP analysis was as described previously (Goldstein et al. 1996). The PCR product (20 µL) was incubated with *Bam*HI (20 U/µL; 1 µL), NEBuffer3 (10x v/v; 3 µL), BSA (0.3 µL) and nuclease-free water (5.7 µL) for 16 hours at 37°C.

*2.2.3.3 CYP2C19*17*

The *CYP2C19*17* RFLP analysis was as described previously (Baldwin et al. 2008). The PCR product (20 µL) was incubated with *Nsi*I (20 U/µL; 1 µL), NEBuffer3 (10x v/v; 2.5 µL) and nuclease-free water (1.5 µL) for 16 hours at 37°C.

2.2.3.4 RFLP analysis: gel electrophoresis

The *CYP2C19*2* and *CYP2C19*17* RFLP reactions were inactivated by heat (65°C and 80°C respectively for 20 minutes). *CYP2C19*3* RFLP reaction was inactivated by adding a stop buffer (6 µL) comprising 50% v/v glycerol, 50 mM EDTA pH 8, 0.05% w/v Orange G. *CYP2C19*2* and *CYP2C19*3* digests were analysed on a 3% agarose gel whereas the *CYP2C19*17* digest was analysed on a 2% agarose gel. *CYP2C19*2* and *CYP2C19*17* RFLP products (10 µL) were mixed with 6x gel loading dye (30% v/v glycerol, 0.25% w/v xylene cyanol, 0.25% w/v bromophenol blue; 2 µL) and loaded onto the gel. *CYP2C19*3* RFLP product with the Orange G stop buffer (15 µL) was loaded directly onto the gel. Gel electrophoresis was carried out in Tris-acetate-EDTA buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0; TAE; 1x v/v) at 100V for 60-90 minutes. The RFLP products were stained with ethidium bromide (0.5 µg/mL in 1x TAE buffer) for 45 minutes and visualised by UV illumination (BioRad Gel imager, BioRad laboratories,

CA, USA). The images were captured and recorded by the Quantity One 4.3.1 Gel Doc software application.

The *CYP2C19*2* mutation (c.681 G>A) results in the loss of the *Sma*I restriction enzyme cutting site. Hence, digestion of a homozygous wild-type (*CYP2C19*1/*1*) sample results in products at 212 and 109 bp, a heterozygous (*CYP2C19*1/*2*) sample will have products at 321, 212 and 109 bp and a homozygous variant (*CYP2C19*2/*2*) will result in a single undigested product at 321 bp.

The *CYP2C19*3* mutation (c.636 G>A) results in the loss of the *Bam*HI restriction enzyme cutting site. Hence, digestion of a homozygous wild-type (*CYP2C19*1/*1*) sample will result in products at 175 and 96 bp, a heterozygous (*CYP2C19*1/*3*) sample will have products at 271, 175 and 96 bp and a homozygous variant (*CYP2C19*3/*3*) will result in a single undigested product at 271 bp.

The *CYP2C19*17* mutation (c.991 A>G) results in the loss of the *Nsi*I restriction enzyme cutting site. Hence, digestion of a homozygous wild-type (*CYP2C19*1/*1*) sample results in a single product at 116 bp, a heterozygous (*CYP2C19*1/*17*) sample will have products at 143 and 116 bp and a homozygous variant (*CYP2C19*17/*17*) will result in a single undigested product at 143 bp.

2.3 Quantification and analysis of omeprazole and 5'hydroxy omeprazole metabolite levels in plasma

In order to determine the CYP2C19 phenotype, omeprazole (20 mg po) was given to individuals and a 2 hour post-dose blood sample was obtained. The concentrations of omeprazole and its 5'hydroxy omeprazole metabolite were determined using the validated assay for omeprazole and 5'hydroxy omeprazole as reported previously (Woolf et al. 1998; Motevalian et al. 1999; Orlando et al. 2003) and was utilised with minor modifications as described below. The reported precision and accuracy of the assay are shown in Table 2.2 (Motevalian et al. 1999).

Analyte	Intra-assay, %CV	Inter-assay , %CV	Minimum detectable concentration, ng/mL
Omeprazole	5.1	4.7	10
5'hydroxy omeprazole	1.5	1.6	15

Table 2.2. ***The reported precision and accuracy of the quantification and analysis of omeprazole and 5'hydroxy omeprazole metabolite levels in plasma (Motevalian et al. 1999).***

Omeprazole and phenacetin were purchased from Sigma-Aldrich, Missouri, USA. The 5'hydroxy omeprazole metabolite was kindly gifted by Dr. Kjell Andersson, AstraZeneca, Mölndal, Sweden. All other chemicals and solvents were of analytical grade.

2.3.1 Preparation of drug standards

Omeprazole and 5'hydroxy omeprazole standards (100 µg/mL) were prepared in alkalinised methanol (0.1% triethylamine) and were further diluted (10 µg/mL) in alkalinised methanol prior to use. The internal standard, phenacetin (1 µg/mL), was prepared in 100% methanol and further dilution was made in alkalinised MilliQ water (0.5% triethylamine, 10 µg/mL). All standards were stored at 4°C prior to use.

2.3.2 Preparation of plasma from whole blood

Blood was collected in a heparinised tube and centrifuged at 1500 *g* for 10 minutes at 4°C. The top plasma layer was aspirated into a clean tube and stored at -20°C until further extraction.

2.3.3 Calibration curve and solid phase extraction

Blank plasma (1 mL) obtained from the NZ Blood Service was spiked with omeprazole and 5'hydroxy omeprazole (0 to 400 ng/mL) and internal standard (phenacetin; 100 µL of 10 µg/mL) as calibration standards. Frozen plasma samples that were prepared from a 2 hour post-dose blood sample as described in section 2.3.2 (1 mL) were allowed to thaw at room temperature, mixed by inversion and then centrifuged (2500 *g*, 5 minutes). Internal standard was added (phenacetin, 100 µL of 10 µg/mL) and the sample was

vortexed for 1 minute. C18 solid phase extraction cartridges (Prevail C18, Alltech) were pre-conditioned by washing twice with 100% methanol (1 mL) and twice with MilliQ water (1 mL). The calibration standards and plasma samples were loaded and passed through the cartridges under vacuum. The cartridges were then washed with MilliQ water (1 mL) twice and once with 10% methanol in water (1 mL). The samples were then eluted with acetonitrile (1 mL) and the eluent was evaporated under vacuum to approximately 100 µL.

2.3.4 HPLC conditions

HPLC analysis of omeprazole and 5'hydroxy-omeprazole was carried out using an Agilent 1200 system with a Zorbax SB-C18 5µ (3.0 × 150 mm) column. The mobile phase consisted of solvent A: 50 mM sodium dihydrogen phosphate (pH 6.0) and solvent B: 90% acetonitrile in methanol at a flow rate of 0.5 mL/min. The initial conditions were 75% solvent A and 25% solvent B. After 5 minutes, solvent B increased linearly to 50% and the flow rate increased linearly to 0.7 mL/min over a period of 2 minutes and was held constant until 15 minutes. Conditions were returned to 25% solvent B and flow rate of 0.5 mL/min by 25 minutes. The total run time was 25 minutes with 5 minutes post-time. Absorbance was monitored with a diode array detector, using a signal of 245 and 302 nm, with a bandwidth of 4 nm. The reference signal was 550 nm with a bandwidth of 50 nm. The retention times of omeprazole, 5'hydroxy omeprazole and phenacetin were 9.9, 5.1 and 7.6 minutes respectively. An example chromatogram and spectrum is shown in Figure 2.1.

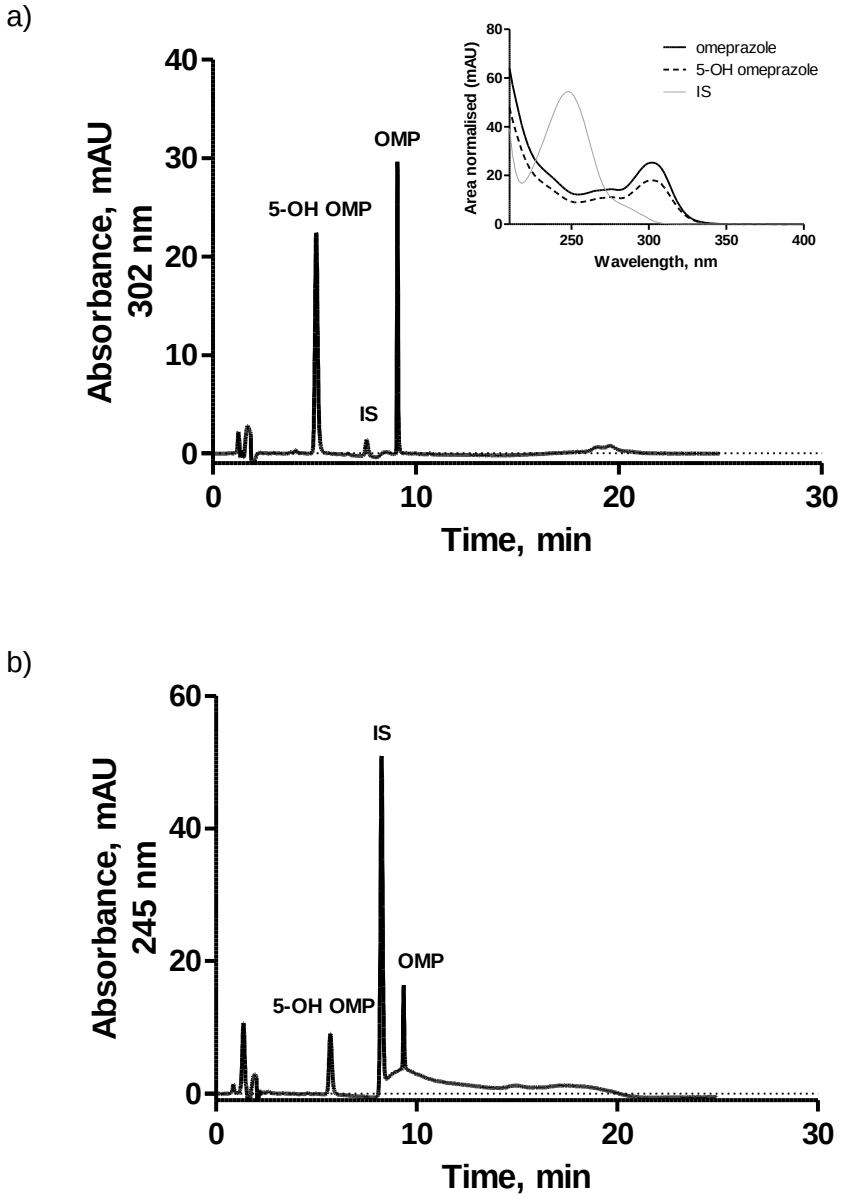

Figure 2.1. ***A representative HPLC chromatogram showing the elution of omeprazole (OMP; Rt 9.9 min), 5-hydroxy omeprazole (5-OH OMP; Rt 5.1 min) and internal standard (IS, phenacetin; Rt 7.6 min*****) at wavelengths of a) 302 nm and b) 254 nm.** Inset shows the UV spectra of omeprazole, 5'hydroxy omeprazole and the internal standard.

Typical calibration curves are shown in Figure 2.2. The linearity was $r^2 = 0.99$ and the lowest concentration of both omeprazole and 5'hydroxy omeprazole determined on the calibration curve was 10 ng/mL.

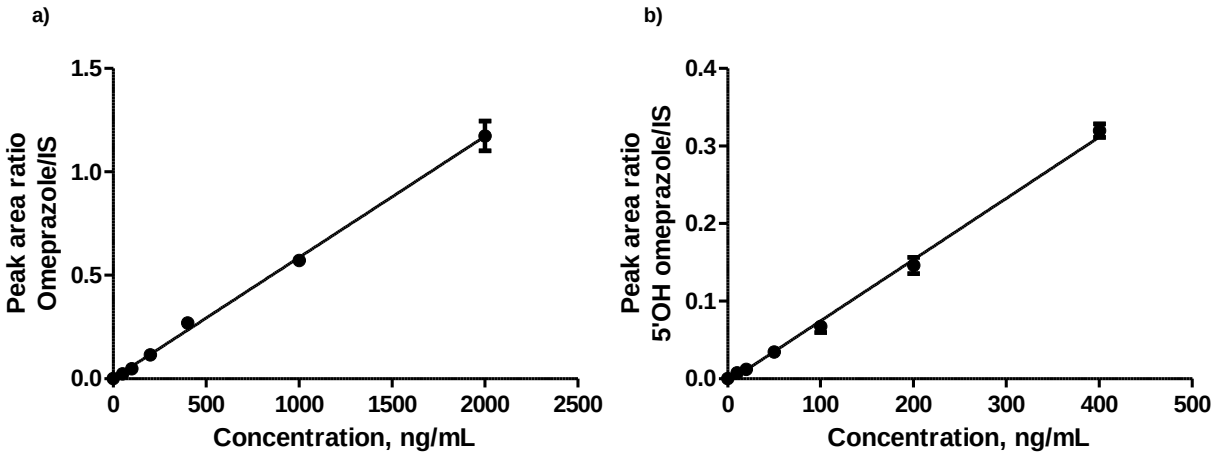

Figure 2.2. ***A typical calibration curve of a) omeprazole*** (0-2000 ng/mL; y = 0.0006x - 0.002; r^2 = 0.99)***.and b) 5'hydroxy omeprazole*** (0-400 ng/mL; y = 0.0008x - 0.005; r^2 = 0.99) ***in triplicate determinations of spiked human plasma.***

The $\log_{10}$ hydroxylation index of omeprazole has been widely reported as a measure of CYP2C19 activity in healthy subjects (Balian *et al.* 1995). The hydroxylation index (HI) was calculated as follows: omeprazole and 5'hydroxy omeprazole concentration were determined from the calibration curves (Figure 2.2) and the ratio of 5'hydroxy omeprazole to omeprazole determined. The $\log_{10}$ of this value was then calculated.

2.4 Cytokine determination

Inflammatory cytokine (IL-1α, IL-1β, IL-6, TNF-α, IFN-γ and TGF-β) concentrations were determined in the serum samples using the commercial Milliplex kit (Millipore Corporation, MA, USA). Assay validation was carried out by the manufacturer with the reported accuracy and precision shown in Table 2.3.

Cytokine	Accuracy, % recovery	Intra-assay, % CV	Inter-assay, % CV	Minimum detectable concentration, pg/mL
IL-1α	103.0	8.2	16.8	3.5
IL-1β	100.1	6.1	7.0	0.4
IL-6	100.0	8.1	11.6	0.3
TNF-α	98.7	10.5	15.9	0.1
IFN-γ	99.2	4.6	5.8	0.1
TGF-β1	91	6	10	10

Table 2.3. ***The reported accuracy and precision of the quantification of cytokines in the commercial Milliplex kit.***

2.4.1 Preparation of reagents

All reagents in the kit were brought to room temperature and mixed gently to bring all salts into solution. The antibody-immobilized bead vials for their respective cytokines were sonicated for 30 seconds and then vortexed for 1 minute. Each antibody bead vial (70 µL) was added to the mixing bottle and brought to a final volume of 3 mL with bead diluent. Both quality controls 1 and 2 were reconstituted with MilliQ water (250 µL) followed by inversion and vortexing and were allowed to sit at room temperature for 10 minutes prior to transfer to microfuge tubes. The 10x wash buffer (30 mL) was diluted to 1x v/v (300 mL) with MilliQ water. The serum matrix was reconstituted with MilliQ water (1 mL) and left at room temperature for at least 10 minutes for complete reconstitution. Prior to use, the human cytokine standard was reconstituted with MilliQ water (250 µL) to give a concentration of 10,000 pg/mL for all cytokine analytes. The standard was inverted and vortexed for 10 seconds and left at room temperature for 10 minutes before transferral into a microfuge tube. Serial dilutions of the standard were made in assay buffer to the following final concentrations: 10,000, 2000, 400, 80, 16, 3.2, 0 pg/mL.

2.4.2 Preparation of serum from whole blood

Blood was collected in a plain tube and stored at 4°C for at least 30 minutes to allow the blood to clot. Subsequent to centrifugation (1500 *g*, 10 min, 4°C), the upper serum layer was aspirated into a clean tube and stored at -20°C prior to analysis.

2.4.3 Immunoassay procedure

The supplied assay plate was set on a plate holder at all times during reagent dispensing and incubation steps to prevent leakage from the bottom of the plate. The plate was covered with the opaque plate lid and aluminium foil during all incubation steps, as antibody-immobilized beads are light sensitive.

To pre-wet the filter plate, assay buffer (200 µL) was added to each well and incubated on the plate shaker for 10 minutes at room temperature before removal by vacuum. Each standard and control (25 µL) was added to their respective wells in duplicates followed by the addition of serum matrix (25 µL) to these wells. The serum samples (25 µL) were added to the plates in duplicate followed by the addition of assay buffer (25 µL) to these wells. The pre-mixed antibody-immobilised beads (25 µL) were vortexed intermittently prior to the addition to each well. The plate was sealed, covered with the opaque lid and foil then incubated with agitation on a plate shaker overnight at 4°C.

The plate was recovered the next day and all fluid was gently removed by vacuum followed by washing twice with the wash buffer (200 µL), with removal of the wash buffer by vacuum between each wash. The detection antibodies (25 µL) were added to each well, the plate was sealed and covered followed by incubation with agitation on a plate shaker for 1 hour at room temperature. Streptavidin-phycoerythrin (25µL) was added to each well, the plate was sealed and covered then incubated with agitation at room temperature for 30 minutes. All fluid was gently removed by vacuum followed by washing twice with wash buffer as before. Sheath fluid (150 µL) was added to all wells and the beads were resuspended on a plate shaker for 5 minutes prior to reading. The plate was ran on the Luminex® system (LINCOplex, LINCO Research Inc, Texas, USA) and the median fluorescent intensity was analysed using a weighted 5-parameter logistic

curve-fitting method as recommended by the manufacturer to calculate cytokine concentrations in the samples.

2.4.4 Single plex assay – TGF-β1

Transforming growth factor Beta (TGF-β) could not be multiplexed as part of the above system as serum samples require pre-treatment prior to the assay. TGF-β concentrations were determined in a separate aliquot of serum.

2.4.4.1 Preparation of reagents

Preparation of reagents was as described previously (section 2.4.1.1) with the following exceptions. The serum matrix was reconstituted with MilliQ water (1 mL) and assay buffer (4 mL) and left at room temperature for at least 10 minutes for complete reconstitution. In a separate microfuge tube, the reconstituted serum matrix (0.1 mL) was further diluted with assay buffer (0.5 mL) to give a working stock solution of 1:30 v/v. Serial dilutions of the TGF-β1 standard (10,000 pg/mL) were made in assay buffer to the following final concentrations: 10,000, 2500, 625, 156.3, 39.1, 9.8, 0 pg/mL.

2.4.4.2 Sample pre-treatment

Initially, serum samples were centrifuged to remove debris and excess lipids. The samples (20 µL) were diluted with sample diluent (80 µL; 1:5 v/v). 8 µL of 1 M HCl was added to the diluted sample so that the pH drops below 3.0 and the mixture was incubated at room temperature for 15 minutes with agitation. The acid-treated serum samples were further diluted with assay buffer (540 µL; 1:6 v/v) immediately prior to addition to sample wells.

2.4.4.3 Immunoassay procedure

The immunoassay procedures were as described in section 2.4.3 with the exception of using the diluted 1:30 v/v serum matrix working stock solution instead of undiluted serum matrix.

2.5 Albumin determination

Serum albumin levels of the plasma samples were determined by using the colorimetric kit, QuantiChrom™ BCG Albumin Assay Kit (BioAssay Systems, CA, USA). The reported minimum detectable concentration by the manufacturer was 0.01 g/dL.

2.5.1 Preparation of standards

The supplied reagent and standard (5 g/dL bovine serum albumin) was brought to room temperature and shaken before use. A serial dilution of the standard was prepared with MilliQ water to the following final concentrations: 5, 4, 3, 2, 1.5, 1.0, 0.5 and 0 g/dL.

2.5.2 Assay procedure

Plasma samples (5 µL) were diluted 2-fold with MilliQ water. The diluted samples and standards (5 µL) were then transferred to a clear bottom 96-well plate in duplicates. The supplied reagent (200 µL) was added to each well and was mixed lightly to avoid bubble formation. The plate was incubated at room temperature for 5 minutes and the absorbance readings were read on a spectrophotometer (SpectraMAX plus, Molecular Devices, Victoria, Australia) at the peak absorbance of 620 nm.

2.5.3 Data analysis

The calibration curve was a hyperbola and is shown in Figure 2.3. The equation was $y = 2.12x / (5.98 + x)$ and the r^2 value was 0.9992.

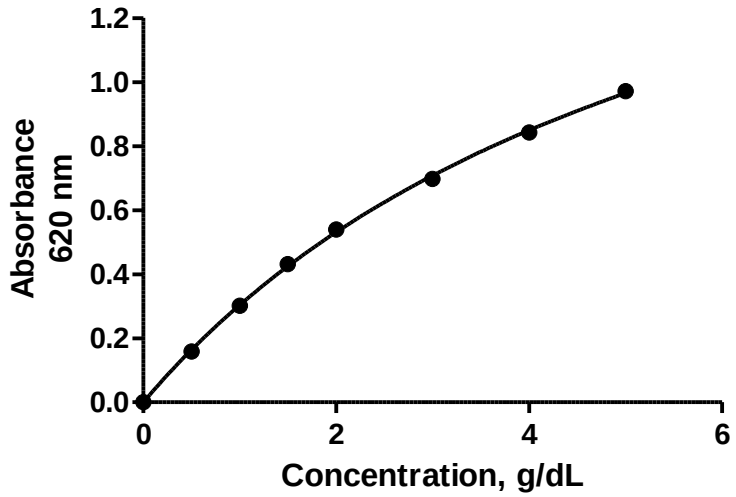

Figure 2.3. ***The calibration curve for the determination of albumin concentration using bovine serum albumin as the standard,*** $y = 2.12x / 5.983 + x$; $r^2 = 0.9992$. Values are the mean ± SD of duplicate determinations.

The average albumin concentration from the samples was calculated using the following formula:

$$\text{Albumin (g/dL)} = \frac{\Delta OD_{sample} \times b \times n}{a - \Delta OD_{sample}}$$

where $\Delta OD_{sample} = (OD_{sample} - OD_{blank})$ and n is the dilution factor; a = 2.12 and b = 5.983.

2.6 Growth hormone determination

Human growth hormone levels in the plasma samples were measured using the commercial kit, Quantikine® Human Growth Hormone Immunoassay (R&D Systems, MN, USA). Assay validation was carried out by the manufacturer with the reported accuracy and precision shown in Table 2.4.

Accuracy, % recovery	Intra-assay, % CV	Inter-assay, % CV	Minimum detectable concentration, pg/mL
108 (101-117)	2.4 – 4.0	6.9 – 9.4	2.10 (0.64 – 7.18)

Table 2.4. ***The reported accuracy and precision of the quantification of growth hormone in the commercial kit.***

2.6.1 Preparation of solutions and standards

All solutions were brought to room temperature and mixed gently to dissolve crystals before use. The wash buffer concentrate (20 mL) was diluted with MilliQ water to prepare 500 mL of wash buffer. The substrate solutions (reagents A and B) were mixed together in equal volumes 15 minutes prior to use. 200 µL of the resultant mixture of substrate was required for each well and this was protected from light. The growth hormone standard was reconstituted with MilliQ water (1 mL) to produce a stock solution of 16,000 pg/mL. The standard was allowed to sit for a minimum of 15 minutes with gentle agitation to ensure complete reconstitution. Serial dilutions of the growth hormone standard were made in the calibrator diluent (RD6-15 for plasma samples) to the following final concentrations: 1600, 800, 400, 200, 100, 50, 25 pg/mL.

2.6.2 Assay procedure

The assay diluent (RD1-57; 100 µL) was added to each well of the supplied 96-well plate followed by the addition of standards (50 µL) and plasma samples (50 µL) to respective wells in duplicates. The plate was covered by the supplied adhesive strip and incubated at room temperature for 2 hours. Each well was aspirated and washed with wash buffer (400 µL) for four times. After the last wash, the plate was inverted and blotted against

clean paper towels to ensure complete removal of wash buffer. Growth hormone conjugate (200 µL) was added to each well and the plate was covered and incubated at room temperature for 2 hours. Again, the plate was aspirated and washed with wash buffer for four times. The substrate solution mixture (200 µL) was added to each well and the plate was protected from the light and incubated at room temperature for 30 minutes. Stop solution (50 µL) was added to each well and the colour change from blue to yellow was recorded by measuring the absorbance at 450 nm and 540 nm (SpectraMAX plus, Molecular Devices, Victoria, Australia).

2.6.3 Data analysis

The individual absorbance readings were corrected for optical imperfections from the plate by subtracting readings at 540 nm from readings at 450 nm for each well. A typical calibration curve is shown in Figure 2.4 and the r^2 value was 0.9989. The mean growth hormone concentration for each individual sample was determined from the calibration curve using the following equation: $y = 0.00045x + 0.017$, $r^2 = 0.9989$.

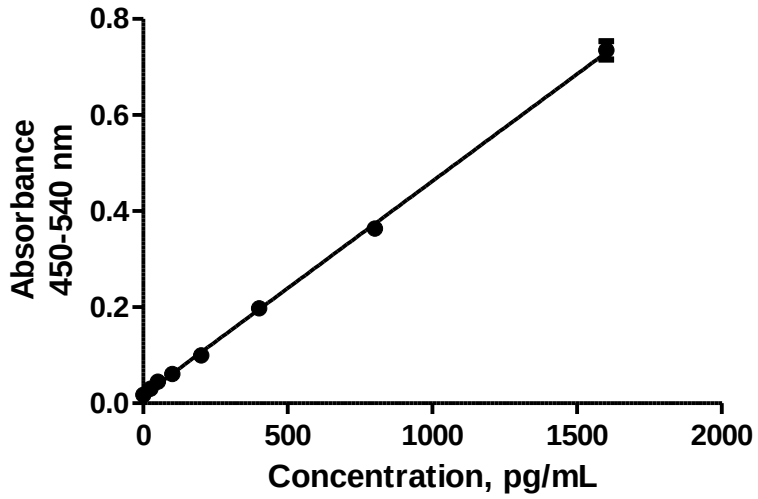

Figure 2.4. ***A typical calibration curve for the determination of human growth hormone concentration,*** $y = 0.00045x + 0.017$; $r^2 = 0.9989$. Values are the mean ± SD of triplicate determinations.

2.7 Quantification and analysis of proguanil and cycloguanil in plasma

An alternative probe drug used in this thesis to measure CYP2C19 activity was proguanil. A 3 hour post-dose blood sample was collected following an oral dose of proguanil (200 mg). The concentrations of proguanil and its metabolites were determined as reported previously (Helsby et al. 1990b) and this was validated after minor modifications as described below. Proguanil, cycloguanil, and the internal standard, chlorcycloguanil were a kind gift from Prof. SA Ward, Liverpool School of Tropical Medicine, Liverpool, UK. All other chemicals and solvents used were of analytical grade. The reported precision and accuracy of the assay are shown in Table 2.5 (Helsby 1991).

Analyte	Intra-assay, %CV	Inter-assay , %CV	Minimum detectable concentration, ng/mL
Proguanil	4.9	3.5	3
Cycloguanil	6	5.4	1

Table 2.5 ***The reported precision and accuracy of the quantification and analysis of omeprazole and 5'hydroxy omeprazole metabolite levels in plasma (Helsby 1991).***

2.7.1 Preparation of drug standards

Stock solutions of proguanil, cycloguanil and chlorcycloguanil (200 µg/mL) were prepared in 100% ethanol and were further diluted to 20 µg/mL in ethanol and stored at 4°C prior to use.

2.7.2 Calibration curve and drug extraction

Calibrated samples were prepared as follows. Blank plasma (0.5 mL) was spiked with proguanil and cycloguanil (0 – 1000 ng/mL) with MilliQ water (0.5mL) and the internal standard, chlorcycloguanil (10 µL of 20 µg/mL) added. Frozen plasma samples that were prepared from a 3 hour post-dose blood sample prepared as described in section 2.3.2 (0.5 mL) were allowed to thaw at room temperature. MilliQ water (0.5 mL) was

added to the sample, vortexed and then centrifuged (2500 *g*, 5 minutes) and the internal standard was added (chlorcycloguanil, 10 µL of 20 µg/mL). C18 solid phase extraction cartridges (Prevail C18, Alltech) were pre-conditioned by washing twice with 100% methanol (1 mL) and twice with MilliQ water (1 mL). Samples were loaded and passed through the cartridges under vacuum. The cartridges were then washed once with MilliQ water (1 mL), followed by methanol (1 mL). The samples were eluted with acidified methanol (1% perchloric acid in methanol; 1 mL) and the eluates were immediately neutralised with sodium hydroxide (0.1 M; 2 mL).

Subsequently, a liquid-liquid extraction was carried out as follows. Ethyl acetate (5 mL) was added to the neutralised eluate and mixed by rotation for 30 minutes. The sample was then centrifuged at 1800 *g* for 10 minutes and the top organic layer was aspirated into a clean tube. The bottom aqueous layer was re-extracted with ethyl acetate using the same procedure. Both the organic layers were combined into a clean tube and evaporated to dryness under vacuum. The resulting residue was resuspended in 60 µL mobile phase (50 mM ammonium acetate pH 4.5 and 100% acetonitrile, 50:50 v/v) and 50 µL was injected onto HPLC.

2.7.3 HPLC conditions

HPLC analysis was carried out using an Agilent 1200 system with an Alltech Altima C8 5µ (3.2 × 150 mm) column. The mobile phase consisted of solvent A: 50 mM ammonium acetate (pH 4.5) and solvent B: 100% acetonitrile with a flow rate of 0.5 mL/min. The initial conditions were 80% solvent A and 20% solvent B with solvent B increasing linearly to 25% by 3 minutes. Solvent B was then increased linearly to 50% at 15 minutes and returned to 20% at 20 minutes. The total run time was 20 minutes with 5 minutes for post-run equilibration. Detection was via a diode array absorbance, using a signal of 245 nm, with a bandwidth of 4 nm. The reference signal was 550 nm with a bandwidth of 50 nm. Retention times of proguanil, cycloguanil, and chlorcycloguanil were 12.4, 7.0 and 9.9 minutes respectively. An example chromatogram and spectrum is shown in Figure 2.5.

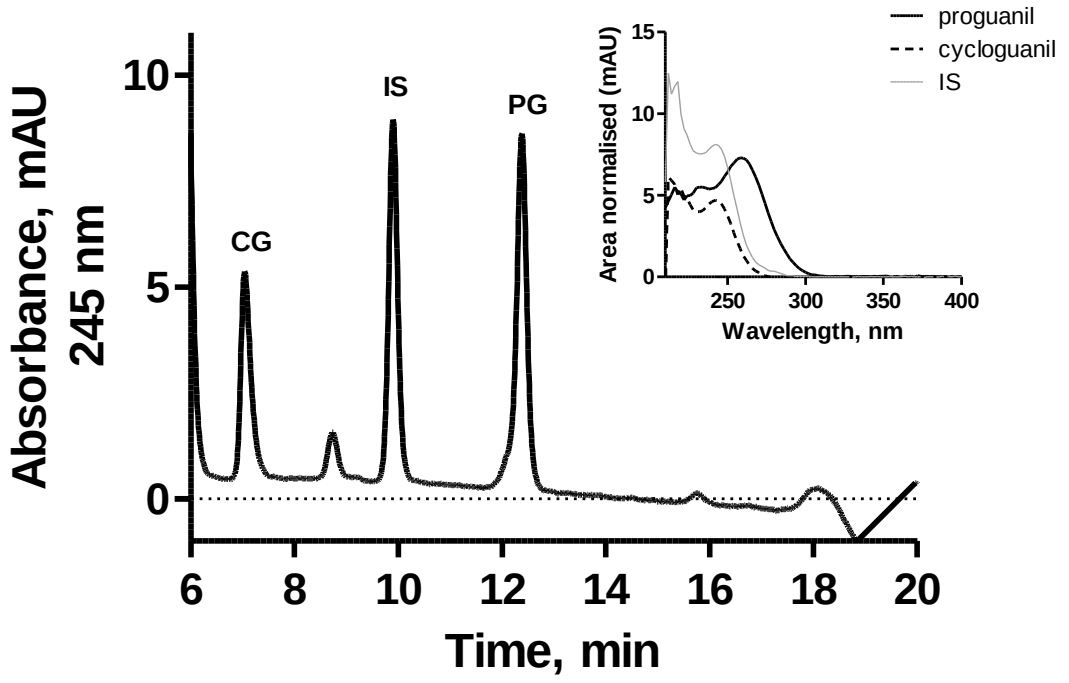

Figure 2.5. ***A representative HPLC chromatogram showing the elution of proguanil (PG; Rt 12.4 min), cycloguanil (CG; Rt 7.0 min) and internal standard (IS, chlorcycloguanil; Rt 9.9 min) at 245 nm***. Inset shows the UV spectra of proguanil, cycloguanil and internal standard.

Typical calibration curves are shown in Figure 2.6. The linearity was $r^2 = 0.99$ and the lowest concentration of both proguanil and cycloguanil determined on the calibration curve was 10 ng/mL.

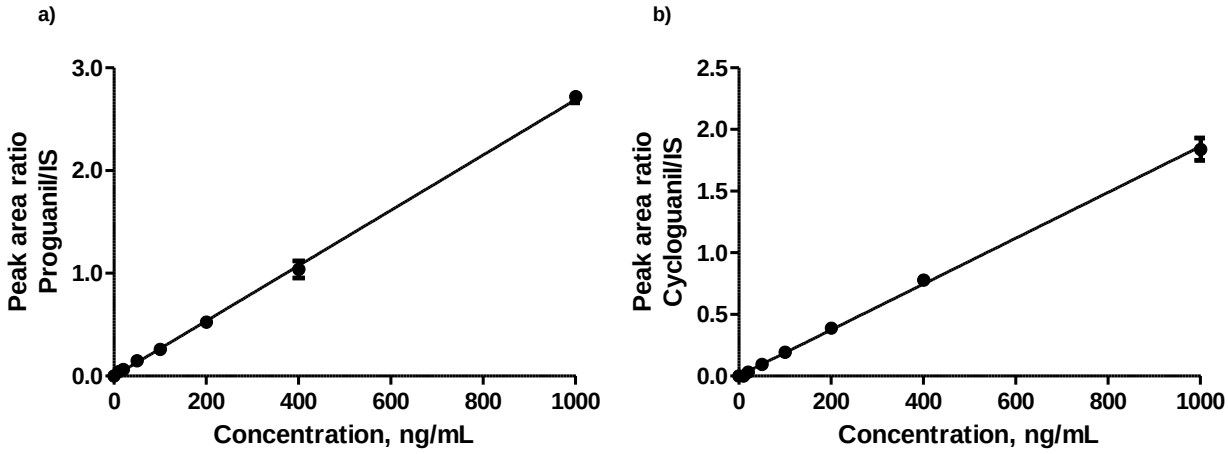

Figure 2.6. ***A typical calibration curve of a) proguanil*** (0-1000 ng/mL; y = 0.0027x - 0.001; $r^2 = 0.998$) ***and b) cycloguanil*** (0-1000 ng/mL; y = 0.0019x + 0.003; $r^2 = 0.997$) ***in triplicate determinations of spiked human plasma.***

The metabolic ratio was calculated as described previously (Ward et al. 1989b; Helsby 1991) and was as follows. Proguanil and the metabolite cycloguanil concentrations were determined from the calibration curves (Figure 2.6) and the ratio of the metabolite cycloguanil to proguanil determined.

2.8 HCT116.*CYP2C19* cell culture

A cell culture system was used as an *in vitro* system to determine the effects nitric oxide on CYP2C19 activity and expression.

α-Minimal Essential Media (α-MEM), fetal calf serum and trypsin-EDTA were purchased from Invitrogen, New Zealand. Puromycin was obtained from Sigma-Aldrich, Missouri, USA. All sterile labware were purchased from BD Biosciences, Massachusetts, USA.

The human colon carcinoma HCT116 cell-line transfected with the *CYP2C19* gene (HCT116.*CYP2C19*) and the negative control HCT116 wildtype (HCT116.WT) cell-line were kindly provided by Dr. Chris Guise and Dr. Adam Patterson (Auckland Cancer Society Research Centre, University of Auckland). HCT116 is a human colorectal carcinoma cell-line (ATCC, Virginia, USA). The transfection of the *CYP2C19* gene into the cell-line is described briefly as follows.

Plasmids encoding human *CYP2C19* cDNA were purchased from OriGene Technologies (Maryland, USA) and cloned into the Gateway compatible vector F527-V5 (Invitrogen, New Zealand). HCT116 cells were transfected using Fugene6 and pooled stable populations were selected with puromycin as previously reported (Ahn et al. 2006; Guise et al. 2010). An example map of the vector showing the relative location of the cloning site and antibiotic resistance is shown in Figure 2.7 (Guise et al. 2010).

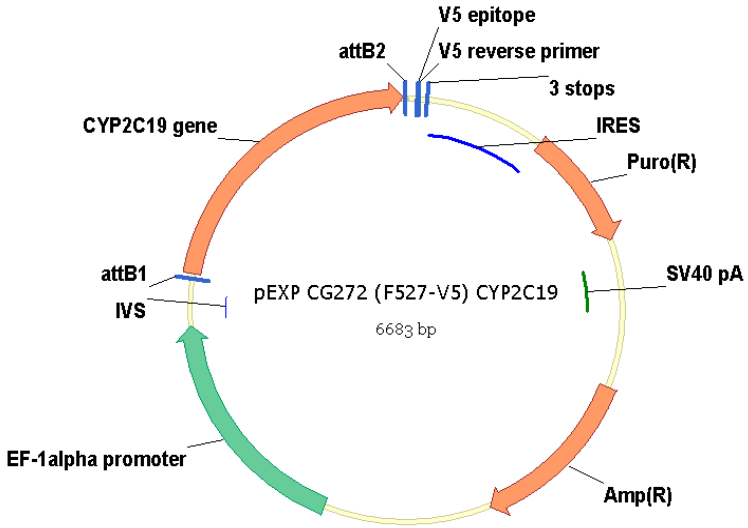

Figure 2.7. ***A map of the vector showing the relative location of the CYP2C19 cloning site and antibiotic resistance in HCT116 cell-line transfected with CYP2C19 (Guise et al. 2010).***

2.8.1 Preparation of cell culture solutions

Heat-inactivated fetal calf serum was required to prevent complement-mediated cell lysis. Frozen serum was defrosted at room temperature and heated at 56°C for 30 minutes and then stored in 50 mL aliquots at -20°C. Culture medium (α-MEM) was supplemented with 5% v/v fetal calf serum for HCT116.WT cell-line and with a further addition of 1 µM puromycin for HCT116.*CYP2C19* cell-line.

2.8.2 Cell culture procedures

Cryovials of HCT116.*CYP2C19* and HCT116.WT cell-line were removed from liquid nitrogen storage and thawed at 37°C for 2-3 minutes. The cell suspensions were transferred to 175 cm^2 flasks containing 40 mL of culture media and incubated in humidified conditions in 5% CO_2 at 37°C.

Both cell-lines were routinely passaged once they reached 70-80% confluency. After removal of old media, flasks were washed with PBS (1.7 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4; 1x v/v; 5 mL) and then incubated with trypsin-EDTA (0.05% v/v; 1 mL) for 5 minutes at room temperature. α-MEM culture medium (10 mL) was added to the flask to inactivate the trypsin and the suspension was gently mixed to break

up any existing cell clumps and subsequently transferred to sterile Falcon tubes (50 mL sized).

Cell density was measured using a Beckman Coulter Z2 Coulter Particle Count and Size Analyzer (California, USA). The cell suspension (100 µL) was transferred to a cuvette containing 9.9 mL of saline and the analyser was adjusted to measure cells ranging between 350-3000 µm in size at a dilution of 100. Cell density was calculated as number of cells per mL of culture medium. An appropriate number of cells were transferred to new flasks containing fresh culture medium for maintenance of the cell-lines. New aliquots of cells were removed from liquid nitrogen storage in every two months to maintain cell phenotype.

2.9 CYP2C19 immunoblot analysis

Immunoreactive CYP2C19 protein in the HCT116.*CYP2C19* cell-line was determined using a standard immunoblot method described below following lysis of cells. RIPA buffer and the protease inhibitor cocktail were both obtained from Sigma-Aldrich, Missouri, USA. All other chemicals and solvents were of analytical grade.

2.9.1 Cell lysis

Cell lysates were prepared from each well on an experimental microplate. After trypsinisation, cells were aspirated into microfuge tubes, pelleted (200 *g*, 5 minutes) and the media removed. The cell pellet was washed with PBS (500 µL) and subsequent to centrifugation and removal of PBS, the cell pellet was resuspended in 100 µL RIPA buffer (with addition of protease inhibitor cocktail, 1% v/v) and incubated on ice for 30 minutes. The cell lysate was spun at 15,800 *g* for 5 minutes, the supernatant was collected and protein concentration determined before use.

2.9.2 Cell-line microsome

A microsomal fraction of the HCT116.*CYP2C19* and wild type cell-line was prepared by ultracentrifugation of confluent cells grown in two 175 cm^2 large flasks as follows. The cells were washed twice with ice-cold PBS prior to the incubation with trypsin/EDTA (0.05%, 1.5 mL) for 5 minutes. Following trypsinisation, the cells were transferred to chilled Falcon tubes and the flasks were washed again with ice-cold PBS (10 mL) to collect any remaining cells and was transferred to the Falcon tube. Cells were pelleted following centrifugation (200 *g*, 5 min at 4°C) followed by a further wash with ice-cold PBS (1.5 mL) and re-pelleted again with centrifugation. The cell-pellet was transferred to a microfuge tube and washed with ice-cold hypotonic nuclear buffer (10 mM HEPES/KOH pH 7.4, 1.5 mM $MgCl_2$, 10 mM KCl, 0.05 mM DTT; 1 mL). After centrifugation (200 *g*, 5 min at 4°C), the cells were resuspended in ice-cold hypotonic nuclear buffer (1.5 mL) and was allowed to incubate at 4°C for 10 minutes. The suspension was sonicated using the Sonicator Ultrasonic Processor (Bandelin Sonorex Technik, Berlin, Germany) three times for 5 seconds with the samples placed on ice and the sonicator probe wiped with 70% ethanol between each sonication. The lysed cellular homogenate was incubated on ice for 10 minutes and was then centrifuged at 700 *g* for 5 minutes and then 9000 *g* for 15 minutes at 4°C to sediment nuclei and mitochondria. The microsomal fraction was obtained by diluting the supernatant (approximately 1:8 v/v) with ice-cold hypotonic nuclear buffer into a 20 mL clean ultracentrifuge tube followed by centrifugation at 100,000 *g* for 1 hour at 4°C. The pellet was resuspended (10 mM $MgCl_2$, 50 mM Tris buffer pH 7.5, 20% glycerol w/v; 500 µL) and then sonicated for 30 seconds and was stored at -80°C prior to further analysis.

2.9.3 Determination of the protein concentration

The protein concentration of cell lysates and microsomal fraction prepared from cells were determined using the BioRad DC assay kit (BioRad Laboratories, California, USA). Serum bovine albumin (BSA; Sigma-Aldrich, Missouri, USA; 4 mg/mL) was used as the standard and was serially diluted to working stocks of 1:10, 1:20, 1:40, 1:80 v/v with 0.1 M NaOH. Cell lysate samples were diluted to 1:2, 1:4, 1:8 v/v in 0.1 M NaOH. Standards and samples (5 µL) were pipetted into a clean microplate. Reagent A (25 µL) and reagent B (200 µL) was added to each well and the plate was incubated at room

temperature for 15 minutes with agitation. The absorbances were determined on a spectrophotometer (SpectraMAX plus, Molecular Devices, Victoria, Australia) at 750 nm. The protein concentration of each sample was calculated from the absorbance values of the sample relative to the calibration curve. An example of a typical calibration curve is shown in Figure 2.8.

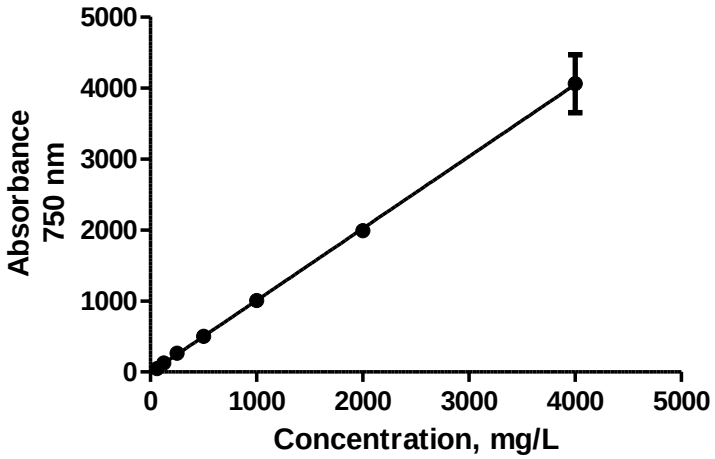

Figure 2.8. ***A typical calibration curve for the determination of protein concentration using bovine serum albumin as a standard,*** y = 1.013x – 6.955, r^2 = 0.9864. Values are mean ± SD of triplicate determinations.

2.9.4 Reagents and buffers

The composition of the reagents and buffers used in immunoblotting are listed in Table 2.2.

Buffer/Gel	Composition
Sample buffer, pH 6.8	100 mM Tris hydrochloride pH 6.8[a] 4% w/v Sodium dodecyl sulfate (SDS)[a] 10% v/v β-mercaptoethanol[b] 20% v/v Glycerol[c] 0.02% Bromophenol blue[d]
Gel electrophoresis buffer	200 mM Glycine[e] 25 mM Tris base[f] 3.5 mM SDS
Transfer buffer	200 mM Glycine 25 mM Tris base 20% v/v Methanol[g]
TBS-Tween buffer, pH 7.5	15 mM Tris hydrochloride 140 mM Sodium chloride[e] 0.1% v/v Tween-20[e]
Stripping buffer	6.9 mM SDS 62.4 mM Tris base 0.009% v/v β-mercaptoethanol
10% separating gel	4.62 mL MilliQ water 3.82 mL 30% Acrylamide/bis[h] 2.86 mL 1.5 M Tris hydrochloride pH 8.8 100 µL 10% w/v Ammonium persulfate[h] 50 µL 20% w/v SDS 15 µL TEMED[h]
6% stacking gel	2.16 mL MilliQ water 0.8 mL 30% Acrylamide/bis 1 mL 0.5 M Tris pH 6.8 10 µL 10% w/v Ammonium persulfate 20 µL 20% w/v SDS 15 µL TEMED

Table 2.6. ***The composition of the immunoblotting buffers used in this study.***
Chemicals and solvents were obtained from: [a] JT Baker, New Jersey, USA; [b] Merck, Darmstadt, Germany; [c] LabServ, Australia; [d] Ajax Finechem, Australia; [e] BDH Laboratory Supplies, Poole, UK; [f] Applichem, Darmstadt, Germany; [g] Scharlau Chemie S.A., Sentmenat, Spain; [h] BioRad Laboratories, California, USA.

2.9.5 SDS-PAGE

Each sample and positive control (CYP2C19 supersomes, BD Gentest™ Supersomes™, New Jersey, USA) was diluted to a protein concentration of 2 mg/mL using phosphate buffer (67mM, pH 7.4) as the diluent. The diluted cell lysate protein was then denatured by the addition of sample buffer (1:1 v/v) to a final protein concentration of 1 mg/mL and heated (95°C for 5 minutes). The denatured protein (10 µg in 10 µL) and the molecular weight marker (5 µL, Precision Plus Protein™ Standards, BioRad Laboratories, California, USA) was loaded onto a 6% stacking gel and separated at 200 volts for approximately 40 minutes on a 10% polyacrylamide gel using the mini-protean apparatus (BioRad Laboratories, California, USA) in gel electrophoresis buffer. Following electrophoresis, the gel was soaked in transfer buffer until the bromophenyl blue band faded.

2.9.6 Protein transfer

Prior to protein transfer, polyvinylidene difluoride (PVDF) membranes (BioRad Laboratories, California, USA) were prepared by pre-soaking in methanol for 20 seconds and in transfer buffer for at least 20 minutes. The transfer sandwich was assembled and placed into the Western transfer tank (BioRad Laboratories, California, USA) according to manufacturer's instructions and immersed in ice cold transfer buffer. Protein transfer was carried out at 110V for one hour on the mini-protean apparatus.

2.9.7 CYP2C19 immunodetection

The PVDF membrane was incubated in blocking solution (5% non-fat milk in TBS-Tween) with agitation for one hour at room temperature followed by washing three times with TBS-Tween for 10 minutes. Rabbit polyclonal anti-human CYP2C19 primary antibody (BD Gentest™, Massachusetts, USA) was diluted in blocking solution (1:5000 v/v) and incubated with the membrane overnight at 4°C, with rocking motion to ensure uniform distribution of antibody. The membrane was washed three times with TBS-Tween for 10 minutes and was further incubated with the horseradish peroxidase conjugated goat anti-rabbit secondary antibody (BD Gentest™, Massachusetts, USA)

diluted in blocking solution (1:2000 v/v) for one hour at room temperature with rocking motion. Following three washes with TBS-Tween for 10 minutes, the membrane was incubated with ECL Advance solution (GE Healthcare UK Ltd, Buckinghamshire, UK; 4 mL) for 5 minutes at room temperature. Excess ECL solution was drained and the membrane was placed in between two layers of transparency. The luminescent image was captured using the LAS-3000 Fujifilm imaging system (Fujifilm Corporation, Japan) and the band intensity was analysed using ImageJ software (National Institutes of Health, Maryland, USA).

2.9.8 Beta-actin immunodetection

The membrane was stripped using stripping buffer for 30 minutes at 50°C and was washed three times in TBS-Tween for 10 minutes. The membrane was then incubated in blocking solution (5% non-fat milk in TBS-Tween) with agitation for one hour at room temperature. Mouse monoclonal anti-actin antibody (Millipore Corporation, Massachusetts, USA) was diluted in blocking solution (1:10,000 v/v) and incubated with the membrane overnight at 4°C with rocking motion. The membrane was then washed three times with TBS-Tween for 10 minutes and was further incubated with the horseradish peroxidase conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology Inc, California, USA) diluted in blocking solution (1:10,000 v/v) for one hour at room temperature with rocking motion. Following three washes with TBS-Tween for 10 minutes, the membrane was incubated with ECL Advance solution for 5 minutes and the image was captured as described previously.

2.10 Real-time polymerase chain reaction (qPCR)

Real-time polymerase chain reaction was used to determine the relative expression of *CYP2C19* mRNA in the HCT116.*CYP2C19* cell-line and is described as follows. The TaqMan® Gene Expression Cells-to-C_T™ kit , TaqMan® Gene Expression assay for *CYP2C19* (Hs00426380_m1) and *GAPDH* (Hs99999905_m1), 384-well PCR plates and the plate covers were obtained from Applied Biosystems, California, USA. All other chemicals and solvents used were of analytical grade.

2.10.1 Cell lysis

Cell lysates were prepared from cells in each well on a 96-well microplate using TaqMan® Gene Expression Cells-to-C_T™ kit. Initially, cells were trypsinised and were pelleted using centrifugation (200 *g*, 5 minutes). The cell pellet was then washed with ice-cold PBS (500 µL), centrifuged and the PBS removed. The resulting cell pellet was resuspended in ice-cold PBS (5 µL). Lysis solution (50µL) supplied in the kit was added to each sample, mixed and incubated at room temperature for 5 minutes. Subsequently, stop solution (5 µL) was added to each sample to terminate the lysis reaction (2 minutes, room temperature). The cell lysates were stored at -20°C prior to further analysis.

2.10.2 Reverse transcription

Solutions and enzymes required for the reverse transcription were supplied in the TaqMan® Gene Expression Cells-to-C_T™ kit. For each reaction, 2x reverse transcription buffer (25 µL), 20x reverse transcription enzyme mix (2.5 µL), nuclease-free water (12.5 µL) and cell lysate (10 µL) was added to a sterile PCR tube, to a final volume of 50 µL in each tube. Reverse transcription was carried out in a thermal cycler and was initially incubated at 37°C for 60 minutes and then at 95°C for 5 minutes to inactivate the reverse transcription enzyme.

2.10.3 Real-time PCR

For each reaction, 2x TaqMan® Gene Expression master mix (5 µL), 20x TaqMan® Gene Expression assay for *CYP2C19* (0.5 µL), nuclease-free water (2.5 µL) and cDNA template (2 µL) was added to each well of 384-well PCR plate and covered with PCR plate cover. Additional wells were tested for no template control, i.e. absence of cDNA as a negative control. The microplate was centrifuged briefly to remove bubbles and to collect the contents at the bottom of the wells. Subsequently, the plate was read in a real-time PCR instrument (7900HT Fast Real-Time PCR System, Applied Biosystems, Singapore) using the following conditions as described in the manufacturer's protocol. It comprised of one cycle of 50°C for 2 minutes for uracil DNA glycosylase (UDG) incubation and 95°C for 10 minutes for enzyme activation followed by 40 repetitions of 95°C for 15 seconds polymerase chain reaction and lastly one repetition of 60°C for 1 minute. The determination of *GAPDH* expression was as described for *CYP2C19* with the exception of using 20x TaqMan® Gene Expression assay for *GAPDH* (0.5 µL).

2.10.4 Data analysis

The data was analysed using relative quantification which relates the PCR signal of the target transcript in a treatment group relative to the control. The derivation of the $2^{-\Delta\Delta C_T}$ equation, including the assumptions, experimental design and validation tests were described in Applied Biosystems User Bulletin No.2 (P/N 4303859) (Livak et al. 2001). *GAPDH* was chosen as the internal reference control.

2.11 Nitrite determination

The total nitrate (nitrite and nitrate) levels in the plasma samples were measured using the fluorometric commercial kit, Nitric Oxide Assay Kit (Calbiochem, Merck KGaA, Darmstadt, Germany). This kit uses the aromatic diamino compound, 2,3-diaminonaphthalene (DAN) as an indicator of nitric oxide formation (Bryan et al. 2007). Nitric oxide is rapidly oxidised almost completely to nitrite followed by a further oxidation to nitrate in whole blood which is both stable in frozen plasma for at least one year (Moshage et al. 1995). Initially, nitrates are converted into nitrites by incubation with nitrate reductase and its cofactors. The presence of nitrites reacts with the relatively non-fluorescent DAN to form the highly fluorescent product, 2,3-naphthotriazole, which can be measured by a fluorometer (Bryan et al. 2007). Details of the assay protocol are described below. The reported minimum detectable concentration by the manufacturer was 0.03 µM.

2.11.1 Sample pre-treatment

Plasma samples collected from patients were stored at -80°C prior to analysis. Samples required deproteinisation prior to determination of nitrite/nitrate levels in patients' plasma or serum and was described as follows (Higuchi et al. 1999). A plasma sample (50 µL) was added to a mixture of MilliQ water (200 µL) and 0.3 M NaOH solution (150 µL). The resultant mixture was incubated at room temperature for 5 minutes before the addition of 5% w/v $ZnSO_4$ solution (150 µL) with vortex mixing, followed by incubation at room temperature for a further 5 minutes. The deproteinised sample was centrifuged at 3800 *g* for 10 minutes and the supernatant was analysed as detailed below.

2.11.2 Preparation of reagents

The supplied buffer concentrate (2 mL) was diluted with MilliQ water to a final volume of 100 mL assay buffer. Both the nitrate reductase and enzyme co-factors were reconstituted with assay buffer (1.2 mL) and kept on ice for the remainder of the experiment. The nitrate standard was reconstituted with assay buffer (1 mL) to produce a stock solution of 2 mM. The standard was mixed gently with vortex to ensure complete

reconstitution and was serially diluted with assay buffer to a final concentration of 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156 and 0.078 µM.

2.11.3 Assay procedure

The standards (30 µL) and samples (20 µL) were added to their respective wells on the 96-well plate. Each well was adjusted to a final volume of 80 µL with assay buffer followed by the addition of the enzyme co-factors (10 µL) and nitrate reductase (10 µL) to each well. The plate was then covered by the supplied adhesive strip and incubated at room temperature for 2 hours with gentle agitation. The fluorometric reagent (2,3-diaminonaphthalene, DAN; 10 µL) was added to each well with a further incubation at room temperature for 10 minutes. Subsequently, NaOH (2.8 M, 20 µL) was added to each well and the fluorescence was measured on a fluorometer (SpectraMAX M2, Molecular Devices, Victoria, Australia) using an excitation wavelength of 375 nm and an emission wavelength of 415 nm.

2.11.4 Data analysis

The calibration curve is shown in Figure 2.9. At lower concentrations, the curve is linear and the r^2 value is 0.9381.

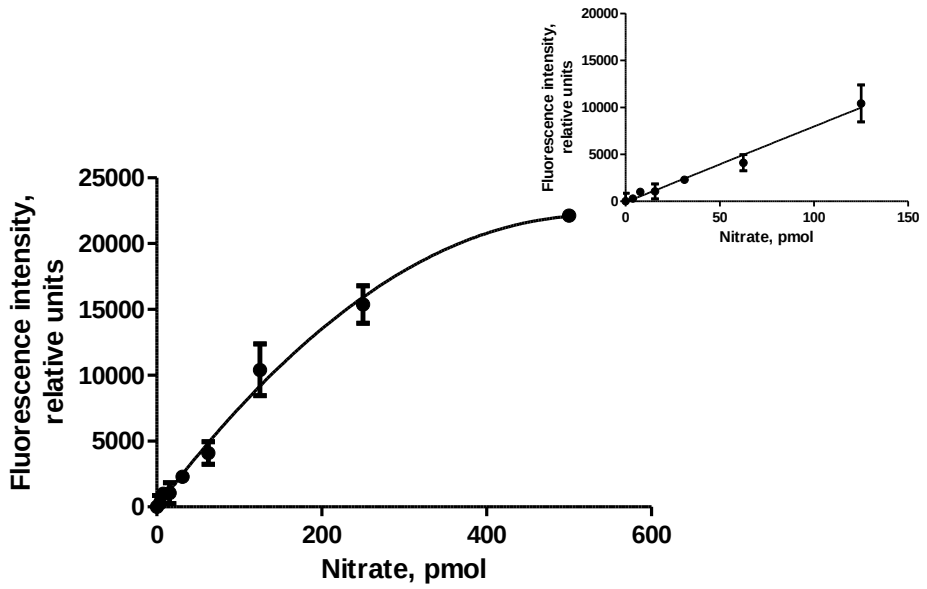

Figure 2.9. ***The calibration curve for the determination of nitrate concentration,*** y = 46.15x + 1189, r^2 = 0.9381. Values are mean ± SD of duplicate determinations.

The concentration of nitrate/nitrite in each sample was determined using the following equation:

$$\text{nitrate + nitrite } (\mu M) = \frac{\text{fluorescence - y intercept}}{\text{slope}} \times \frac{1}{\text{volume of sample } (\mu L)} \times \text{dilution}$$

CHAPTER 3

Compromised CYP2C19 activity in advanced cancer

3.1 Introduction

A number of animal studies have demonstrated that the presence of extra-hepatic tumours can influence the drug metabolising capacity of the liver due to altered expression of CYP enzymes (Rivory et al. 2002; Scripture et al. 2005; Charles et al. 2006). However, very few studies have attempted to determine whether neoplastic disease can also influence CYP activity in a clinical setting. A study of CYP2C19 activity in a small group of advanced cancer patients reported that 25% of patients had poor CYP2C19 metabolising activity despite having a wildtype *CYP2C19* genotype (Williams et al. 2000). Recently it has been reported that CYP3A4 activity is also decreased in advanced cancer patients (Rivory et al. 2002), and the inflammatory response to the tumour was proposed as a mechanism since C-reactive protein correlated with decreased CYP3A4 activity in the cancer patients. In addition, CYP2C19 activity was reported to correlate inversely with elevated pro-inflammatory TNF-α and IL-6 in patients with congestive heart failure (Frye et al. 2002).

A number of proinflammatory cytokines can down-regulate CYP isozymes in human hepatocyte cultures (Aitken et al. 2007). Indeed, *CYP2C19* mRNA expression was down-regulated by IL-6 and TGF by 35% and 50% respectively (Aitken et al. 2007).

A number of proinflammatory cytokines are produced as autocrine growth signals by tumour cells and also in response to the tumour by the surrounding stromal cells (Nicolson 1993; Lázár-Molnár et al. 2000). These elevated cytokines are associated with paraneoplastic disorders such as cachexia (Matthys et al. 1997). Pro-inflammatory cytokines such as TNF-α and IL-6 are also known to elicit an acute phase response in the liver which leads to increased production of proteins such as C-reactive protein (CRP) and decreased synthesis of albumin (Fearon et al. 1998; Bolayırlı et al. 2007). Hence, cytokines associated with neoplastic disease may elicit a hepatic acute phase response and this may also influence the hepatic expression and activity of CYP enzymes, such as CYP2C19 and CYP3A4 (Figure 3.1).

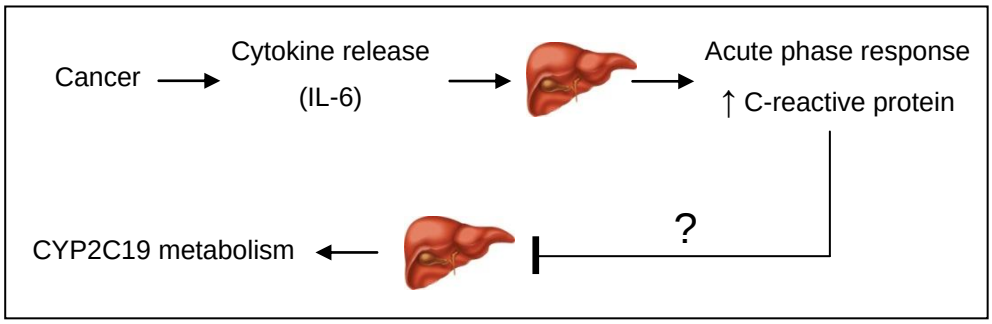

Figure 3.1. ***The potential relationship between cancer-associated inflammation and CYP2C19 activity.*** Cancer-associated inflammation, also described in Figure 1.3, leads to increased release of inflammatory cytokines which will induce the hepatic acute phase response; together this may influence expression or activity of CYP enzymes.

Low CYP3A4 activity was shown to be associated with an increase in chemotherapy-associated toxicity (Slaviero et al. 2003). Hence, if CYP2C19 is compromised in cancer, this may also result in therapeutic failure due to lack of bioactivation or clearance of a drug. However, it is not known whether the highly inflammatory state in the cancer patients may play a role in the compromised metabolism by CYP2C19.

Recent studies have shown that growth hormone (GH) may also affect the hepatic CYP-mediated drug metabolism in humans. In one study, a randomised placebo-controlled trial of 30 healthy elderly men showed that growth hormone inhibited CYP2C19 (Jürgens et al. 2002; Sarlis et al. 2005). The *S/R* ratio of mephenytoin exhibited a significant elevation after GH treatment compared with placebo which indicates an inhibition of CYP2C19 activity (Jürgens et al. 2002). In another study conducted in lung cancer patients, it was demonstrated that serum GH levels were raised in patients with extensive disease and with elevated IL-6 concentration (Martin et al. 1999). Moreover, a significant relationship was found between GH and nutritional variables as percentage of weight loss and body mass index which suggests that GH increases with the impairment of the nutritional status (Martin et al. 1999). Furthermore, GH was also suggested to increase in relation with an enhancement of acute phase response (Martin et al. 1999). It may be possible that the increase in GH levels in cancer may be able to further downregulate CYP2C19 activity.

The hypothesis is that there is a discordance between genotype and phenotype and this may be due to inflammatory response to the tumour.

The aims of this study were to (a) confirm the discordance between the genotype and CYP2C19 activity in patients with advanced cancer previously reported by Williams and

colleagues (Williams et al. 2000) and (b) to determine if there is a relationship between CYP2C19 metaboliser status and inflammatory markers, such as cytokines and acute phase response proteins, as well as growth hormone concentration.

3.1.1 Study design

The study was approved by the New Zealand Health and Disability Multi-Regional Ethics Committee (NTX/05/07/083). Patients with advanced incurable (terminal) cancer who were not receiving any chemotherapy or hormone therapy for at least 4 weeks prior to the study were recruited. Patients on any medications that are known inducers or inhibitors of the CYP2C19 enzyme were excluded from the study. All patients were over 18 years of age with adequate hepatic and renal function (creatinine levels < 0.12 mmol/L, AST/ALT < 90 U/L, ALP < 300 U/L, bilirubin < 20 µmol/L). An initial estimate of sample size was 50 patients in order to detect with 80% power an incidence of 25% poor metabolisers compared with an expected incidence of 3% in a healthy Caucasian population.

At baseline, a total of 20 mL of blood was obtained. This included 5 mL in a heparinised tube which was sent to the local clinical laboratory service (LabPLUS) for measurement of C-reactive protein, 5 mL in a plain tube for serum preparation to determine cytokine concentrations (as described in section 2.4) and 8.5 mL in a PAXgene™ blood DNA tube for storage prior to preparation of genomic DNA for *CYP2C19* genotype determination (as described in section 2.2). Patients were given a single oral dose of omeprazole (20 mg) and 2 hour post-dose blood sample (10 mL) was collected in a heparinised tube. Plasma was prepared and analysed for omeprazole and 5'hydroxy omeprazole concentrations (as described in section 2.3). The plasma sample was also used to determine levels of albumin (section 2.5) and growth hormone (section 2.6).

Statistical analyses were determined using SigmaPlot (Version 11, Systat Software Inc, Germany) and GraphPad Prism (Version 5, GraphPad Software Inc, USA). Continuous data were reported as the median and interquartile range (25%-75% quartile; IQR). Associations between continuous variables were assessed using Spearman's rank correlation, and Mann-Whitney rank-rum tests were used to compare continuous variables across two groups. The discordance between *CYP2C19* genotype and

phenotype was assessed using McNemar's test and proportions were compared using Fisher's exact test. Two-sided *P*-values < 0.05 were considered statistically significant.

3.2 Results

3.2.1 Demographics

Following full informed written consent, 33 patients with advanced incurable cancer were recruited into the study. The demographics of the patients are listed in Table 3.1. There were 18 male and 15 female patients with a median age of 62 years (IQR 57-69) and median weight of 72.9 kg (IQR 64.2-82). Patients had a wide range of primary tumour types and the majority of the patients had metastatic disease (27 out of 33). Of the patients with metastatic disease, seven patients had liver metastases (Table 3.1).

Study #	Gender	Ethnicity	Age (yrs)	Height (cm)	Weight (kg)	BMI	Primary neoplasm	Metastases[a]	Liver metastases[b]
401	F	English	58	173.5	91.7	30.6	Breast	Y	N
402	M	NZ European	61	180.5	72.3	22.1	Gall bladder	N	N
403	M	NZ European	64	176.5	73	23.3	Squamous cell carcinoma	N	N
405	F	Eurasian	33	165	55	20.2	Breast	Y	Y
406	F	NZ European	68	167	79	28.3	Breast	Y	N
407	F	NZ European	53	169	53	18.6	Breast	Y	Y
408	F	English	47	164	82	30.5	Breast	Y	N
409	M	NK	69	172	71.8	24.3	Mesothelioma	N	N
410	M	NZ European	57	174	71.7	23.7	Mesothelioma	Y	N
411	M	NZ European	71	166	62	22.5	Colorectal	Y	Y
412	F	NZ European	65	168.5	104	36.4	Lung	Y	N
413	F	NK	59	162	62.3	23.7	Ovary	Y	N
414	F	NZ European	43	162	70	26.7	Breast	Y	N
415	M	NK	69	168	60.9	21.6	NK	N	N
417	M	NZ European	62	168	83.6	29.6	Rectum	Y	N
418	M	NZ European	74	170	78.8	27.3	Mesothelioma	Y	N
419	M	NZ European	66	177.5	62.1	19.6	Pancreas	N	N
423	M	NZ European	67	177	92	29.4	Colorectal	Y	Y
424	M	NZ European	60	183.5	34.2	19	Colon	Y	Y
425	M	Chinese	54	159	64.5	25.5	Hodgkin’s lymphoma	Y	Y
426	F	NZ European	60	166	72.9	26.5	Non-small cell lung cancer	Y	N
427	M	NZ European	68	175	92	30	Rectum	Y	N
428	F	NZ European	69	166	65	23.6	Colorectal	Y	N
429	F	NZ European	71	164.4	93.3	34.7	Peritoneum	Y	N
430	M	NZ European	55	191.3	100.7	27.7	Kidney	Y	N
431	F	NZ European	55	148	51.8	23.6	Breast	Y	Y
432	M	NZ European	79	171.5	75.5	25.5	Lung	Y	N
433	M	NZ European	61	165	69.5	25.5	Mesothelioma	Y	N
434	F	NZ European	69	161.2	48.9	18.9	NSCLC	Y	N
435	F	NZ European	72	164.5	76.2	28.	Lung	Y	N
436	M	NZ European	59	169	100.5	35.4	Lung	Y	N
437	M	NZ European	63	167	80.8	29	Melanoma	Y	N
438	F	NZ European	49	166	80.4	29.2	Breast	N	N

Table 3.1. ***The demographics of the patients with advanced incurable cancer.*** BMI = Body mass index. [a] metastases present: Y = Yes, N = No. [b] Liver metastases present: Y = Yes, N = No. NK = unknown

3.2.2 *CYP2C19* genotype

Following isolation of genomic DNA, the *CYP2C19* genotype was determined using PCR-RFLP analysis of the **2* and **3* variant alleles (as described in section 2.2). One patient (# 425) was homozygous for the *CYP2C19*3* variant allele and was classified as a genotypic poor metaboliser. This homozygous variant patient was self identified as Eurasian and the *CYP2C19* variant allele was more common in the Asian population (de Morais et al. 1994a; Bertilsson 1995). The *CYP2C19*3* variant was not observed in any other patients. The remaining 32 patients were *wt/wt* (*n* = 20) or *wt/var* (*n* = 12) for the *CYP2C19*2* allele and hence were all classified as extensive metabolisers (genotypic).

The Hardy-Weinberg equilibrium of the variant allele frequency was assessed using the equation ($p^2 + 2pq + q^2 = 1$), where p is the number of wildtype alleles (*CYP2C19*1)* and q is the number of variant alleles (either *CYP2C19*2* or *CYP2C19*3)*. The observed and expected allele totals and frequencies for *CYP2C19*2* and *CYP2C19*3* are shown in Table 3.2.

Genotype	****1/*1***	****1/*2***	****2/*2***	**Total**
Observed *n*	21	12	0	33
Observed frequency	0.636	0.343	0	1
Expected frequency	0.669	0.298	0.033	1
Expected *n*	22.09	9.82	1.09	33
Genotype	****1/*1***	****1/*3***	****3/*3***	**Total**
Observed *n*	32	0	1	33
Observed frequency	0.970	0	0.030	1
Expected frequency	0.940	0.059	0.001	1
Expected *n*	31.03	1.94	0.03	33

Table 3.2. ***Genotype totals and frequencies for CYP2C19*2 and CYP2C19*3 in patients with advanced incurable cancer.*** *CYP2C19*2* was in agreement with Hardy-Weinberg equilibrium ($\chi^2 = 1.63$, $P = 0.443$), but *CYP2C19*3* was not ($\chi^2 = 33$, $P < 0.0001$).

The distribution of allele frequencies for *CYP2C19*2* was in agreement with Hardy-Weinberg equilibrium ($\chi^2 = 1.63$, $P = 0.443$). However, the distribution of *CYP2C19*3* was not in agreement with the Hardy-Weinberg equilibrium ($\chi^2 = 33$) with a *P*-value < 0.0001.

3.2.3 CYP2C19 metabolic activity

CYP2C19 activity was determined using omeprazole as the probe substrate (as described in section 2.3) and the log omeprazole hydroxylation index (HI) was used to categorise patients as either phenotypic extensive (HI < 1) or poor metaboliser (HI ≥ 1) (Balian et al. 1995; Kortunay et al. 1997b). Two patients (#411 and #414) had no detectable levels of drug or metabolite in the plasma, which may indicate compliance issues, and these patients were excluded from any further analysis.

The omeprazole log hydroxylation index of each patient compared with their genotype is shown in Table 3.3. The one patient (# 425) who was homozygous variant (*var/var*) for *CYP2C19*3* had no detectable metabolite and was assigned a log hydroxylation index of 2, to indicate null metabolism.

Patient #	*CYP2C19* genotype	Predicted phenotype	Log omeprazole hydroxylation index	Actual phenotype	Genotype-phenotype discordance?
401	**1/*1*	EM	0.83	EM	N
402	**1/*1*	EM	0.27	EM	N
403	**1/*2*	EM	1.59	PM	Y
405	**1/*2*	EM	0.59	EM	N
406	**1/*2*	EM	2.00	PM	Y
407	**1/*2*	EM	1.48	PM	Y
408	**1/*2*	EM	0.80	EM	N
409	**1/*1*	EM	1.93	PM	Y
410	**1/*1*	EM	0.45	EM	N
411	**1/*1*	EM	nd	nd	ND
412	**1/*1*	EM	0.49	EM	N
413	**1/*2*	EM	2.00	PM	Y
414	**1/*1*	EM	nd	nd	ND
415	**1/*2*	EM	1.15	PM	Y
417	**1/*1*	EM	0.73	EM	N
418	**1/*1*	EM	0.19	EM	N
419	**1/*1*	EM	0.16	EM	N
423	**1/*1*	EM	1.22	PM	Y
424	**1/*1*	EM	0.11	EM	N
425[a]	**3/*3*	PM	2.00[a]	PM	N[a]
426	**1/*2*	EM	0.92	EM	N
427	**1/*1*	EM	0.07	EM	N
428	**1/*1*	EM	2.00	PM	Y
429	**1/*2*	EM	0.61	EM	N
430	**1/*2*	EM	0.49	EM	N
431	**1/*1*	EM	1.32	PM	Y
432	**1/*2*	EM	0.50	EM	N
433	**1/*1*	EM	-0.10	EM	N
434	**1/*1*	EM	1.40	PM	Y
435	**1/*1*	EM	0.42	EM	N
436	**1/*1*	EM	1.21	PM	Y
437	**1/*1*	EM	0.38	EM	N
438	**1/*2*	EM	0.77	EM	N

Table 3.3. ***The CYP2C19 genotype-phenotype discordance in individual advanced incurable cancer patients.*** EM = extensive metaboliser; PM = poor metaboliser. nd = not-detectable; ND = not determined. Y = Yes; N = No. [a] Patient #425 was a homozygous variant poor metaboliser.

The measured log omeprazole hydroxylation index in the 31 patients ranged from -0.1 to 2 and the frequency distribution of the log hydroxylation index was clearly not bimodal (Figure 3.2).

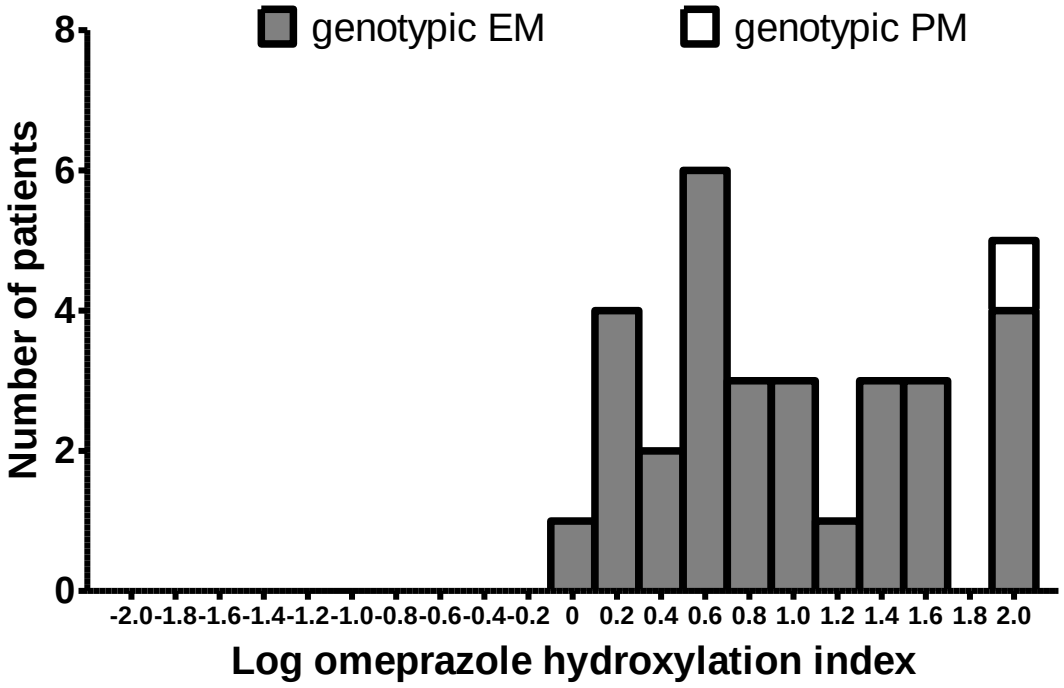

Figure 3.2. ***The frequency distribution of log omeprazole hydroxylation index of the advanced incurable cancer patients.*** The white bar indicates the one patient (#425) who was homozygous variant. The bimodal shape that was observed in a healthy population was absent.

The median log hydroxylation index for CYP2C19 *wt/wt* and *wt/var* patients were 0.47 (IQR 0.19-1.22) and 0.86 (IQR 0.60-1.54) respectively and the distribution of the metabolic activity for each genotype category are shown in Figure 3.3. The distribution of both the *wt/wt* and *wt/var* groups passed the Shapiro-Wilk normality test ($P > 0.05$). Phenotypic poor metabolisers were observed in both the *wt/wt* and *wt/var* groups. Of the 18 patients who were *wt/wt,* 33.3% had a log hydroxylation index >1 and of the 12 patients who were *wt/var,* 41.6% had a log hydroxylation index >1. The proportions of phenotypic poor metabolisers in the two genotypic extensive metaboliser groups were not significantly different from each other ($P = 0.6$) (Figure 3.3).

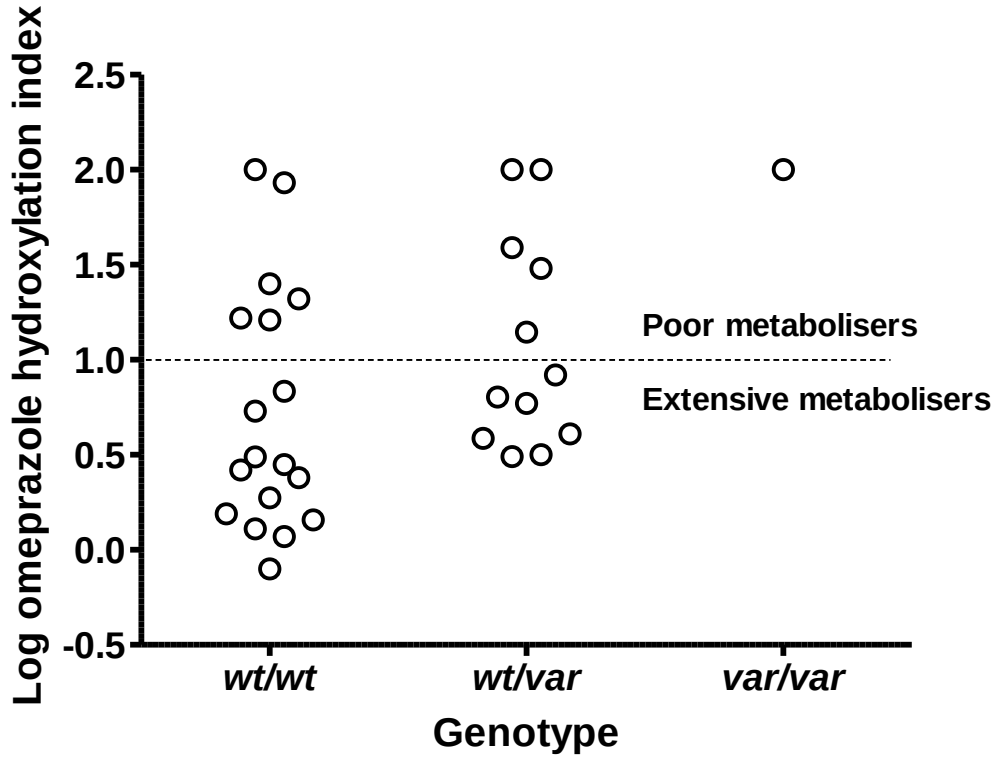

Figure 3.3. ***The distribution of CYP2C19 metabolic activity in advanced incurable cancer patients with known CYP2C19 genotype.*** The difference in the proportions of phenotypic poor metabolisers in *wt/wt* or *wt/var* category were not significant (*P = 0.06).*

Of the 30 patients who were genotypic extensive metabolisers (either *wt/wt* or *wt/var*), 19 were classified as phenotypic extensive metabolisers with a log hydroxylation index < 1 and the remaining 12 were phenotypic poor metabolisers (log hydroxylation index > 1). To determine the discordance between genotype predicted CYP2C19 metaboliser status and phenotype, the one patient (# 425) who was homozygous variant was excluded from further analysis. Hence, of the 30 patients that were genotypic extensive metabolisers,

11 (37%) had a poor metaboliser phenotype. The discordance was significantly different from the expected proportion of zero ($P < 0.0005$).

3.2.4 Relationship with inflammatory status

To assess whether compromised CYP2C19 activity correlated with inflammatory cytokines, circulating levels of IL-1α, IL-1β, IL-6, TNF-α, TGF-β were measured in the patient samples. The concentrations of these cytokines were highly variable across the patient group and some, such as IL-1α and IL-6, were elevated (Figure 3.4a) compared with reported normal values (Table 1.10). The concentration of the acute phase response protein, C-reactive protein (CRP), ranged between 0.2 to 208 mg/L in the patient group and was highly elevated in some patients (Figure 3.4b).

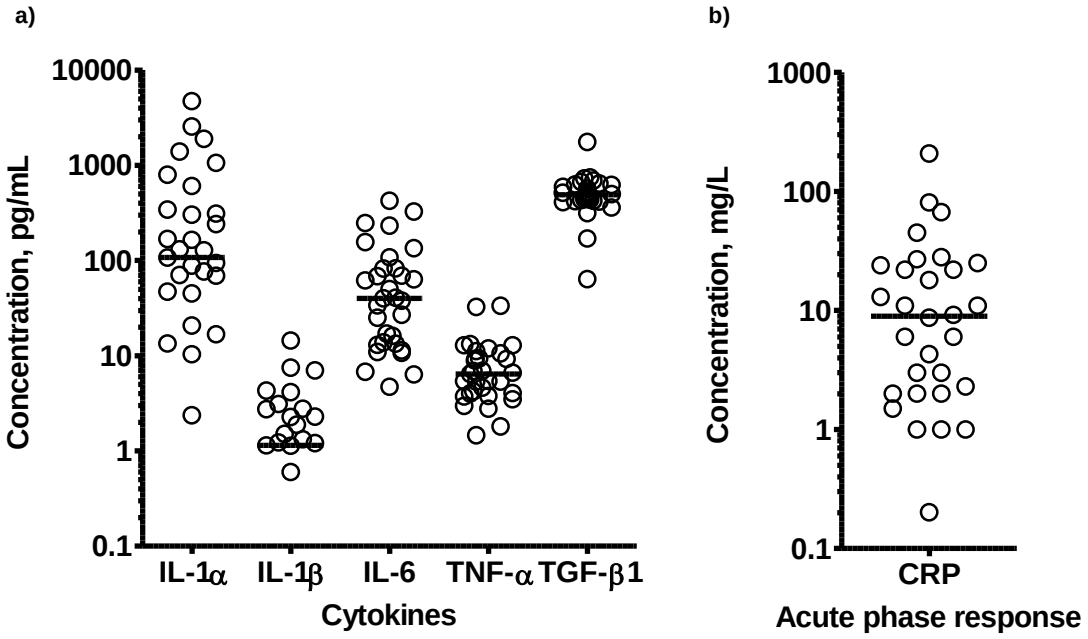

Figure 3.4. ***a) The cytokine concentrations and b) the CRP concentration of each advanced incurable patient.*** Bar shows the median concentration observed in the patients. The normal concentrations of the cytokines and CRP are shown in Table 1.10.

However, there were no statistically significant correlations between any of the individual cytokines, or CRP and CYP2C19 metabolic activity (log omeprazole hydroxylation index) in the patients (Figure 3.5).

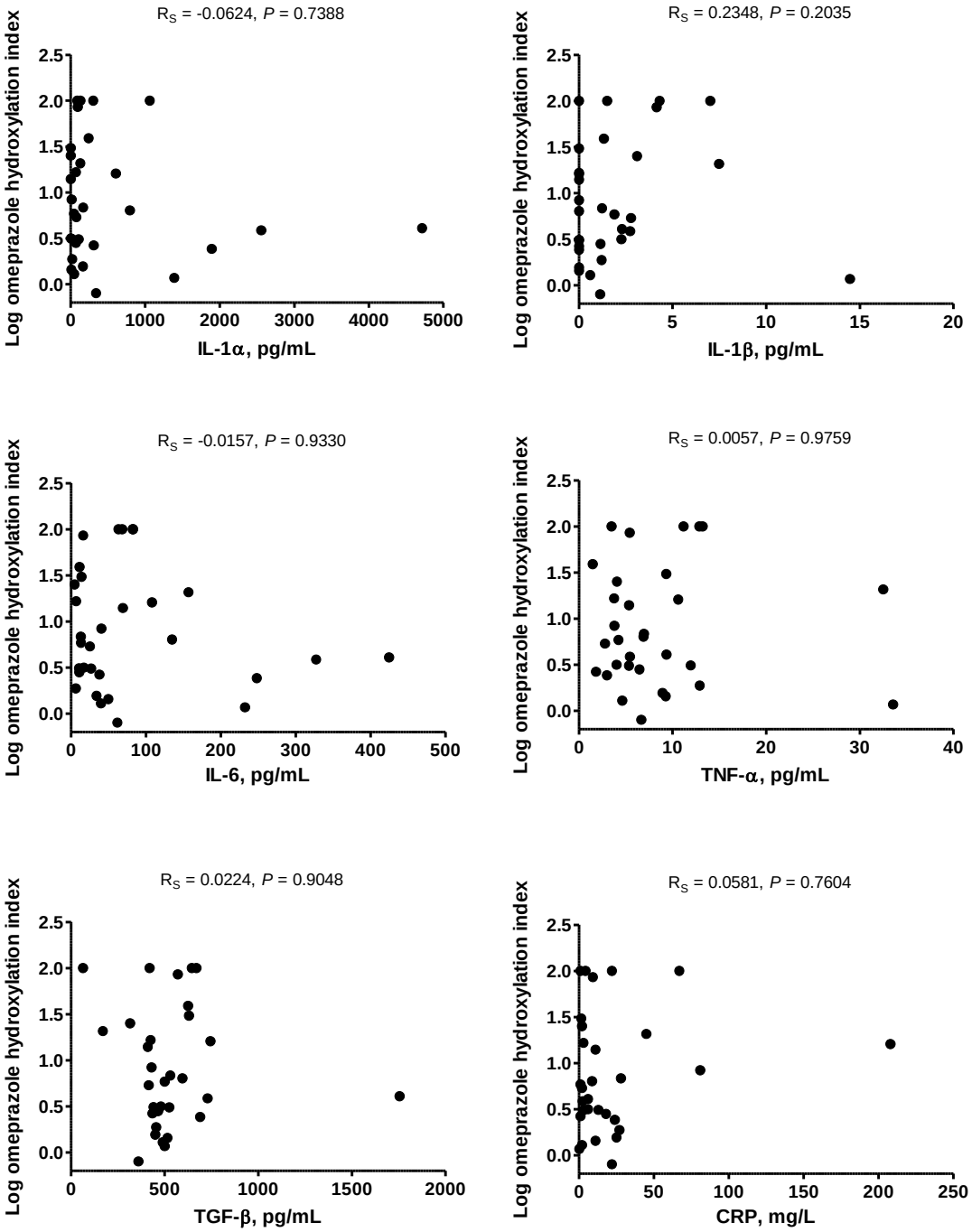

Figure 3.5. ***The lack of relationship between CYP2C19 activity and the levels of inflammatory cytokines and CRP in advanced incurable cancer patients.*** The *P*-values from Spearman's rank correlation test are shown on the graphs.

The hepatic acute phase response to inflammation is also associated with decreased albumin levels (Mariani et al. 1976; Fearon et al. 1998). The median albumin concentration in the patients was 44.8 g/L (IQR 43.7-48.8) and this was within the normal healthy range (35-50 g/L) (Kyle 2008) with the exception of two patients (# 410 and # 436) who were hypoalbuminemic (less than 35 g/L). One patient had a low concentration of albumin, just higher than the standard cut off value for hypoalbuminemia (# 428 = 35.4 g/L). There was no correlation between CYP2C19 metabolic activity and levels of albumin in the patients (Figure 3.6a). In addition, there was no relationship between levels of CRP and albumin in the cancer patients (Figure 3.6b), despite the well characterised negative correlation reported between CRP and albumin (McMillan et al. 2001a; Al-Shaiba et al. 2004).

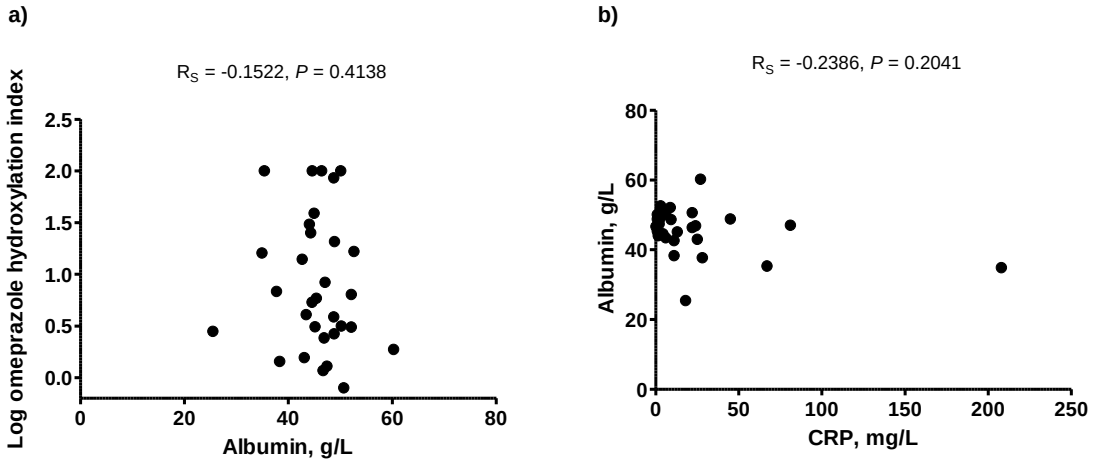

Figure 3.6. ***a) The lack of relationship between CYP2C19 activity and albumin levels and b) the lack of relationship between CRP and albumin levels in advanced incurable cancer patients.*** The *P*-values from Spearman's rank correlation test are shown on the graphs.

The relationship between growth hormone concentrations and CYP2C19 activity was also assessed. Growth hormone concentrations in the patients were highly variable (median = 955.7 pg/mL, IQR 496.3-4117.2 pg/mL), although no patients had levels above normal values (> 24 µg/L) (Kyle 2008). Furthermore, there was no significant correlation between growth hormone levels and the CYP2C19 metabolic activity of the patients (Figure 3.7).

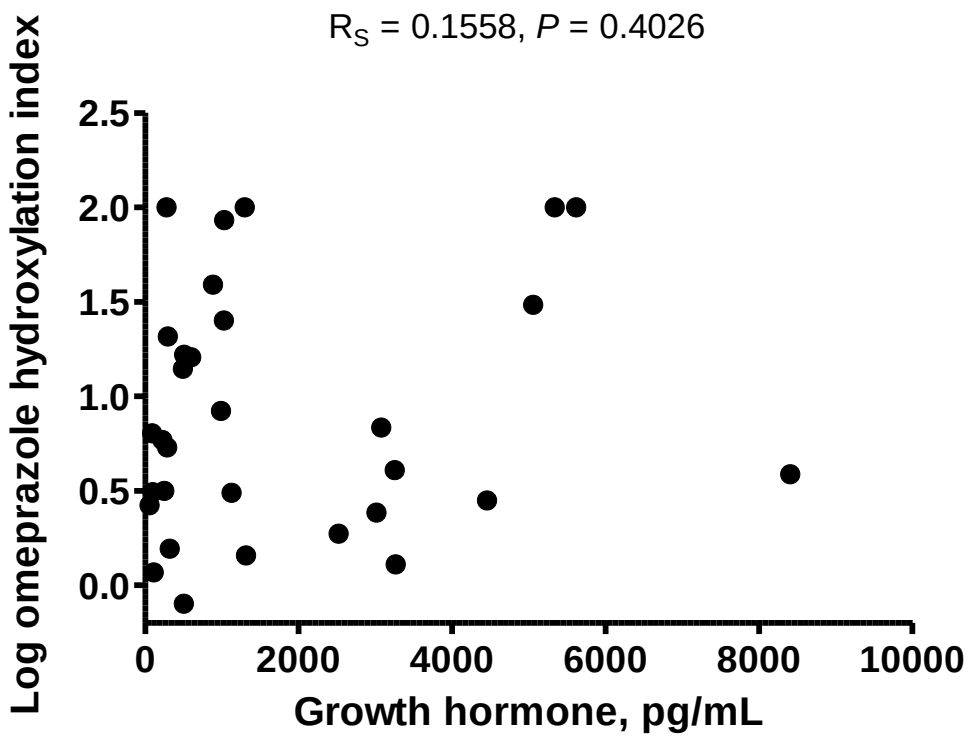

Figure 3.7. ***The lack of relationship between CYP2C19 activity and plasma growth hormone concentrations in advanced incurable cancer patients.*** Spearman r = 0.1558, *P* = 0.4026.

To confirm that there was no relationship between biological markers of inflammation and CYP2C19 activity, the data were also analysed based on the metaboliser category (Table 3.4)

	Phenotype		
	Extensive metabolisers median (IQ range) *n* = 19	***Poor metabolisers*** median (IQ range) *n* = 12	***P*-value**
IL-1α (pg/mL)	107.2 (20.8, 794.5)	111.2 (17.7, 287.3)	0.61
IL-1β (pg/mL)	1.1 (0, 39.2)	1.4 (0, 39.2)	0.35
IL-6 (pg/mL)	38.0 (13.4, 134.9)	65.9 (12.0, 82.7)	0.97
TNF-α (pg/mL)	6.5 (4.0, 9.3)	7.4 (3.8, 12.5)	0.57
TGF-β (pg/mL)	490 (440, 553)	497.5 (338.8, 641.3)	0.63
CRP (mg/L)	8.7 (2.0, 24.0)	9.1 (2.3, 39.3)	0.76
Albumin (g/L)	44.8 (43.0, 48.9)	46.9 (43.4, 50.2)	0.41
Growth hormone (pg/mL)	955.7 (496.3, 4117.2)	988.8 (222.5, 3075)	0.44

Table 3.4. ***Comparison of the inflammatory markers and growth hormone concentration between phenotypic extensive and poor metabolisers of advanced incurable cancer patients.*** *P*-values were calculated using Mann-Whitney rank-sum test.

There was no significant difference in any of the measured biological markers between extensive or poor metaboliser patients.

Advanced cancer is associated with cachexia and this is associated with elevated cytokines (Tisdale 2001). Whilst cachexia was not directly assessed in the present study, the body mass index (BMI) could be determined from the patient's height and weight at the time of recruitment. Of the 13 patients with a BMI of less than 25 kg/m^2, 62% (n = 8) patients were phenotypic poor metabolisers whereas only 4 out of 18 (22%) patients with a BMI of greater than 25 kg/m^2 were phenotypic poor metabolisers. There was a significant difference (P = 0.03) in proportions of poor metabolisers in each of these BMI categories.

3.3 Discussion

This larger and more extensive study has confirmed the previous observation (Williams et al. 2000) that CYP2C19 metabolic activity was compromised in patients with advanced incurable cancer. There was a significant discordance between CYP2C19 genotype and metabolic activity. Of the patients that were genotypic extensive metaboliser (*wt/wt* and *wt/var*), 37% had compromised CYP2C19 metabolic activity which is similar to the 25% reported in the previous study. The difference in incidence between the two studies could be due to the relatively small sample sizes or due to differences in the disease stage or tumour type between the two patient groups.

The expected incidence of CYP2C19 poor metabolisers in a healthy Caucasian population is 3% (Wilkinson et al. 1989; Bertilsson 1995) and there is a good correlation between genotype (homozygous variant) and poor metaboliser phenotype in healthy populations (Xie et al. 1999b). The cancer patient population in this study was predominantly Caucasian with one patient self identified as Eurasian, three patients with no recorded ethnicity and one patient who was Chinese. The patient in the present study that self identified as Chinese was a homozygous variant genotype for the *CYP2C19*3* allele, which is common in Asian populations (de Morais et al. 1994a; Bertilsson 1995).

The log omeprazole hydroxylation index (HI) of genotypic extensive metabolisers in a healthy population was reported to range between -0.55 and 0.40 (Balian et al. 1995). However, in the present study of the genotypic extensive metabolisers of the cancer patients, the bimodality was lost and there appeared to be a generalised decrease in CYP2C19 activity in advanced cancer with the log hydroxylation index ranging from -0.10 to 2.0.

There are many clinical factors that may influence hepatic drug metabolism including co-medications. Polypharmacy is common in cancer patients due to the complex treatment regimes. However, the inclusion criteria for the present study excluded patients that were on any medication. Furthermore, abnormalities or disease in liver and kidney function may contribute to decreased hepatic metabolism (Andersson et al. 1993b; Elston et al. 1993; Frye et al. 1996; Frye et al. 2006); however none of the patients in the current study had obvious compromised liver or kidney function as the inclusion criteria included patients with adequate liver and kidney function as determined by standard

clinical measures. Hence compromised CYP2C19 function is unlikely to be directly due an overall decrease in liver function. This is in contrast to the previous study (Williams et al. 2000), where a number of the patients had elevated ALP and two patients had hepatitis.

In the present study, although five patients had liver metastases, these subjects did not have a phenotypic poor metaboliser status. In addition, there was no correlation between the location/type of the primary tumour and poor metaboliser status.

Previous studies have suggested that inflammation may play a role in compromised CYP metabolising activity. However, in the present study, no relationship was observed between CYP2C19 metabolic activity and levels of the measured inflammatory cytokines or acute phase response proteins. The levels of cytokines observed in the present study were in the same range as the data reported previously in cancer patients (Rivory et al. 2002) and in congestive heart failure (Frye et al. 2002). However, the concentrations of IL-6 and TGF-β required to significantly down-regulate *CYP2C19* mRNA expression *in vitro* (Aitken et al. 2007) were approximately 100-fold higher than the levels observed in the cancer patients in the present study. The circulating half-life of cytokines is relatively short (Beutler et al. 1985; Castell et al. 1988; Klapproth et al. 1989) and the presence of soluble receptors may underestimate free circulating cytokine levels (Fernandez-Botran 1991; Rose-John et al. 1994). In addition, levels of cytokines can oscillate throughout the day (Munoz et al. 1991; Togo et al. 2009) . Moreover, alternative cytokines or other inflammatory mediators that were not measured in this study may play a role in the down-regulation of CYP2C19 activity. CYP3A4 activity has been found previously to be inversely correlate with CRP levels in cancer patients (Rivory et al. 2002). However, there was no significant correlation between the CYP2C19 metabolic activity and CRP in the present study. Nevertheless, only five patients had CRP levels > 40 mg/L in this study and hence CRP concentrations were relatively low in comparison with the CYP3A4 study (Rivory et al. 2002). Hypoalbuminemia also occurs as an effect of elevated acute phase response and albumin levels negatively correlate to CRP levels (McMillan et al. 2001a; McMillan et al. 2001b; Al-Shaiba et al. 2004). There was no relationship between the albumin and CRP levels in the patients of this study, but this relationship only appears to occur when there are relatively high levels of CRP. Hence although no correlation was demonstrated in the present study, a role for inflammatory mediators cannot be disregarded.

Administration of human growth hormone has been reported to decrease CYP2C19 activity in healthy volunteers (Jürgens et al. 2002) and growth hormone has been reported to be elevated in cancer (Dülger et al. 2004). Although the growth hormone levels in the cancer patients were highly variable, there was no correlation with decreased CYP2C19 metabolic activity.

Cachexia, a paraneoplastic syndrome associated with selective loss of skeletal muscle and progressive weight loss which is associated with elevated inflammatory cytokines, particularly IL-6 and TNF-α, decreased albumin production, as well as decreased growth hormone levels (Dülger et al. 2004). An arbitrary cut-off value of BMI = 25 kg/m^2 defines individuals as overweight in the current World Health Organization classification (2006), and interestingly, CYP2C19 poor metabolism was associated with patients with a BMI of less than 25 in this study. However BMI is not a good measure of cachexia particularly in a late stage disease, as progressive weight loss due to increased muscle and fat catabolism may be obscured by weight gain due to ascites. Furthermore, decreasing body mass index may also be a consequence of chemotherapy-associated side effects such as vomiting and diarrhoea.

In conclusion, this study confirmed the previous finding that CYP2C19 activity is compromised in cancer patients with up to 37% of subjects categorised as poor metabolisers. Although this preliminary study could not demonstrate a relationship between inflammatory markers, this mechanism cannot be discounted. Notably compromised CYP2C19 activity in cancer patients may be of clinical importance as it may affect the patient's response to chemotherapeutic drugs that are substrates for CYP2C19.

CHAPTER 4

CYP2C19 metabolic status at various stages of cancer

4.1 Introduction

In the previous clinical study described in chapter 3, a discordant relationship was found between the CYP2C19 genotype and phenotype in a population of patients with advanced incurable (terminal) cancer. 37% of these patients had compromised CYP2C19 functional activity despite having an extensive metaboliser genotype (*wt/wt, wt/var*) (Helsby et al. 2008).

It was hypothesised that the compromised CYP2C19 metabolism was due to the inflammatory status of the cancer patients. However as reported in chapter 3, there was no clear relationship between the measured inflammatory mediators and the CYP2C19 metabolic status in that cohort of the patients.

To determine whether the compromised CYP2C19 activity observed in the initial studies (chapter 3) occurs in patients at an earlier stage of cancer progression, a second clinical study was designed. This second clinical study also included additional measurements based on the findings from the initial study (chapter 3).

Interestingly, the discordant relationship observed in the initial study (chapter 3) was associated with the patient's body mass index (BMI). There were significantly more poor metabolisers (62% vs 22%) in the group of patients that had a BMI less than 25 kg/m^2 in comparison to those patients with a BMI greater than 25 kg/m^2 (P = 0.03). Progressive loss of weight may be associated with cancer cachexia, which involves wasting of adipose tissue and lean body mass in the patients (Tisdale 2001). Inflammatory cytokines are associated with the progression of cancer cachexia (Matthys et al. 1997). However, BMI is not a good measure of cachexia as body weight can also be influenced by ascites, or poor nutritional status due to chemotherapy induced vomiting. A more common method to assess the nutritional status of individuals is arm anthropometry (Jelliffe 1966), where the muscle circumference and cross-sectional muscle and fat areas of an individual can be calculated following the measurement of the arm circumference and triceps skin-fold thickness (Gurney et al. 1973; Norman et al. 2006). To investigate further the possible role of the change in body mass as a correlate with CYP2C19 compromised metabolic status, more detailed anthropometry using skin-fold thickness measurements was undertaken in this second study.

Omeprazole was used as the probe drug in the determination of CYP2C19 metabolic activity in the previous clinical study (chapter 3). However, omeprazole is commonly prescribed to the patients on chemotherapy who may experience epigastric disturbance due to chemotherapy induced nausea and vomiting. Multiple dosing with omeprazole has the ability to induce CYP1A2 (Rost et al. 1992), and this precludes the use of this drug as a selective probe of CYP2C19 activity in subjects on chronic therapy. Time to recruit patients into the previous study was limited by this widespread use of omeprazole as these patients were specifically excluded from the study.

To increase the recruitment rate in the study described in this chapter, an alternative CYP2C19 probe drug, proguanil, was used. The major metabolic route of proguanil *in vivo* is catalysed by CYP2C19 (Funck-Brentano et al. 1997) and the *CYP2C19* genetic polymorphism influences the pharmacokinetics of proguanil and formation of cycloguanil with a clear gene-dose effect (Herrlin et al. 2000). In order to prevent any co-inhibition of CYP2C19 activity in patients who may have been prescribed omeprazole, these individuals were advised to withhold the use of omeprazole for at least three half-lives (i.e. > 12 h) before the probe test with proguanil.

The previous study (chapter 3) was undertaken in cancer patients who had been heavily pretreated with chemotherapy but did not respond to treatment and were no longer on treatment and had no further therapeutic options. The consequences of compromised hepatic drug metabolising enzyme function will however be particularly important during first-line chemotherapy. It is therefore imperative to determine whether CYP2C19 function is also compromised in patients at earlier stages of treatment and disease progression. In order to undertake such a study, patients with colorectal carcinoma were recruited as detailed below.

Management of colorectal carcinoma involves surgical resection of the primary tumour followed by a course of chemotherapy to prevent recurrence of the disease in those patients with no evidence of metastases. However, metastatic colorectal carcinoma is often present, with hepatic lesions common, thus management of these patients following resection of the primary tumour involves more extensive chemotherapy to control disease progression. The study comprised patients with stage IV colorectal carcinoma who had resection of the primary tumour (where possible). Patients were then sub-categorised into those with absence of primary tumour and metastases (no-

evaluable disease, NED) and those with metastatic burden with or without primary tumour (stage IV).

The hypothesis is that discordance also occurs at earlier stages of disease.

The aims of this study were firstly to determine whether CYP2C19 functional activity is also compromised in a different population of cancer patients with earlier stage of disease. Secondly to investigate further the potential role of cancer-associated inflammation and the nutritional status of the patients with respect to CYP2C19 function. In addition, the relationship between tumour burden and any potential confounding effects of chemotherapy on the CYP2C19 activity were also studied.

A preliminary analysis of the data from this ongoing study is described in this chapter.

4.1.1 Study design

The study was approved by the Northern X Regional Ethics Committee (NTX08/07/060). Two separate groups of gastrointestinal cancer patients with either stage IV or no-evaluable disease (resected) were recruited into the study. All patients were over 18 years old and gave full informed written consent. All patients had adequate hepatic and renal function (creatinine levels < 0.12 mmol/L, AST/ALT < 90 U/L, ALP < 300 U/L, bilirubin < 20 µmol/L). Patients receiving medication that is a known inhibitor or inducer of CYP2C19, and where a washout period was not clinically feasible were excluded from the study.

The study aimed to recruit at least 25 eligible patients in each group (a total of 50 patients) in order to detect with 80% power an incidence of at least 20% poor metabolisers compared with an expected incidence of 3% in a healthy Caucasian population (Wilkinson et al. 1989). However, at the date of analysis for submission of this thesis, recruitment was n = 15 for stage IV disease and n = 14 for no-evaluable disease.

The disease stage of each patient was categorised using the TNM staging criteria (Greene et al. 2001; O'Connell et al. 2004). The tumour burden was determined by an adaption of the RECIST criteria (Response Evaluation Criteria in Solid Tumours)

(Therasse et al. 2000; Eisenhauer et al. 2009). In this study, the linear sum of the six largest tumour deposits was measured from the patient's CT scan or X-ray.

Patients were initially dispensed with the probe drug, proguanil, and were advised to take a single oral dose (200 mg) of proguanil three hours in advance of their next clinic visit for routine tumour and biochemistry assessment. Thus proguanil was administered between the scheduled chemotherapy cycles. A total of 28.5 mL of blood was obtained. This included 5 mL in a plain tube which was sent to the local clinical laboratory service (LabPLUS) for measurement of C-reactive protein, 5 mL in a plain tube for serum preparation (as described in section 2.4.2), 10 mL in a heparinised tube for plasma preparation (as described in section 2.3.2) and 8.5 mL in a PAXgene™ blood DNA tube for storage prior to preparation of genomic DNA to determine *CYP2C19* genotype (as described in section 2.2). Plasma was analysed for proguanil and cycloguanil metabolite concentrations (as described in section 2.7) whilst serum was used to determine levels of various proinflammatory cytokines (section 2.4). CYP2C19 activity was reported as a continuous variable (drug/metabolite ratio) and as a categorical variable (extensive or poor metaboliser).

Triceps skin-fold thickness, measured using callipers, and arm circumference measurements were also taken at the clinic visit. However, these two measurements alone are not a precise diagnosis of the nutritional status of the patients. This requires calculation of the cross-sectional fat and muscle areas using the following equations based on arm circumference (C) and triceps skin-fold thickness (T) (Gurney et al. 1973; Reid et al. 1992; Norman et al. 2006).

$$\text{Fat area } (mm^2) = \frac{T \times C}{2} - \frac{\pi T^2}{4} \qquad \text{Muscle area } (mm^2) = \frac{(C - \pi T)^2}{4\pi}$$

Statistical analyses were determined using GraphPad Prism (Version 5.02, GraphPad Software Inc, USA) and SigmaPlot (Version 11.0, Systat Software Inc, Germany). Continuous data were reported as the median and interquartile range (25%-75% quartile; IQR). Associations between continuous variables were assessed using Spearman's rank correlation, whilst Mann-Whitney rank-rum tests were used to compare continuous variables across two groups. The proportions were compared using Chi-square and Fisher's exact test. Two-sided *P*-values < 0.05 were considered statistically significant.

4.2 Results

4.2.1 Demographics

Following full informed written consent, a total of 29 patients with gastrointestinal cancer were recruited into the study. Of these, 15 had stage IV disease and 14 had no-evaluable disease. The demographics of the patients are listed in Table 4.1. Arm circumference measurements were not recorded for patients #1001, #1002 and #1003 at the time of sample collection due to collection error.

There were 19 male and 10 female patients with a median age of 66 years (IQR 52-77) and a median weight of 76 kg (IQR 63.9-86.0). These ages and weights were similar to the values observed in the previous study in patients with advanced incurable cancer (Table 3.1).

Of the 15 patients with stage IV disease, 11 were male and 4 female, with a median age of 73 years (IQR 58-77) and a median weight of 77.7 kg (IQR 63.5-93.3). There were 8 males and 5 females with no-evaluable disease with a median age of 60.5 years (IQR 47.8-77.3) and a median weight of 74.3 kg (IQR 64.0-84.6). There was no significant difference in the age and weight between both groups ($P > 0.05$).

The body mass indices of both categories of cancer patients were also similar (median 26.8, IQR 24.3-31.5 in stage IV; median 25.9, IQR 24.7-27.4 in no-evaluable disease). Similarly, there was no difference in the triceps skin-fold thickness (median 11.5 mm, IQR 10.0-13.0 in stage IV; median 10.6 mm, IQR 7.0-17.5 in no-evaluable disease) and arm circumference measurements (median 316.0 mm, IQR 276.3-334.8 in stage IV; median 292.0 mm, IQR 268.5-319.5 in no-evaluable disease) between both groups of patients. There was also no difference in the cross-sectional arm fat area (median 1509 mm^2, IQR 1177-1746 versus median 1108 mm^2, IQR 819-2010) or muscle area (median 6074 mm^2, IQR 4684-7036 versus median 5205 mm^2, IQR 4709-6264) between the patients with stage IV or no-evaluable disease respectively. None of the differences between the two groups were of statistical significance ($P > 0.05$).

Patient #	Stage	Gender	Ethnicity	Age (yrs)	Height (cm)	Weight (kg)	BMI (kg/m^2)	Triceps skin-fold (mm)	Arm circumference (mm)	Arm fat area (mm^2)	Arm muscle area (mm^2)
1001	IV	M	NZ European	75	172.0	93.3	31.5	10.8	ND	ND	ND
1002	IV	M	Dutch	79	177.5	77.7	24.8	7.2	ND	ND	ND
1003	IV	F	NZ European	83	149.0	62.3	28.1	20.4	ND	ND	ND
1004	IV	M	NZ European	82	168.0	105.4	37.3	13.8	322	1751.9	6178.7
1005	IV	M	NZ European	58	175.0	75.0	24.3	12.2	287	1383.5	4920.9
1006	IV	M	NZ European	73	174.0	84.7	28	13.4	335	1801.5	6827.1
1008	IV	M	NZ European	48	180.0	87.0	26.9	13	330	1728.0	6653.7
1009	IV	M	English	77	179.0	79.9	24.9	10	275	1128.3	4721.6
1013	IV	M	English	65	181.0	116.0	35.4	10	392	1713.3	10346.7
1020	IV	F	NZ European	66	160.0	46.5	18.2	10	225	878.3	2982.1
1021	IV	M	NZ European	53	171.0	76.0	26	11.2	334	1560.9	7105.5
1022	IV	F	NZ European	74	168.0	63.5	22.5	8	240	802.1	3673.9
1027	IV	F	NZ European	42	161.0	61.8	23.8	12	280	1324.7	4672.0
1028	IV	M	NZ European	74	166.0	73.9	26.8	11.5	310	1456.2	5968.8
1029	IV	M	NZ European	65	179.0	115.0	35.9	13	340	1793.0	7121.9
1007	NED	F	NZ European	37	158.0	63.7	25.5	15	288	1604.8	4617.2
1010	NED	M	NZ European	79	178.0	84.3	26.6	6.8	307	929.7	6492.6
1011	NED	F	NZ European	48	168.0	96.2	34.1	32.2	380	3559.7	6187.3
1012	NED	M	Indian	79	169.0	75.0	26.3	6.2	289	801.1	5780.7
1014	NED	F	English	59	157.0	61.5	25	13.6	290	1515.6	4865.7
1015	NED	M	NZ European	77	178.0	84.9	26.8	7.6	316	1058.3	6790.9
1016	NED	M	Filipino	47	171.0	97.4	33.3	14.4	346	1979.6	7198.4
1017	NED	M	English	58	189.0	84.5	23.7	7.6	294	974.7	5806.5
1018	NED	F	NZ European	43	167.0	81.2	29.1	19	315	2101.8	5187.1
1019	NED	M	NZ European	78	169.0	72.3	25.3	7	264	803.1	4660.7
1023	NED	M	English	69	168.0	73.5	26	7	270	824.1	4894.7
1024	NED	F	NZ European	51	161.0	48.8	18.8	17	220	1156.9	2208.5
1025	NED	M	NZ European	75	168.0	64.1	22.7	5.2	260	609.3	4724.7
1026	NED	F	NZ European	62	162.0	67.8	25.8	23.5	330	2514.9	5222.2

Table 4.1. ***The demographics of the cancer patients with stage IV or no-evaluable disease.*** NED = no-evaluable disease. BMI = Body mass index. ND = not determined

4.2.2 *CYP2C19* genotype

Following isolation of genomic DNA, the *CYP2C19*2*, *CYP2C19*3* and *CYP2C19*17* variant alleles of *CYP2C19* were determined using PCR-RFLP as described in section 2.2.

None of the patients were genotypic poor metabolisers (*CYP2C19*2/*2*, **2/*3*, **3/*3*). Sixteen patients had *CYP2C19*1/*1* genotype, four were *CYP2C19*1/*17*, three *CYP2C19*17/*17*, one *CYP2C19*17/*2,* four *CYP2C19*1/*2* and one *CYP2C19*1/*3*. All these patients were classified as genotypic extensive metabolisers. Individual patient genotypes are shown in Table 4.2.

Patient #	Disease stage	*CYP2C19* genotype	Predicted phenotype	Metabolic ratio (drug/metabolite)	Measured phenotype	Genotype-phenotype discordant?
1001	IV	**17/*2*	EM	4.01	EM	N
1002	IV	**1/*1*	EM	3.13	EM	N
1003	IV	**1/*1*	EM	6.72	EM	N
1004	IV	**1/*1*	EM	16.47	PM	Y
1005	IV	**17/*17*	EM	150.57	PM	Y
1006	IV	**1/*17*	EM	10.01	PM	Y
1008	IV	**1/*1*	EM	4.48	EM	N
1009	IV	**1/*1*	EM	12.22	PM	Y
1013	IV	**17/*17*	EM	6.15	EM	N
1020	IV	**1/*1*	EM	2.12	EM	N
1021	IV	**1/*1*	EM	3.27	EM	N
1022	IV	**1/*2*	EM	3.35	EM	N
1027	IV	**1/*1*	EM	0.88	EM	N
1028	IV	**1/*1*	EM	3.85	EM	N
1029	IV	**17/*17*	EM	2.64	EM	N
1007	NED	**1/*17*	EM	2.32	EM	N
1010	NED	**1/*1*	EM	7.97	EM	N
1011	NED	**1/*1*	EM	22.74	PM	Y
1012	NED	**1/*1*	EM	3.24	EM	N
1014	NED	**1/*17*	EM	3.60	EM	N
1015	NED	**1/*2*	EM	6.32	EM	N
1016	NED	**1/*3*	EM	5.33	EM	N
1017	NED	**1/*17*	EM	7.38	EM	N
1018	NED	**1/*1*	EM	13.87	PM	Y
1019	NED	**1/*1*	EM	1.06	EM	N
1023	NED	**1/*1*	EM	1.28	EM	N
1024	NED	**1/*2*	EM	0.93	EM	N
1025	NED	**1/*1*	EM	0.93	EM	N
1026	NED	**1/*2*	EM	2.14	EM	N

Table 4.2. ***The CYP2C19 genotype and phenotype of individual cancer patients with stage IV or no-evaluable disease.*** NED = no-evaluable disease. EM = extensive metaboliser, PM = poor metaboliser. Y = yes, N = no.

The Hardy-Weinberg equilibrium of the variant allele frequency was assessed using the equation ($p^2 + 2pq + q^2 = 1$), where p is the number of wildtype alleles and q is the number of variant alleles (either *CYP2C19*2* or *CYP2C19*3)*. The observed and expected allele totals and frequencies for *CYP2C19*2*, *CYP2C19*3* and *CYP2C19*17* are shown in Table 4.3.

Genotype	***1/*1***	***1/*2***	***2/*2***	**Total**
Observed *n*	24	5	0	29
Observed frequency	0.828	0.172	0	1
Expected frequency	0.835	0.158	0.007	1
Expected *n*	24.22	4.57	0.22	29
Genotype	***1/*1***	***1/*3***	***3/*3***	**Total**
Observed *n*	28	1	0	29
Observed frequency	0.966	0.034	0	1
Expected frequency	0.966	0.034	0	1
Expected *n*	28.01	0.98	0.01	29
Genotype	***1/*1***	***1/*17***	***17/*17***	**Total**
Observed *n*	22	4	3	29
Observed frequency	0.759	0.138	0.103	1
Expected frequency	0.685	0.285	0.030	1
Expected *n*	19.86	8.28	0.86	29

Table 4.3. ***Genotype totals and frequencies for CYP2C19*2*, *CYP2C19*3*** **and** ***CYP2C19*17 in patients with stage IV or no-evaluable disease.*** All alleles were in agreement with Hardy-Weinberg equilibrium (χ^2 = 0.24, *P* = 0.8867 for *CYP2C19*2*; χ^2 = 0.01, *P* = 0.9949 for *CYP2C19*3*; χ^2 = 2.788, *P* = 0.2481 for *CYP2C19*17*).

The distribution of allele frequencies for *CYP2C19*2*, *CYP2C19*3* and *CYP2C19*17* were all in agreement with Hardy-Weinberg equilibrium (χ^2 = 0.24, *P* = 0.8867 for *CYP2C19*2*; χ^2 = 0.01, *P* = 0.9949 for *CYP2C19*3*; χ^2 = 2.788, *P* = 0.2481 for *CYP2C19*17*).

Two patients (#1012 and 1016) were not of Caucasian origin. Patient #1012 was of Indian ethnicity and had *CYP2C19*1/*1* genotype. Patient #1016 was of Filipino origin and had *CYP2C19*1/*3* genotype. The *CYP2C19*3* variant is extremely rare in Caucasian population (de Morais et al. 1994a) and this Filipino individual was the only patient to carry this variant allele.

4.2.3 CYP2C19 metabolic activity

The CYP2C19 metabolic activity in each individual patient volunteer was determined using proguanil as the probe drug (as described in section 2.7). The metabolic ratio (drug/metabolite) was used to categorise patients as extensive metaboliser (metabolic ratio < 10) or poor metaboliser (metabolic ratio ≥ 10), as previously described (Ward et al. 1989b; Helsby et al. 1990b).

The median metabolic ratio in the group of patients with stage IV disease was 4.01 (IQR 3.13-10.01) and was similar to that observed in the patients with no-evaluable disease (median 3.42, IQR 1.23-7.53; *P* = 0.4194).

Importantly, there were patients that were genotype-phenotype discordant in both the stage IV and no-evaluable disease group despite the presence of normal function genes. For example, patient #1009 with stage IV disease had poor metabolic activity (metabolic ratio = 12.22) and patient #1018 with no-evaluable disease had a metabolic ratio of 13.87 and was also a poor metaboliser. Both these patients had a wildtype *CYP2C19*1/*1* genotype (Table 4.2). 4 out of 15 (27%) of the patients with stage IV disease were discordant while 2 out of 14 (14%) patients with no-evaluable disease were poor metabolisers (Table 4.2). The difference between the proportion of discordant patients between both disease categories was not significant (*P* = 0.6513).

This genotype-phenotype discordance was only observed in individuals who were homozygous wildtype (*CYP2C19*1/*1, *1/*17, *17/*17*; Figure 4.1). However, only small numbers (n = 2 and n = 4) were heterozygote carriers (*CYP2C19*1/*2, *1/*3* and **17/*2)* in stage IV and no-evaluable disease category respectively.

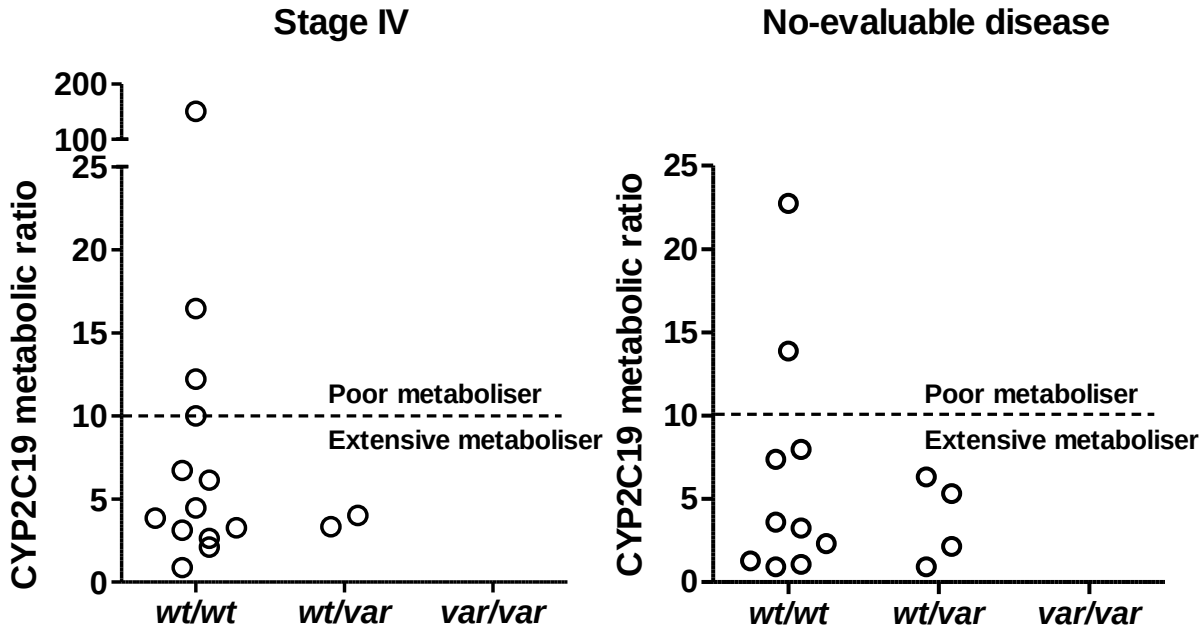

Figure 4.1. ***The distribution of CYP2C19 metabolic activity in cancer patients with stage IV or no-evaluable disease relative to genotype.*** The difference in the proportions of phenotypic poor metabolisers in both disease categories was not significant (*P* = 0.6513). *wt/wt = CYP2C19*1/*1, *1/17, *17/17*; *wt/var = CYP2C19*1/2, *1/*3, *17/2* and *var/var = CYP2C19*2/*2* and **3/*3.*

Thus it appears that compromised CYP2C19 activity also occurs in cancer patients with stage IV and no-evaluable disease. This confirms the presence of discordance observed previously in advanced incurable terminal disease (chapter 3).

Comparison of the incidence of phenotypic poor metabolisers across the two clinical studies was possible since there was a reported direct correlation (Spearman r = 0.87, *P* < 0.01) between the metabolic ratios of both probe drugs, omeprazole and proguanil (Herrlin et al. 2000). The antimode to categorise individuals as poor metabolisers of omeprazole and proguanil agreed with each other (omeprazole log hydroxylation index ≥ 1 and proguanil/cycloguanil ratio ≥10 respectively). Individuals who have a ratio greater than the antimode (≥ 10) for proguanil have previously been demonstrated to be homozygous for loss-of-function alleles (Herrlin et al. 2000).

There was a lower proportion of poor metabolisers in the group of patients with no-evaluable disease (14%) and in patients with stage IV disease (27%) compared with the group with advanced incurable cancer (37%) previously reported in chapter 3 (Figure 4.2). There was a general decrease in the incidence of genotype-phenotype discordance with disease severity, however this is not statistically significant (*P* = 0.065), possibly due to the small sample size for this preliminary analysis.

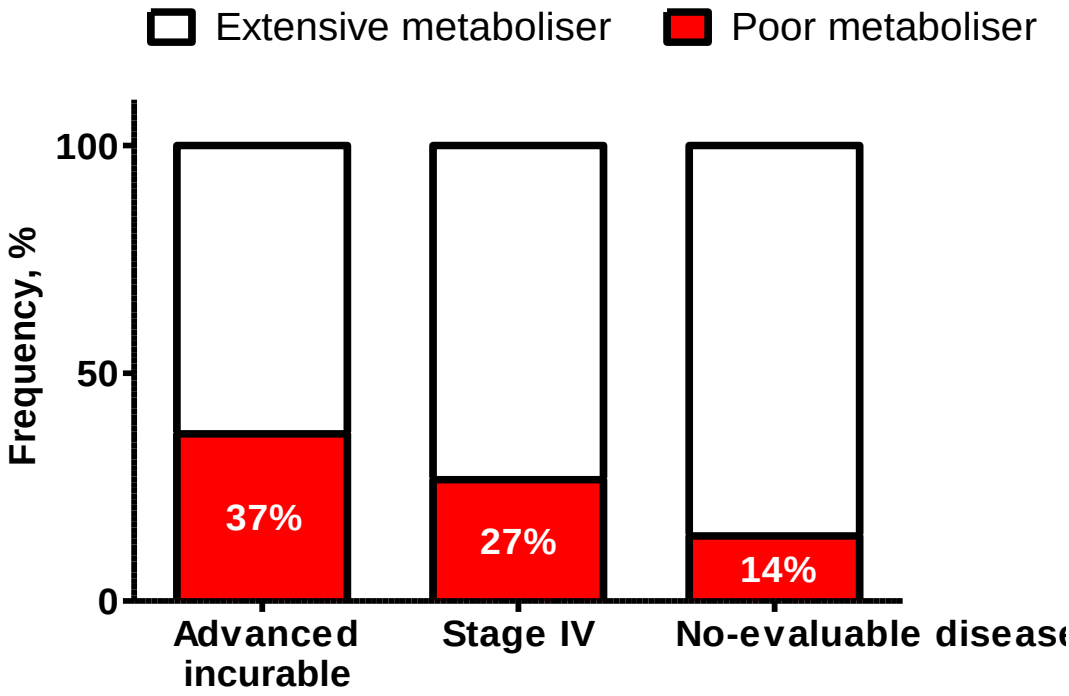

Figure 4.2. ***Proportion of CYP2C19 phenotypic poor metabolisers in different patient groups.***
There was a general decrease in the incidence of genotype-phenotype discordance with disease severity, however this was not statistically significant (*P* = 0.065). The metabolic ratio of the advanced incurable cancer group (n = 30) was measured using omeprazole as the probe drug (log hydroxylation index ≥ 1 is indicative of a poor metaboliser). Proguanil was used as the probe drug to determine the metabolic ratio of stage IV (n = 14) and no-evaluable disease group (n = 15). A proguanil metabolic ratio ≥ 10 is indicative of a poor metaboliser.

4.2.4 Tumour burden

To investigate whether total tumour burden was related to CYP2C19 activity, the tumour burden in patients with stage IV disease was determined from CT scans or X-rays using RECIST evaluation as described in the study design (section 4.1.1). CT scans or X-rays were often unavailable at the same time as the proguanil phenotyping test, hence only the CT scan or X-ray closest to the phenotype test in each patient was analysed for this part of the study. An example of how the tumour burden from a CT scan was measured that of patient #1004 is shown in Figure 4.3. Patient #1004 had four liver metastatic deposits; these were 53, 79, 68 and 46 mm in the largest dimension. Hence the patient had a total tumour burden of 246 mm.

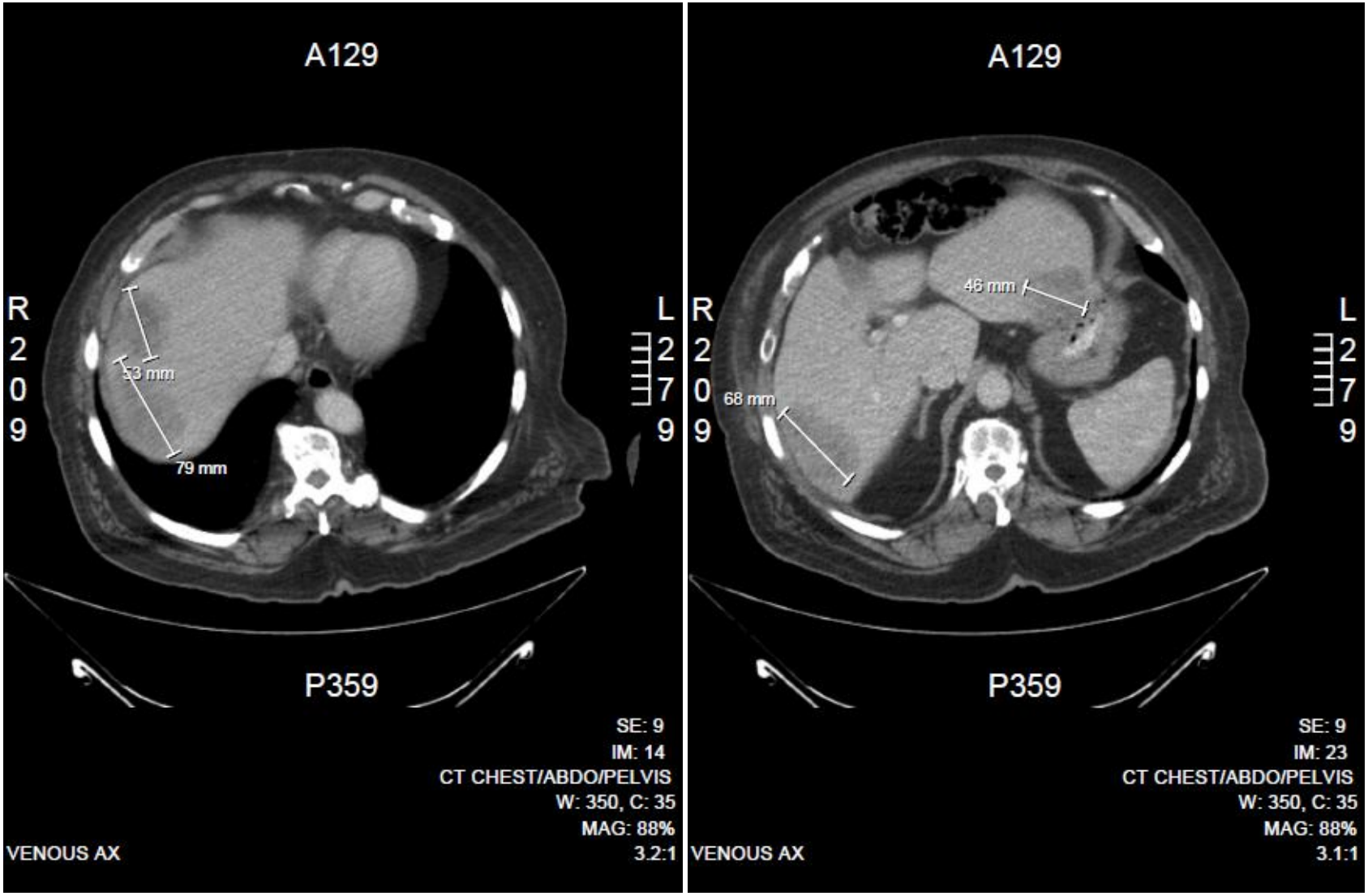

Figure 4.3. ***An example of RECIST performed on a CT scan of patient #1004.*** Dimensions were taken on the longest axis and measured in millimetres.

The tumour burden and the location of the colorectal metastases in each patient with stage IV disease are shown in Table 4.4. The majority of the stage IV patients in this study had the primary colorectal tumour removed but had colorectal metastases which were mainly in the liver. There were two patients (#1009 and #1027) who still had a primary tumour (burden of 37.1 mm and 100 mm respectively) but did not have liver colorectal carcinoma metastases (Table 4.4). Although many of the patients with stage IV disease had colorectal metastases in the liver, all patients in the study had adequate liver and renal function determined by standard clinical measures due to the inclusion criteria of the study and hence compromised CYP2C19 activity is unlikely to be due to disturbance of normal liver function.

Patient #	Date of CYP2C19 test	Metabolic ratio (drug/metabolite)	Date of CT scan	Tumour burden, mm	Location of metastases
1001	17/04/2009	4.01	17/04/2009	131	Liver
1002	07/04/2009	3.13	22/05/2009	291	Liver
1003	01/05/2009	6.72	14/04/2009	183	Liver
1004	01/10/2009	16.47	10/11/2009	246	Liver
1005	14/09/2009	150.57	28/09/2009	178	Liver
1006	27/08/2009	10.01	01/09/2009	136	Liver
1008	08/09/2009	4.48	25/08/2009	59	Liver
1009	15/09/2009	12.22	17/08/2009	37.1	None
1013	03/09/2009	6.15	14/09/2009	85	Lymph nodes
1020	14/09/2009	2.12	05/08/2009	124	Liver
1021	16/09/2009	3.27	06/10/2009	131	Liver
1022	03/05/2010	3.35	30/04/2010	72	Liver
1027	09/06/2010	0.88	07/04/2010	100	None
1028	24/06/2010	3.85	17/07/2010	73	Liver
1029	16/07/2010	2.64	21/06/2010	83	Liver

Table 4.4. ***The tumour burden of the patients with stage IV disease determined from CT scans or X-rays using RECIST.*** Dates are shown as DD/MM/YYYY.

Although there was a higher tumour burden (median 157 mm, IQR 61.8-229.0) in four poor metaboliser patients compared with extensive metabolisers (n = 11, median 100 mm, IQR 73.0-131.0), this was not significant (*P* = 0.4723) (Figure 4.4). This lack of significance may relate to the small number of patients with poor metaboliser phenotype in this preliminary analysis.

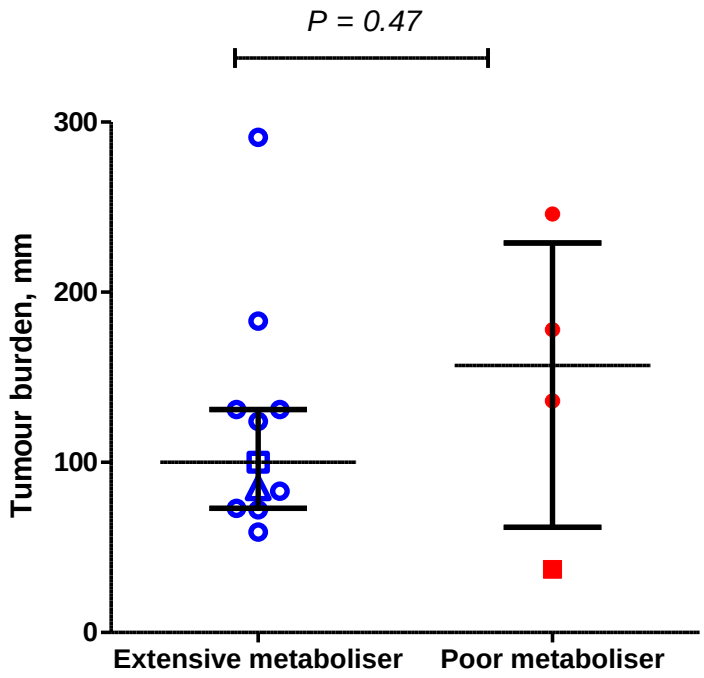

Figure 4.4. The association between higher tumour burden in stage IV patients who were poor metabolisers compared to *extensive metabolisers, although not significant (P = 0.47).* Circle represents those patients that have liver metastases, square represents absence of metastases (primary tumour only) and triangle represent metastases at lymph nodes. Data are shown as median (interquartile range).

When CYP2C19 activity was determined as a continuous variable, there was no apparent correlation with increasing tumour burden (Spearman r = 0.1769, *P* = 0.5281) (Figure 4.5).

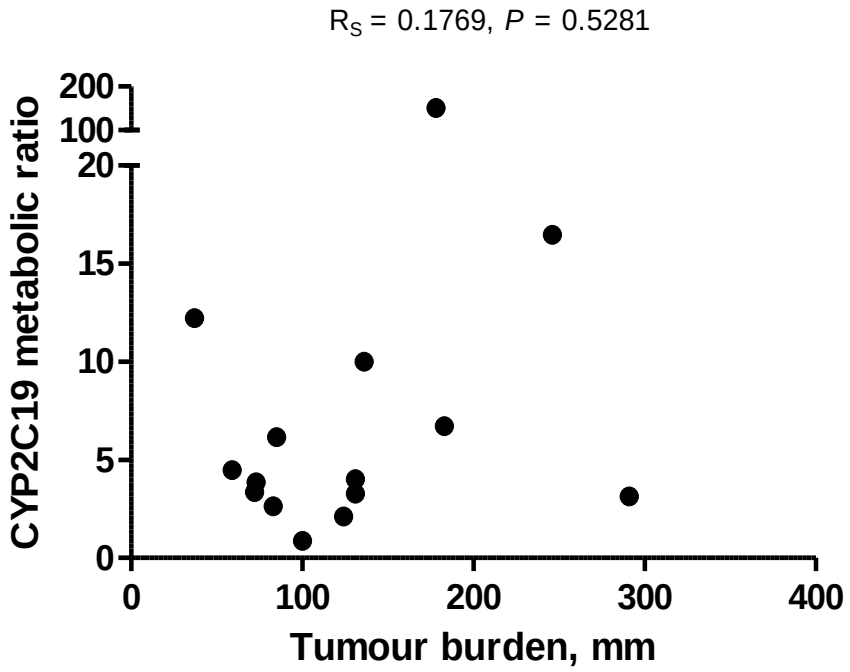

Figure 4.5. ***The lack of relationship between CYP2C19 metabolic ratio and the tumour burden in patients with stage IV disease.*** Spearman r = 0.1769, *P* = 0.5281.

In summary, there was a trend to a higher tumour burden in phenotypic poor metabolisers compared with extensive metabolisers, but this was not significant. However, the relatively small sample size (n = 15) at this stage of recruitment may limit detection of any potential direct relationship between CYP2C19 activity and tumour burden.

4.2.5 Cancer chemotherapy

One potential factor which may lead to differences in the proportion of poor metabolisers in the different patient groups (Figure 4.2) is the different chemotherapeutic history. Patients with advanced incurable cancer (chapter 3) were heavily pre-treated with chemotherapy and were only enrolled into the study when no further treatment options were available so were no longer receiving active chemotherapy. In contrast, patients in the current clinical study were given chemotherapy as part of their normal clinical treatment. The phenotype probe test was determined during the chemotherapy washout period. However, chemotherapy may be a possible contributing factor to the genotype-phenotype discordance observed in these patients in the current study.

Example schedules of the chemotherapy regimens of the patients in this study and the temporal relationship to the CYP2C19 phenotype tests are shown in Figure 4.6.

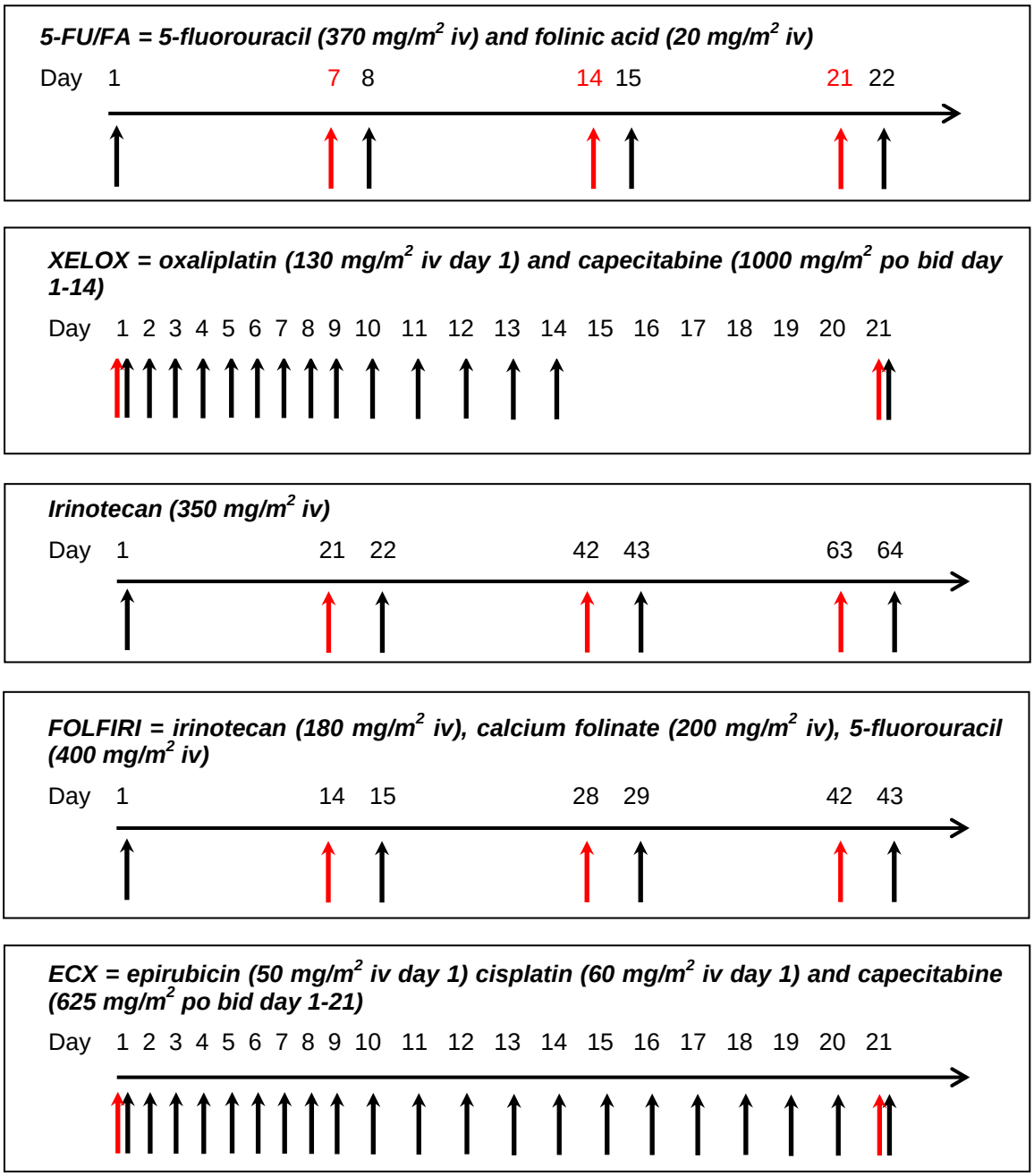

Figure 4.6. ***Typical chemotherapy regimens of the patients with stage IV or no-evaluable disease and the relationship to the wash-out period prior to phenotype test.*** Black arrow indicates time of chemotherapy and red arrow indicates time of phenotype test. Additional chemotherapy regimens include: capecitabine (1250 mg/m^2 po bid, day 1-14), and gemcitabine (1000 mg/m^2 iv day 1, 8, 15) where the phenotype test was undertaken during cycle intervals (i.e. day 7).

Table 4.5 indicates the treatment schedules of each patient and the relationship to the date of the phenotype test. Most of the patients with no-evaluable disease (NED) were on the XELOX (n = 7) or 5-FU/FA regimen (n = 5) with the exception of one patient on ECX regimen and one that had not initiated any chemotherapy regime at the time of the phenotype test. Capecitabine is a prodrug of 5-fluorouracil (Miwa et al. 1998) and therefore 13 of the NED patients had been pre-treated with a 5-fluorouracil containing therapy for at least 7 days prior to the phenotype test.

There were 5 patients with stage IV disease on the XELOX regimen and 2 patients on capecitabine, 3 on FOLFIRI, 2 on irinotecan and 1 patient receiving either gemcitabine or ECX. Three patients were chemotherapy naïve prior to the phenotype test. Thus 9 patients had been pre-treated with a 5-fluorouracil containing regimen for at least 7 days prior to the phenotype test. 5-fluorouracil pre-treatment did not appear to predispose to poor metaboliser status in stage IV patients. 11% (1/9) of the stage IV patients on a 5-fluorouracil containing regimen were poor metabolisers compared with 50% of poor metabolisers (3/6) who were not receiving 5-fluorouracil or were treatment naïve.

All the patients with no-evaluable disease were on regimens containing 5-fluorouracil and the incidence of poor metabolisers in the 13 patients who received the drug prior to the phenotype test was 15% (2/13).

The different chemotherapy regimens did not appear to influence the patient's CYP2C19 metabolic activity. For example, both patients #1009 and #1018 were poor metabolisers and were on schedules which contained capecitabine bid (ECX and XELOX) for 21 and 14 days respectively (Table 4.5). However, other patients were on regimens which contained this drug but did not have a poor metabolic status. In addition, the poor metaboliser patient #1004, although scheduled to take 5-fluorouracil, was treatment naïve when tested and the other two poor metabolisers with stage IV disease (#1005 and #1006) had been treated with irinotecan.

Patient #	Disease stage	Date of MR analysis	Metabolic ratio	Date of last chemotherapy	Chemotherapy regimen[a]	Time between phenotype test and chemotherapy
1001	IV	17/04/2009	4.01	28/03/2008	XELOX	> 1 yr
1002	IV	07/04/2009	3.13	30/03/2009	capecitabine	7 days
1003	IV	01/05/2009	6.72	04/05/2009	capecitabine	Not started yet
1004	IV	01/10/2009	16.47	29/12/2009	5-FU/FA	Not started yet
1005	IV	14/09/2009	150.57	25/08/2009	irinotecan	20 days
1006	IV	27/08/2009	10.01	06/08/2009	irinotecan	21 days
1008	IV	08/09/2009	4.48	26/08/2009	FOLFIRI	13 days
1009	IV	15/09/2009	12.22	27/08/2009	ECX	19 days
1013	IV	03/09/2009	6.15	11/8/2009	XELOX	23 days
1020	IV	14/09/2009	2.12	07/09/2009	gemcitabine	7 days
1021	IV	16/09/2009	3.27	02/09/2009	FOLFIRI	14 days
1022	IV	03/05/2010	3.35	19/01/2010	XELOX	> 4 months
1027	IV	09/06/2010	0.88	04/10/2010	XELOX	Not started yet
1028	IV	24/06/2010	3.85	20/04/2010	XELOX	2 months
1029	IV	16/07/2010	2.64	14/07/2010	FOLFIRI	2 days
1007	NED	15/09/2009	2.32	26/08/2009	XELOX	20 days
1010	NED	02/09/2009	7.97	26/08/2009	5-FU/FA	7 days
1011	NED	15/09/2009	22.74	27/08/2009	XELOX	19 days
1012	NED	03/09/2009	3.24	27/08/2009	5-FU/FA	7 days
1014	NED	08/09/2009	3.60	01/09/2009	5-FU/FA	7 days
1015	NED	03/09/2009	6.32	23/07/2009	5-FU/FA	7 days
1016	NED	02/09/2009	5.33	05/08/2009	XELOX	28 days
1017	NED	03/09/2009	7.38	13/08/2009	XELOX	21 days
1018	NED	24/09/2009	13.87	03/09/2009	XELOX	21 days
1019	NED	18/09/2009	1.06	04/09/2009	5-FU/FA	7 days
1023	NED	03/05/2010	1.28	01/03/2010	XELOX	> 2 months
1024	NED	04/06/2010	0.93	18/05/2010	ECX	17 days
1025	NED	26/05/2010	0.93	16/06/2010	5-FU/FA	Not started yet
1026	NED	14/06/2010	2.14	04/08/2009	XELOX	10 months

Table 4.5. ***The chemotherapy regime of each patient with stage IV or no-evaluable disease.*** The patients with discordant CYP2C19 activity are highlighted in grey. [a] XELOX = capecitabine and oxaliplatin; 5FU = 5-fluorouracil; FOLFIRI = 5-fluorouracil, folinic acid and irinotecan; 5-FU/FA = 5-fluorouracil and folinic acid; ECX = epirubicin, cisplatin and capecitabine. Dates are shown as DD/MM/YYYY.

4.2.6 Nutritional status

In the previous clinical study (chapter 3), there was preliminary evidence of an association between body mass index (BMI) and CYP2C19 activity in the patients with advanced incurable cancer. There were significantly more poor metabolisers in patients with a BMI of less than 25 kg/m^2. We hypothesised that cachexia may have a role in the compromised CYP2C19 metabolism. However the body mass index is not a good indicator for cachexia therefore additional anthropometric measurements were used in the current study to measure the nutritional status of individual patients.

The majority of the patients in the current study had a BMI greater than 25 kg/m^2 in both patient categories (9/15 and 11/14 patients in stage IV and no-evaluable disease category respectively; Table 4.6). Six patients had a BMI greater than 30 kg/m^2 and would be classed as obese by the World Health Organisation (2006). In contrast to the previous study in advanced incurable cancer patients, there was no relationship between the proportions of extensive or poor metabolisers and body mass index in either disease categories (P > 0.05; Table 4.6).

	Stage IV			**No-evaluable disease**		
	BMI < 25	BMI > 25	*p-value*	BMI < 25	BMI > 25	*p-value*
Extensive metaboliser	4	7		3	9	
Poor metaboliser	2	2		0	2	
Proportion of poor metaboliser	0.33	0.22	*0.91*	0	0.18	*0.89*

Table 4.6. ***The lack of association between the proportion of poor metabolisers with stage IV or no-evaluable disease in relation to their body mass index (BMI;*** **kg/m^2*).** *P*-values were calculated using Fisher's exact test.

However, there was a significant positive correlation between body mass index and the CYP2C19 metabolic ratio (Spearman r = 0.4953, P = 0.0063) (Figure 4.7). This was particularly the case for the group of patients with no-evaluable disease (Spearman r = 0.7129, P = 0.0042; Figure 4.7b).

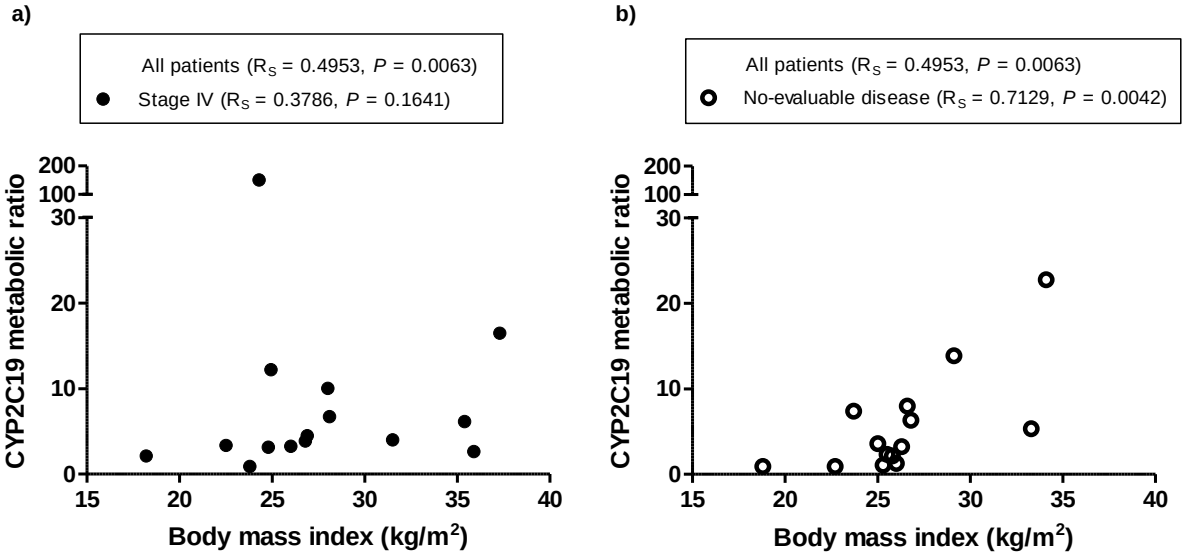

Figure 4.7. ***The relationship between the body mass index and CYP2C19 metabolic ratio in patients with a) stage IV or b) no-evaluable disease.*** The body mass index was calculated as weight/height^2 (kg/m^2). The *P*-values from Spearman's rank correlation test are shown on the graphs.

Further determination of nutritional status was undertaken using arm anthropometric measurements. There was no relationship between the triceps skin-fold thickness and the CYP2C19 metabolic ratio in all the patients (Spearman r = 0.2611, P = 0.1713) and this was also not observed in sub-category analysis (P > 0.05).

In contrast, there was a significant positive correlation between the arm circumference and CYP2C19 metabolic ratio in the patients (Spearman r = 0.5041, *P* = 0.0086) (Figure 4.8). In particular, this positive association was only seen in patients with no-evaluable disease (Spearman r = 0.7877, *P* = 0.0008; Figure 4.8b).

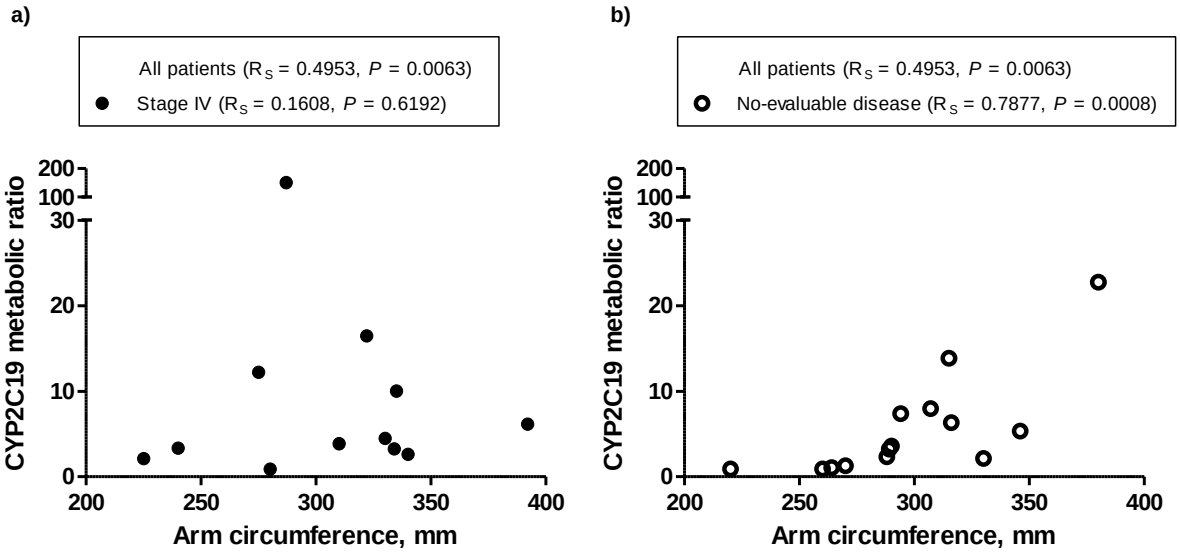

Figure 4.8. ***The relationship between the arm circumference and CYP2C19 metabolic ratio in patients with a) stage IV or b) no-evaluable disease.*** The *P*-values from Spearman's rank correlation test are shown on the graphs.

By arm anthropometric calculations, the arm fat and arm muscle area were determined from the patients' triceps skin-fold thickness and arm circumference as described in section 4.1.1. The CYP2C19 metabolic ratio in each patient was positively associated with the arm fat area (Spearman r = 0.4230, *P* = 0.0313; Figure 4.9a). Similarly, there was a positive association between increasing CYP2C19 metabolic ratio with increasing arm muscle area (Spearman r = 0.4801, *P* = 0.0131; Figure 4.9b). Again, there was a highly significant positive correlation in the patients with no-evaluable disease (Spearman r = 0.7173, *P* = 0.0039).

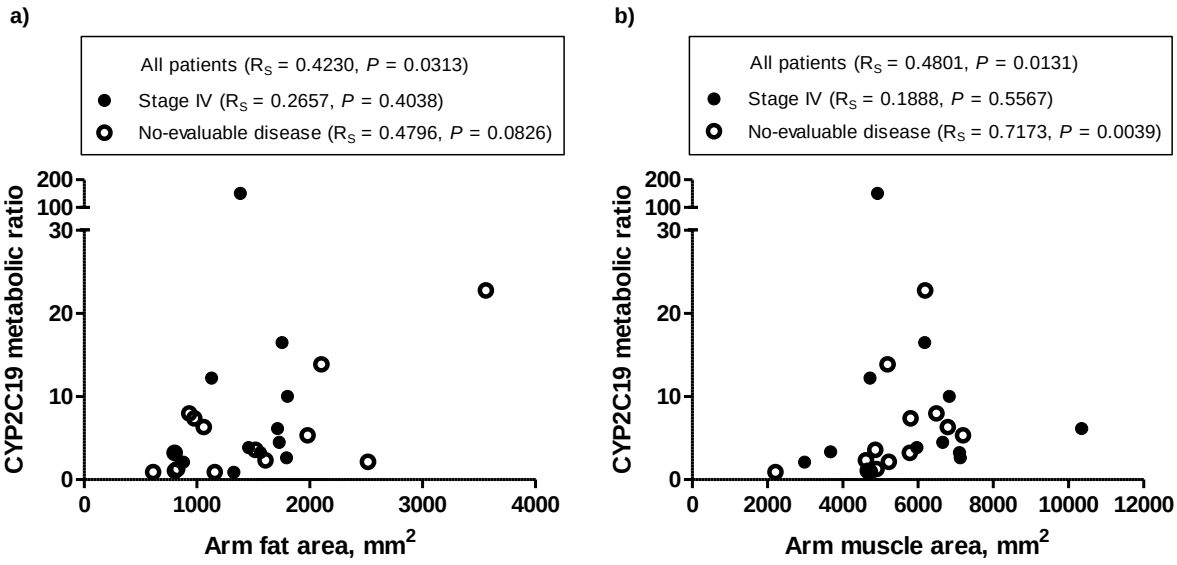

Figure 4.9. ***The relationship between CYP2C19 metabolic function and a) arm fat area and b) arm muscle area in the patients.*** The *P*-values from Spearman's rank correlation test are shown on the graphs.

It appears that patients with increased anthropometric measures, i.e. greater arm circumference, fat or muscle area, had a lower CYP2C19 metabolic activity. Since obesity is associated with liver inflammation (Ferrante 2007; Marchesini et al. 2008; Buechler et al. 2011), there may be an additional, non-cancer related inflammation which may influence the down-regulation of CYP2C19 in those patients with high body mass index and no primary or metastatic tumour burden (no-evaluable disease).

In summary, it appears that patients with no-evaluable disease had a lower CYP2C19 activity as their nutritional status increased. Whether the 14% genotype-phenotype discordance in this group of patients was due to obesity-associated inflammation requires further study.

4.2.7 Relationship with inflammatory status

A number of studies (both experimental and clinical) suggest that inflammatory cytokines play a prominent role in the down-regulation of drug metabolism. To investigate further the possible role of pro-inflammatory mediators in the compromised activity of CYP2C19, the concentrations of various inflammatory cytokines were measured in the patients with stage IV or no-evaluable disease (Table 4.7).

Inflammatory marker	Stage IV (n = 15)	No-evaluable disease (n = 14)	*p*-value
IL-1α	3.2 (3.2-132.1)	3.2 (3.2-108.9)	*0.94*
IL-1β	3.2 (3.2-3.2)	3.2 (3.2-3.2)	*ND*
IL-6	7.36 (3.2-15.66)	3.2 (3.2-10.89)	*0.19*
TNF-α	6.39 (4.65-8.11)	7.15 (4.50-11.48)	*0.59*
TGF-β	775.2 (573.7-850.6)	836.3 (692.8-951.2)	*0.31*
IFN-γ	3.2 (3.2-7.62)	4.985 (3.388-144.9)	*0.07*
CRP	2.20 (1-14.40)	1.45 (1-2.975)	*0.22*
Albumin	41.0 (40.0-43.0)	40.0 (38.5-42.5)	*0.34*

Table 4.7. ***A summary of the inflammatory markers concentrations in each patient group (stage IV or no-evaluable disease).*** Median concentrations (interquartile range) are reported. All units are in pg/mL, except for CRP (mg/L) and albumin (g/L). ND = not determined. *P*-values were calculated using Mann-Whitney rank-sum test

There appears to be a trend towards increased inflammation in patients with stage IV compared with no-evaluable disease. The concentrations of IL-6 and C-reactive protein were higher in the group of patients with stage IV disease compared with the group with no-evaluable disease (Table 4.7). However, this was not significantly different ($P > 0.05$). In contrast, IFN-γ and TGF-β were lower in stage IV patients than in those with no-evaluable disease, although not significantly different. The concentrations of the other cytokines and proteins that were measured in this study were similar ($P > 0.05$) between both groups (Table 4.7).

Interestingly, the patients who were genotype-phenotype discordant (metabolic ratio > 10) had significantly elevated C-reactive protein compared with those patients with a metabolic ratio < 10 ($P = 0.0449$; Figure 4.10a). A lower concentration of TGF-β was also significantly associated with compromised CYP2C19 activity in the discordant patients ($P = 0.0292$; Figure 4.10b).

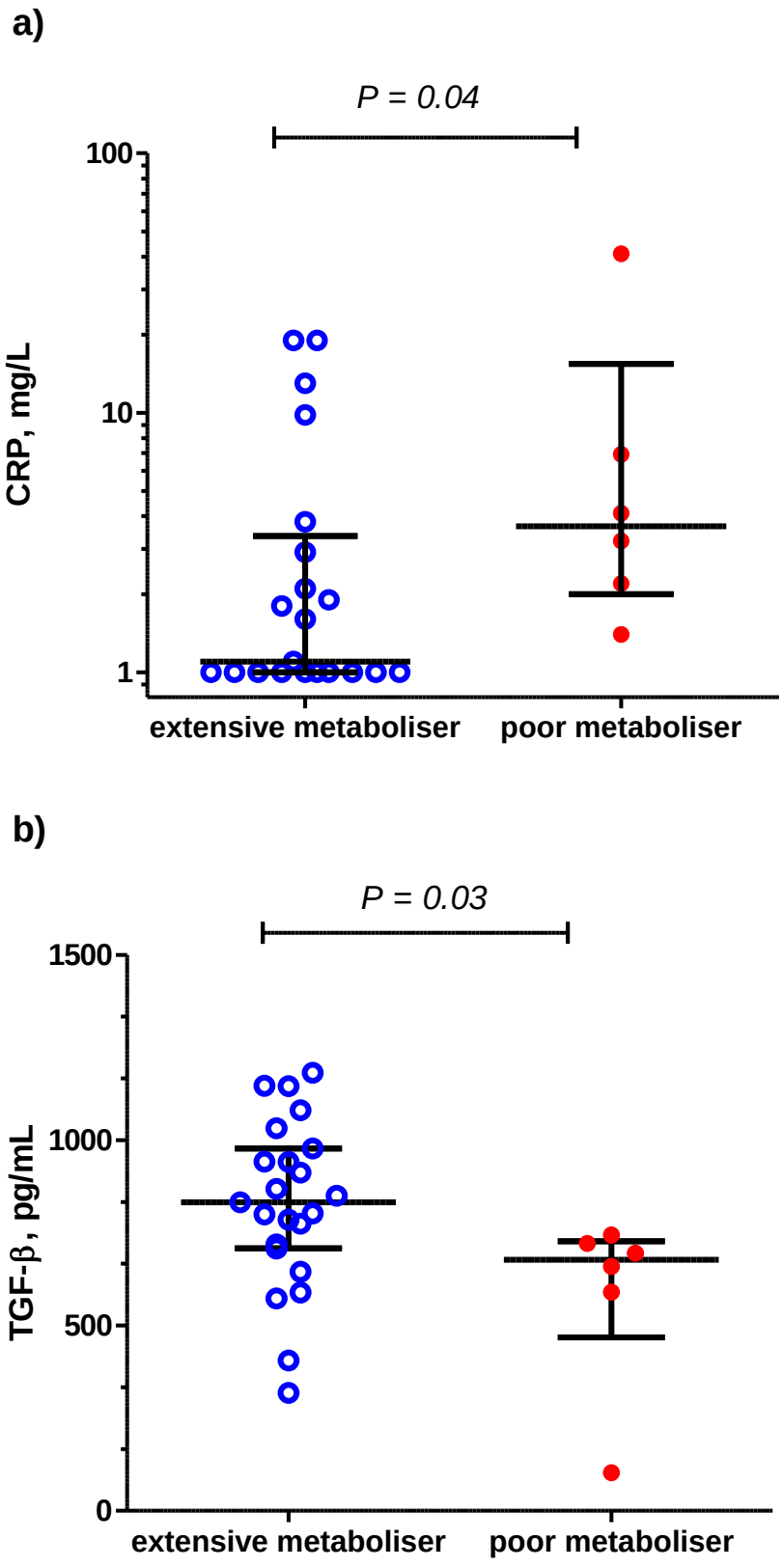

Figure 4.10. ***The relationship between a) C-reactive protein and b) TGF-β in extensive and poor metabolisers from both stage IV and no-evaluable disease.*** Data are shown as median and interquartile range.

When the data were analysed separately for each patient category, there was no statistical difference between extensive and poor metaboliser patients for any of the inflammatory markers that were measured (Table 4.8). However, the median concentration of C-reactive protein was higher in the discordant subjects in both the stage IV and no-evaluable disease patient categories. In contrast, the median concentration of TGF-β was lower in the discordant subjects in both disease categories.

	Phenotype					
	Stage IV			***No-evaluable disease***		
	Extensive metaboliser (n = 11)	Poor metaboliser (n = 4)	*p-value*	Extensive metaboliser (n = 14)	Poor metaboliser (n = 2)	*p-value*[a]
CRP	1.6 (1.0-14.4)	4.6 (1.6-32.5)	*0.39*	1.1 (1.0-2.1)	3.7 (3.2-4.1)	ND
IL-6	7.4 (3.2-15.7)	7.2 (3.2-32.8)	*0.95*	3.2 (3.2-12.5)	3.2 (3.2-3.2)	ND
IL-1α	3.2 (3.2-132.1)	3.2 (3.2-180.5)	*0.88*	3.2 (3.2-314.4)	3.2 (3.2-3.2)	ND
IL-1β	3.2 (3.2-3.2)	3.2 (3.2-13.99)	*0.85*	3.2 (3.2-3.2)	3.2 (3.2-3.2)	ND
TNF-α	6.4 (4.7-8.1)	6.3 (4.3-9.1)	*0.95*	7.9 (5.0-12.5)	3.2 (3.2-3.2)	ND
TGF-β	800.5 (573.7-1033)	677.9 (242.1-715.3)	*0.10*	891.3 (711.3-968.7)	667.3 (590.2-744.5)	ND
IFN-γ	3.2 (3.2-7.8)	3.2 (3.2-4.8)	*0.72*	5.0 (3.5-169.5)	8.6 (3.2-13.9)	ND
Albumin	42.0 (40.0-44.0)	40.5 (36.3-42.5)	*0.26*	40.0 (39.0-43.0)	40.0 (38.0-42.0)	ND

Table 4.8. ***A summary of the inflammatory marker concentrations in extensive or poor metabolisers in each disease category.*** All units were in pg/mL, except for CRP (mg/L) and albumin (g/L). *P*-values were calculated using Mann-Whitney rank-sum test.[a] ND = not determined, *p*-values could not be calculated for the no-evaluable disease group as the sample size of the discordant group was too small. Values reported as median (interquartile range).

Although a positive correlation was also observed between the C-reactive protein concentration and the CYP2C19 metabolic ratio (Spearman r = 0.3069), this was not significant (*P* = 0.1194). C-reactive protein is a secondary biomarker of inflammation as its synthesis is stimulated by the inflammatory cytokine IL-6 (Castell et al. 1989). A significant positive relationship was observed between IL-6 and C-reactive protein in the patients with stage IV disease (Spearman r = 0.7232, *P* = 0.0052). However, IL-6 did not correlate with the CYP2C19 metabolic ratio in the patients (Spearman r = -0.04576, *P* = 0.8137).

Importantly, in addition to the relationship between TGF-β and poor metaboliser category, there was also a significant negative relationship between TGF-β concentrations and CYP2C19 metabolic ratio (Figure 4.11). An increase in TGF-β concentration was associated with an increase in the CYP2C19 activity, i.e. lower metabolic ratio (Spearman r = -0.5331, *P* = 0.0029). Sub group analysis indicated that this negative correlation was statistically significant in the patients with stage IV disease (*P* = 0.0172) and close to significance (*P* = 0.0534) in those patients with no-evaluable disease (Figure 4.11).

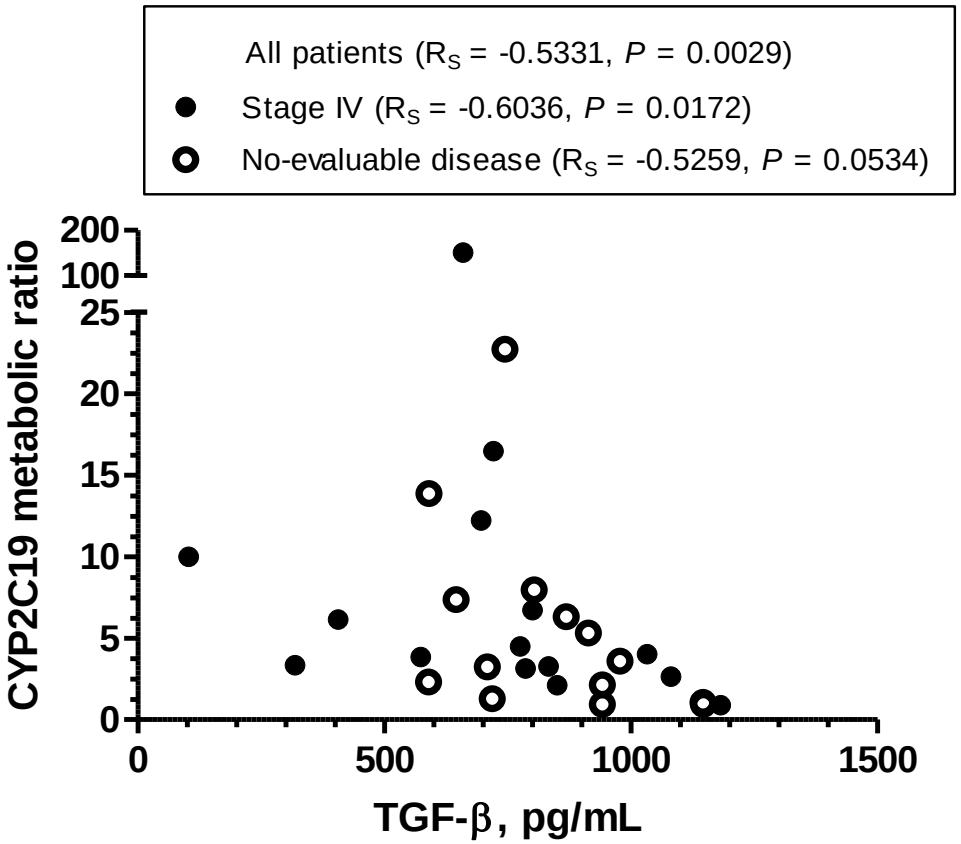

Figure 4.11. ***The relationship between TGF-β and CYP2C19 metabolic ratio in all patients in the study.*** The *P*-values from Spearman's rank correlation test are shown on the graphs.

Similarly, the serum concentrations of TNF-α (Figure 4.12) were negatively correlated with CYP2C19 metabolic ratio (Spearman r = -0.3596, *P* = 0.0554) and this was statistically significant in the patients with no-evaluable disease (Spearman r = -0.7467, *P* = 0.0022).

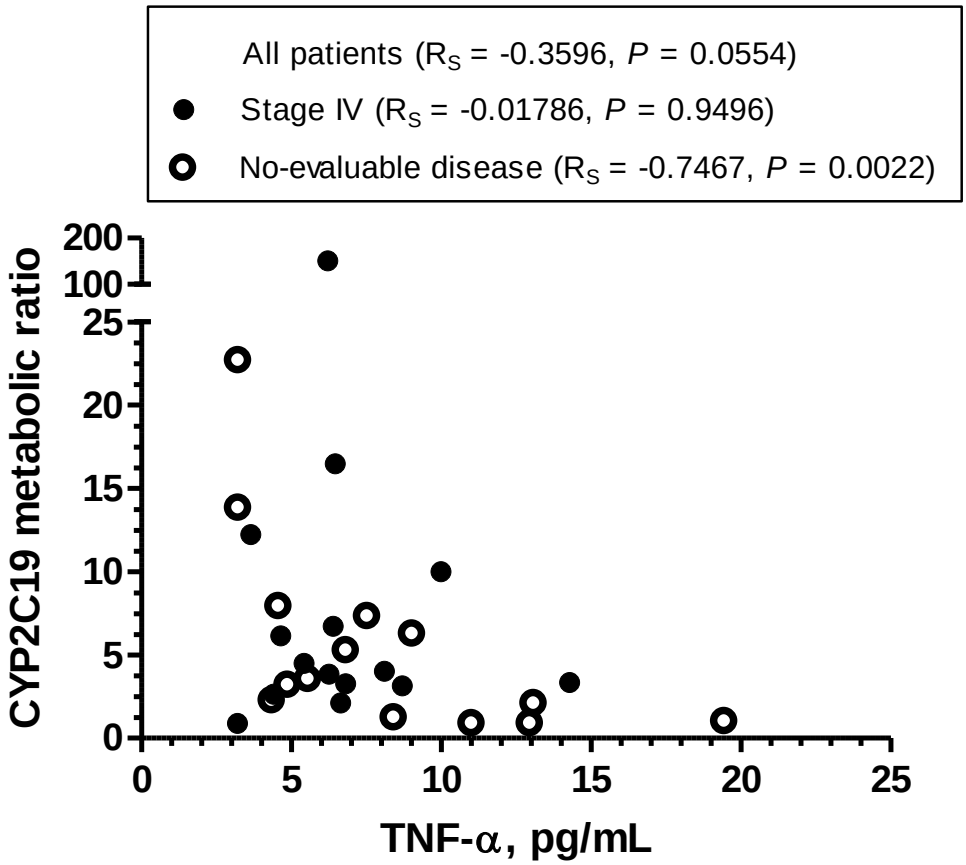

Figure 4.12. ***The relationship between TNF-α and CYP2C19 metabolic ratio in all patients in the study.*** The *P*-values from Spearman's rank correlation test are shown on the graphs.

Of the other inflammatory mediators that were measured in the patients, there were no relationships between the concentrations of the inflammatory cytokines (IL-1α, IL-1β and IFN-γ) or albumin with the patients' CYP2C19 metabolic ratio (Figure 4.13).

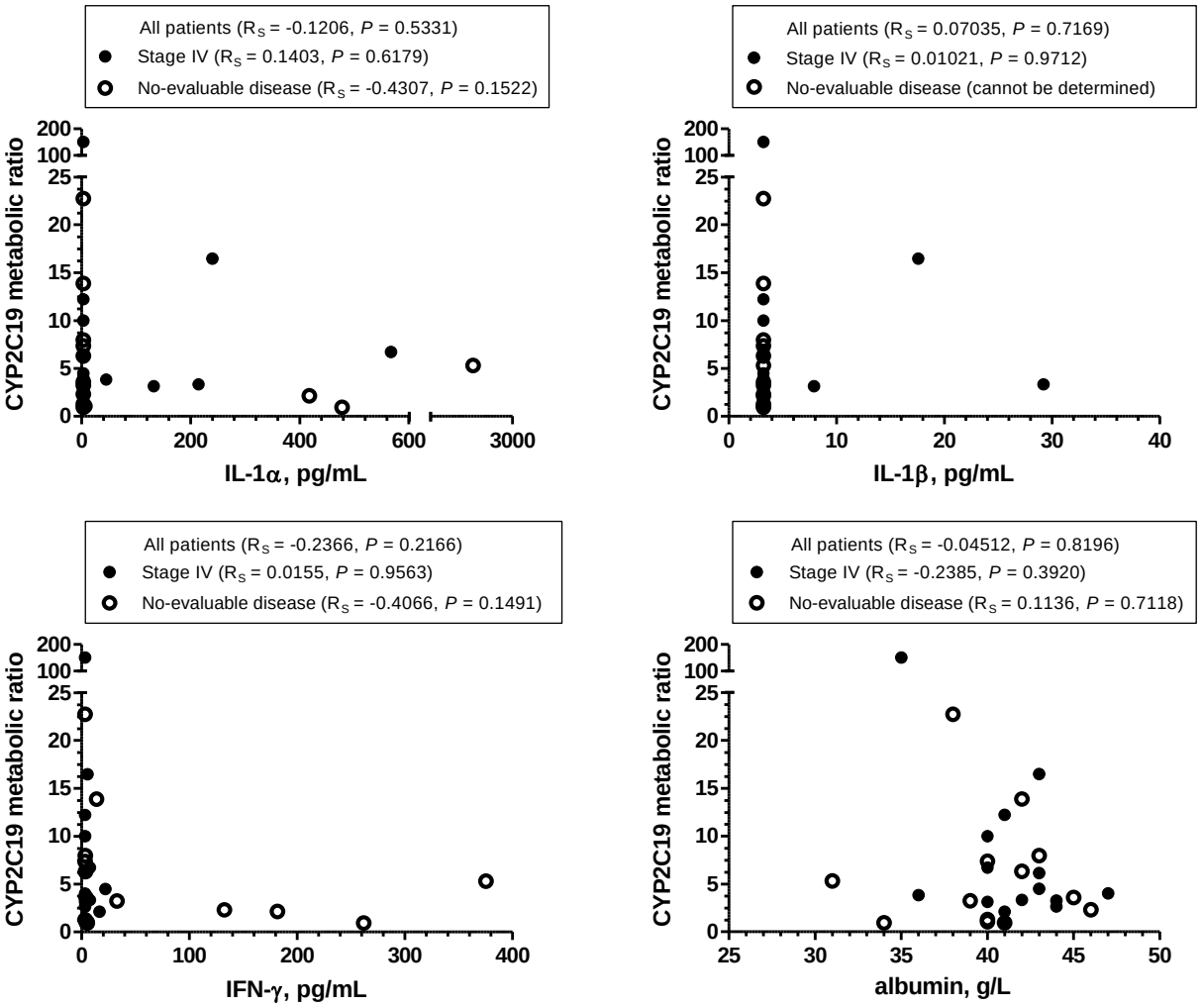

Figure 4.13. ***The relationship between CYP2C19 activity and the levels of inflammatory mediators.*** The *P*-values from Spearman's rank correlation test are shown on the graphs.

In summary, this small study suggests that the inflammatory status is associated with differences in CYP2C19 activity in individuals. Patients with a genotype-phenotype discordant poor metaboliser status have significantly higher C-reactive protein and lower TGF-β concentrations than concordant extensive metaboliser individuals. In addition, circulating concentrations of TGF-β appear to correlate negatively with CYP2C19 activity. Hence, perturbations in cytokines in each category of patients (stage IV versus no-evaluable disease) potentially driven by different disease-associated inflammatory mechanisms (cancer or obesity) may underlie the changes in CYP2C19 activity. Whether the patients with higher body mass index have steatohepatitis and associated inflammation is not known.

The relationships between body mass index and the inflammatory markers were investigated in the patients (Table 4.9).

	All patients		**Stage IV**		**No-evaluable disease**	
	R_S	*P-value*	R_S	*P-value*	R_S	*P-value*
IL-1α	0.0083	*0.97*	0.0999	*0.72*	-0.1046	*0.72*
IL-1β	-0.0722	*0.71*	-0.0919	*0.74*	ND	*ND*
IFN-γ	-0.1396	*0.47*	-0.2072	*0.46*	-0.1082	*0.71*
CRP	-0.1156	*0.57*	-0.3264	*0.28*	0.1716	*0.56*
IL-6	-0.1819	*0.35*	-0.2877	*0.30*	-0.1404	*0.63*
TGF-β	-0.1328	*0.49*	-0.0036	*0.99*	-0.3934	*0.16*
TNF-α	-0.3458	*0.07*	-0.1321	*0.64*	-0.5699	*0.03*

Table 4.9. ***The relationship between body mass index and the inflammatory markers of patients in this study.*** The Spearman rank value (R_S) and *P*-values are reported.

There was a lack of relationship between body mass index and the various inflammatory markers determined in the patients ($P > 0.05$). In particular, although there was an inverse correlation between TGF-β concentration and CYP2C19 activity (Figure 4.11), there was no association between body mass index and TGF-β concentrations ($P > 0.05$; Table 4.9). However, there was an inverse correlation between body mass index and TNF-α concentrations (Spearman r = -0.3458, $P = 0.0661$) and this association was only observed in the no-evaluable disease category (Spearman r = -0.5699, $P = 0.0334$; Table 4.9).

4.2.8 Repeated phenotyping test

To further assess the changes and discordance in CYP2C19 activity, the patients were phenotyped with the probe drug proguanil on a further two occasions during their routine clinical visits. Each phenotype test was conducted during the patients' chemotherapy washout period as shown in Figure 4.6. Details of the probe testing occasions and phenotype metabolic ratio results are shown in Table 4.10.

Patient #	Disease stage	Metabolic ratio (drug/metabolite)						
		Test 1	*Days between test 1 & 2*	*Test 2*	*Days between test 2 & 3*	*Test 3*	*Mean ± sd*	*CV%*
1001	IV	4.01	21	4.18	67	3.74	3.98 ± 0.22	5.58
1002	IV	3.13	21	3.50	28	3.30	3.31 ± 0.19	5.60
1003	IV	6.72	21	14.31	21	13.12	11.38 ± 4.08	35.86
1004	IV	16.47	42	14.48	28	40.13	23.69 ± 14.27	60.22
1005	IV	150.57	21	67.35	18	31.30	83.07 ± 61.17	73.63
1006	IV	10.01	19	8.90	21	8.93	9.28 ± 0.63	6.81
1008	IV	4.48	14	3.27	14	4.18	3.98 ± 0.63	15.85
1013	IV	6.15	21	5.91	21	6.77	6.28 ± 0.44	7.07
1020	IV	2.12	7	1.44	24	2.88	2.15 ± 0.72	33.56
1021	IV	3.27	13	6.39	21	3.12	4.26 ± 1.85	43.34
1022	IV	3.35	21	4.49	22	14.14	7.33 ± 5.93	80.91
1027	IV	0.88	21	1.43	22	4.51	2.27 ± 1.96	86.06
1028	IV	3.85	21	2.39	43	2.86	3.03 ± 0.75	24.57
1029[a]	IV	2.64	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]
1009[b]	IV	12.22					4.62 ± 6.59[b]	142.47[b]
% poor metaboliser		**27%**		**23%**		**31%**		
1009[b]	NED		84	0.50	10	1.15		
1007	NED	2.32	21	3.21	21	3.41	2.98 ± 0.58	19.47
1010	NED	7.97	7	45.26	7	20.17	24.47 ± 19.01	77.71
1011	NED	22.74	23	10.24	21	10.47	14.48 ± 7.15	49.38
1012	NED	3.24	7	2.85	7	6.28	4.12 ± 1.88	45.54
1014	NED	3.60	7	10.45	7	8.85	7.63 ± 3.58	46.94
1015	NED	6.32	7	4.27	7	5.24	5.28 ± 1.03	19.43
1016	NED	5.33	21	6.45	26	9.24	7.01 ± 2.01	28.74
1017	NED	7.38	7	6.03	19	6.37	6.59 ± 0.70	10.65
1018	NED	13.87	28	7.61	21	5.50	8.99 ± 4.35	48.40
1019	NED	1.06	7	1.66	6	2.49	1.74 ± 0.72	41.35
1023	NED	1.28	28	1.45	17	3.67	2.13 ± 1.33	62.51
1024	NED	0.93	31	1.47	28	1.97	1.46 ± 0.52	35.71
1025	NED	0.93	7	1.45	7	2.37	1.58 ± 0.73	46.05
1026	NED	2.14	38	3.76	28	3.72	3.21 ± 0.92	28.81
% poor metaboliser		**14%**		**20%**		**13%**		

Table 4.10. ***The CYP2C19 metabolic ratios of patients with stage IV and no-evaluable disease determined on three separate occasions.***
[a] Patient #1029 withdrew from the study after the first phenotype test
[b] Patient #1009 originally had stage IV disease but had tumour resection after the first phenotype test

Many patients had a relatively stable metabolic ratio over time. However in the group of patients with stage IV disease, patients #1003, 1004, 1005, 1009 and 1022 had considerable variation in the CYP2C19 metabolic ratio over the three separate occasions. Similarly, of those patients with no-evaluable disease, patients #1010, 1011, 1014, and 1018 had large variation in their three separate tests of CYP2C19 activity (Figure 4.14).

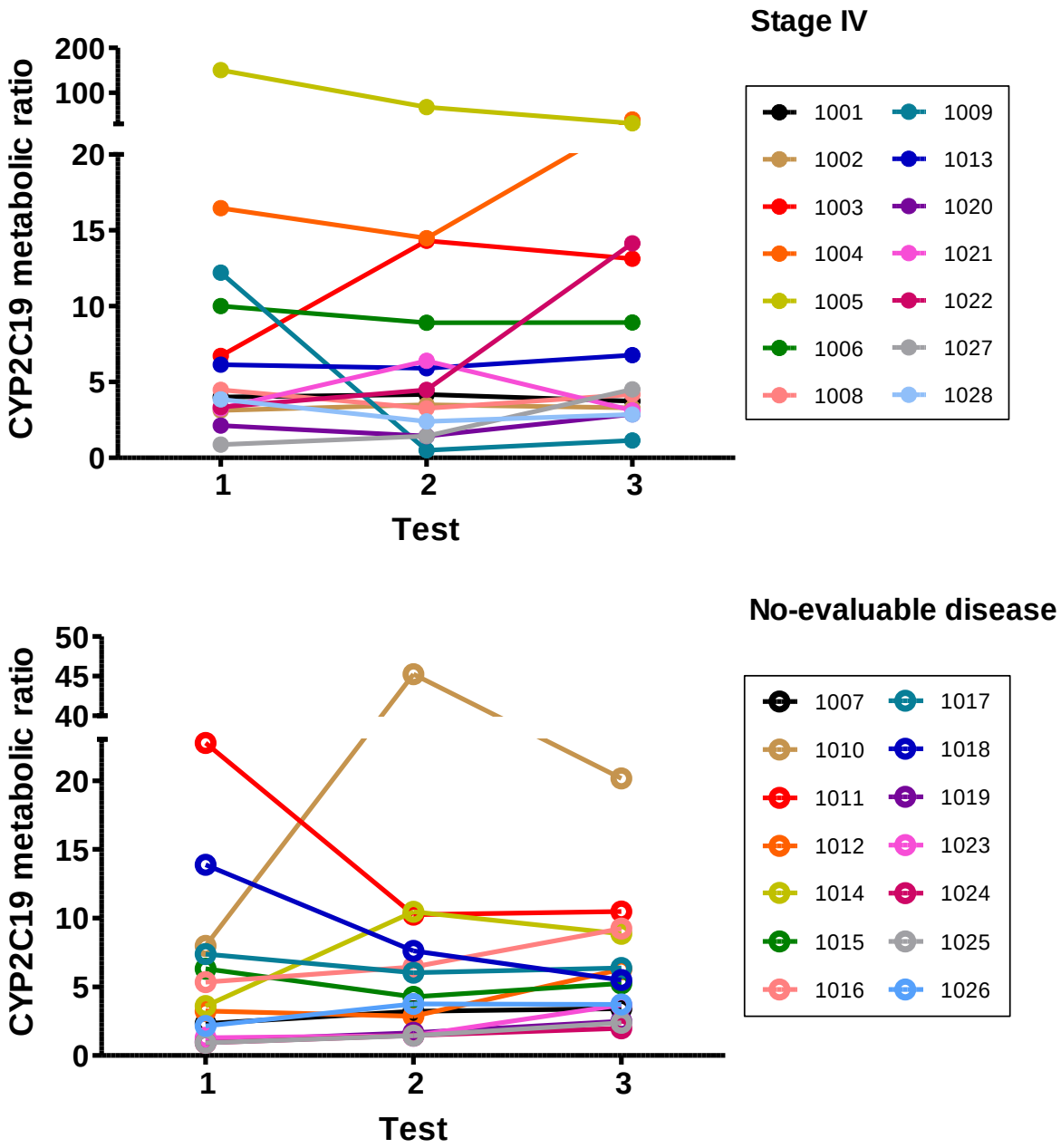

Figure 4.14. ***The variability of the CYP2C19 metabolic ratio in individual patients with stage IV or no-evaluable disease over three separate occasions.***

This temporal variability in CYP2C19 activity resulted in sufficient changes in activity for six patients to change phenotype categories (i.e. from extensive to poor metaboliser or from poor to extensive metaboliser) over the test period. The proportion of poor metabolisers was always higher in the patients with stage IV disease (23%-31%) compared with the no-evaluable disease category (13-20%; Table 4.11).

One subject of particular note is patient #1009, who had stage IV disease and a metabolic ratio of 12.22 at the first test occasion. The second proguanil probe test was undertaken 84 days later and the drug/metabolite ratio as a marker of CYP2C19 activity had improved to 0.50. This patient had undergone colorectal tumour resection following test 1 and at test 2, this patient had no-evaluable disease. Indeed, when tested a further 94 days later, the metabolic ratio in this individual was relatively stable at 1.15. This suggests a possible influence of tumour burden on CYP2C19 activity.

To investigate whether changes in tumour burden were associated with the temporal changes in metabolic ratio, the RECIST data was determined from CT scans or X-rays closest to the date of the additional probe drug tests (Table 4.11). Patient #1027 only had one CT scan, hence a change of tumour burden could not be determined. In addition, patient #1029 withdrew from the study; therefore both of these patients were excluded from this part of the study.

Tumour burden increased in seven patients, indicating disease progression. Four patients responded to treatment and the tumour burden decreased. There was no change in tumour burden in two patients over the period of phenotype testing indicating stable disease (Table 4.11). No obvious relationship between response to treatment or disease progression and the changes in metabolic ratio was observed (Table 4.11). However, the proportion of patients with a poor metaboliser status at the final test was lower in those patients who had responded to therapy compared with those whose tumour burden had increased or remained stable (0.25 versus 0.29 and 0.5 respectively).

Patient #	Change in tumour burden	Metabolic ratio[a]	Metaboliser status	Proportion of PM at test 3	Comments
1013	Decrease	Constant	EM	0.25	Surgery after first probe test
1021	Decrease	Constant	EM		
1009	Decrease	Decrease	PM → EM		
1003	Decrease	Increase	EM → PM		
1004	Constant	Increase	PM	0.5	
1008	Constant	Constant	EM		
1001	Increase	Constant	EM	0.29	
1002	Increase	Constant	EM		
1006	Increase	Constant	PM → EM		
1020	Increase	Constant	EM		
1028	Increase	Constant	EM		
1005	Increase	Decrease	PM		
1022	Increase	Increase	EM → PM		

Table 4.11. Changes in *tumour burden in relation to the stage IV patient's CYP2C19 metabolic ratio over the three test periods.* EM = extensive metaboliser, PM = poor metaboliser.

To investigate whether changes in inflammatory status were associated with the variations in the CYP2C19 metabolic ratio, the concentrations of the inflammatory mediators were also measured in the blood samples collected on each of the three separate test occasions (Table 4.12 and Table 4.13).

	Stage IV								
	Patient #	Metabolic ratio	Inflammatory mediators						
			IL-1α	*IL-1β*	*IL-6*	*TNF-α*	*IFN-γ*	*TGF-β*	*CRP*
Increasing metabolic ratio	1003-1	6.72	567.05	3.20	34.40	6.39	7.62	800.47	19
	1003-2	14.31	559.51	3.20	35.78	6.51	3.20	665.52	ND
	1003-3	13.12	603.98	3.20	29.51	5.49	3.62	721.71	14
	1004-1	16.47	239.60	17.58	11.11	6.47	5.38	721.64	6.9
	1004-2	14.48	207.78	17.17	16.92	5.86	3.51	749.50	52
	1004-3	40.13	186.09	17.94	11.56	4.47	13.65	729.57	11
	1022-1	3.35	214.37	29.22	15.43	14.29	7.77	318.74	9.8
	1022-2	4.49	173.14	22.87	15.61	11.72	46.07	344.36	17
	1022-3	14.14	177.61	22.71	20.69	11.69	72.99	363.94	15
Decreasing metabolic ratio	1005-1	150.57	3.20	3.20	40.01	6.22	3.20	659.70	41
	1005-2	67.35	3.20	3.20	260.66	7.91	3.20	760.17	ND
	1005-3	31.30	3.20	3.20	27.81	5.43	6.11	691.51	ND
	1009-1	12.22	3.20	3.20	3.20	3.65	3.22	696.10	1.4
	1009-2	0.50	3.20	3.20	3.20	3.20	3.20	905.12	<1
	1009-3	1.15	3.20	3.20	3.20	3.20	3.20	809.88	<1
Constant metabolic ratio	1001-1	4.01	3.20	3.20	3.20	8.11	3.20	1033.25	ND
	1001-2	4.18	3.20	3.20	3.20	7.62	3.20	976.16	ND
	1001-3	3.74	3.20	3.20	3.20	5.52	3.20	851.82	ND
	1002-1	3.13	132.06	7.89	15.66	8.70	3.20	786.03	ND
	1002-2	3.50	131.71	8.74	13.35	15.49	3.20	701.09	ND
	1002-3	3.30	146.98	9.32	13.53	12.71	3.20	919.80	ND
	1006-1	10.01	3.20	3.20	3.20	9.99	3.20	102.93	2.2
	1006-2	8.90	3.20	3.20	3.20	9.16	6.50	738.60	4.5
	1006-2	8.93	3.20	3.20	3.20	10.02	5.10	900.66	4.7
	1008-1	4.48	3.20	3.20	7.99	5.43	22.13	775.18	<1
	1008-2	3.27	3.20	3.20	3.20	5.13	11.76	740.17	<1
	1008-3	4.18	3.20	3.20	9.42	6.17	24.42	804.60	<1
	1013-1	6.15	3.20	3.20	7.36	4.65	3.20	405.88	<1
	1013-2	5.91	3.20	3.20	4.37	5.71	3.20	614.75	1.6
	1013-3	6.77	3.20	3.20	3.20	5.10	3.20	494.96	1.4
	1020-1	2.12	3.20	3.20	31.45	6.64	16.55	850.62	19
	1020-2	1.44	3.20	3.20	34.52	6.92	10.73	521.11	20
	1020-3	2.88	3.20	3.20	31.97	10.31	11.91	1233.69	34
	1021-1	3.27	3.20	3.20	3.20	6.81	4.70	832.95	3.8
	1021-2	6.39	3.20	3.20	3.20	4.36	41.94	825.50	4.7
	1021-3	3.12	3.20	3.20	3.20	6.49	6.64	945.66	6
	1023-1	1.28	3.20	3.20	3.20	8.39	3.20	719.10	13
	1023-2	1.45	3.20	3.20	3.20	8.79	9.68	1248.54	2.4
	1023-3	3.67	3.20	3.20	3.20	8.05	3.20	1089.82	1.6
	1027-1	0.88	3.20	3.20	3.20	3.20	3.20	1182.25	<1
	1027-2	1.43	3.20	3.20	3.20	3.20	3.30	1082.32	<1
	1027-3	4.51	3.20	3.20	3.20	3.29	3.20	1150.31	<1
	1028-1	3.85	45.17	3.20	4.34	6.25	3.20	573.73	1.6
	1028-2	2.39	ND	ND	ND	ND	ND	ND	1.8
	1028-3	2.86	ND	ND	ND	ND	ND	ND	17

Table 4.12. ***The variability in inflammatory mediators relative to changes in the patient's CYP2C19 metabolic ratio with stage IV disease*.** All units in pg/mL except CRP mg/L. ND = not determined.

	No-evaluable disease								
	Patient #	Metabolic ratio	Inflammatory mediators						
			IL-1α	*IL-1β*	*IL-6*	*TNF-α*	*IFN-γ*	*TGF-β*	*CRP*
Increasing metabolic ratio	1010-1	7.97	3.20	3.20	3.20	4.55	3.45	803.50	<1
	1010-2	45.26	3.20	3.20	3.20	4.19	3.20	467.80	<1
	1010-3	20.17	3.20	3.20	3.20	4.73	3.20	588.92	<1
	1014-1	3.60	3.20	3.20	3.20	5.54	4.03	977.51	1.8
	1014-2	10.45	3.20	3.20	3.20	7.00	3.20	1075.54	2.2
	1014-3	8.85	3.20	3.20	3.20	4.87	3.20	893.87	2.0
Decreasing metabolic ratio	1011-1	22.74	3.20	3.20	3.20	3.20	3.20	744.46	4.1
	1011-2	10.24	3.20	3.20	9.64	6.26	4.40	172.42	3.3
	1011-3	10.47	3.20	3.20	3.20	7.42	3.20	464.07	5.1
	1018-1	13.87	3.20	3.20	3.20	3.20	13.93	590.21	3.2
	1018-2	7.61	3.20	3.20	3.20	3.20	11.80	647.69	2.6
	1018-3	5.50	3.20	3.20	3.20	3.34	5.99	597.05	3.2
Constant metabolic ratio	1007-1	2.32	3.20	3.20	10.06	4.33	132.62	589.65	<1
	1007-2	3.21	3.20	3.20	3.08	4.54	107.48	513.14	<1
	1007-3	3.41	3.20	3.20	3.20	4.11	63.20	448.40	<1
	1012-1	3.24	3.20	3.20	3.20	4.86	32.68	708.72	<1
	1012-2	2.85	3.20	3.63	4.68	5.18	26.04	639.52	<1
	1012-3	6.28	3.20	3.84	4.77	6.39	22.86	658.64	ND
	1015-1	6.32	3.20	3.20	3.20	9.01	3.65	869.05	1.1
	1015-2	4.27	3.20	3.20	3.20	7.91	3.20	753.98	<1
	1015-3	5.24	3.20	3.20	3.20	9.25	3.20	906.88	1
	1016-1	5.33	2759.18	3.20	13.37	6.80	375.37	913.45	<1
	1016-2	6.45	633.89	3.20	3.66	4.84	95.62	961.69	<1
	1016-3	9.24	210.08	3.20	3.20	4.65	30.07	933.12	1.1
	1017-1	7.38	3.20	3.20	3.20	7.50	3.20	645.21	1.90
	1017-2	6.03	3.20	3.20	3.20	8.73	3.20	849.98	2.6
	1017-3	6.37	3.20	3.20	3.20	8.36	3.20	949.89	2.8
	1019-1	1.06	6.14	3.20	3.20	19.42	4.69	1146.64	<1
	1019-2	1.66	3.20	3.20	3.20	16.05	3.22	1169.98	<1
	1019-3	2.49	3.20	3.20	3.20	16.71	3.20	960.68	<1
	1023-1	1.28	3.20	3.20	3.20	8.39	3.20	719.10	13
	1023-2	1.45	3.20	3.20	3.20	8.79	9.68	1248.54	2.4
	1023-3	3.67	3.20	3.20	3.20	8.05	3.20	1089.82	1.6
	1024-1	0.93	477.57	3.20	34.25	12.94	261.88	1147.11	1
	1024-2	1.47	3.20	3.20	3.20	5.44	16.40	986.46	<1
	1024-3	1.97	3.20	3.20	3.20	4.65	3.20	977.45	<1
	1025-1	0.93	3.20	3.20	3.20	10.99	5.28	942.09	2.1
	1025-2	1.45	3.20	3.20	3.20	9.12	3.77	915.57	1.7
	1025-3	2.37	3.20	3.20	3.20	9.96	3.20	665.07	<1
	1026-1	2.14	417.20	3.20	50.91	13.06	181.80	942.37	2.9
	1026-2	3.76	ND	ND	ND	ND	ND	ND	1.2
	1026-3	3.72	ND	ND	ND	ND	ND	ND	<1

Table 4.13. ***The variability in inflammatory mediators relative to changes in the patient's CYP2C19 metabolic ratio with no-evaluable disease.*** All units in pg/mL except CRP mg/L. ND = not determined.

The decrease in circulating TGF-β levels associated with low CYP2C19 activity at test 1 (section 4.2.7, Figure 4.10b and Figure 4.11) was also observed (Table 4.12 and Table 4.13).

For example, patient #1003 with stage IV disease had increased metabolic ratio (6.72, 14.31 and 13.12) over the three tests and there was an associated decrease in TGF-β concentration (800.47, 665.52 and 721.71 pg/mL; Table 4.12). Similarly, patient #1010 with no-evaluable also showed an associated decrease in TGF-β concentration (803.50, 467.80 and 588.92 pg/mL) with poor metabolic ratio (7.97, 45.26 and 20.17; Table 4.13). Moreover, there was an inverse relationship between TGF-β concentration and CYP2C19 metabolic ratio (Spearman r = -0.4432, P = < 0.0001). This was seen in both the stage IV and no-evaluable disease patient categories (Spearman r = -0.4731, P = 0.0024 and spearman r = -0.4523, P = 0.0026 respectively).

Elevated C-reactive protein was associated with poor CYP2C19 activity at test 1 (section 4.2.7, Figure 4.10a), although it was not of statistical significance. There was no obvious change in C-reactive protein concentration with variability in the patient's metabolic ratio (Table 4.12 and Table 4.13). However, a significant positive correlation between C-reactive protein concentration and CYP2C19 activity was observed (Spearman r = 0.3409, P = 0.0028). In particular, this relationship was observed in the no-evaluable disease category (Spearman r = 0.3294, P = 0.0310).

The inverse correlation observed between increasing TNF-α concentrations and decreasing CYP2C19 metabolic in the no-evaluable disease category at test 1 (section 4.2.7, Figure 4.12) was also observed after further phenotype tests (Spearman r = -0.3828, P = 0.0123).

The other inflammatory cytokines did not correlate to the changes in the CYP2C19 activity (P > 0.05).

Since some associations between TGF-β, body mass index and tumour burden with CYP2C19 activity/status were observed at test 1 (section 4.2.7), the relationship between metabolic ratio, cytokines, arm anthropometry, tumour burden and chemotherapy in two individual patients on three test occasions are discussed in detail below.

Patient #1009 (Figure 4.15) had stage IV disease and was a poor metaboliser (metabolic ratio = 12.22). At this time, the patient had a TGF-β concentration of 696 pg/mL, which was similar to the median concentration (688 pg/mL) observed in all discordant (poor metaboliser) patients at test 1. At day 31, the patient underwent tumour resection and was reassessed to have no-evaluable disease. The patient was phenotyped again at day 84 at which the CYP2C19 activity had improved (metabolic ratio = 0.50) along with an increase in plasma TGF-β concentration (905 pg/mL). Subsequently, the patient was phenotyped again at day 94 and the metabolic ratio was relatively stable at 1.15 with a TGF-β concentration of 810 pg/mL. The TGF-β concentration in patient #1009 at the second and third test was similar to the median concentration (833 pg/mL) observed in all extensive metaboliser patients at test 1. Moreover, the body mass index decreased as the patient's CYP2C19 activity improved (24.9, 21.5 and 21.7 at each test) which is in agreement of the positive relationship observed previously (Figure 4.7), between increasing body mass index and low CYP2C19 activity.

In contrast, patient #1010 had no-evaluable disease and was an extensive metaboliser (metabolic ratio = 7.97) at the time of admission into the study (Figure 4.15). At the second phenotype test, the patient had a large decrease in CYP2C19 activity (metabolic ratio = 45.26) and at day 10, the patient presented with hand-and-foot syndrome. This is a relatively common cutaneous side-effect of capecitabine chemotherapy and, as the name suggests, is associated with inflammation of the hand and foot (Narasimhan et al. 2004; Janusch et al. 2006). This individual case has been reported in detail elsewhere (Helsby et al. 2010d). At day 14, although the CYP2C19 activity improved, the patient was still categorised as a poor metaboliser (metabolic ratio = 20.17). Similar to patient #1009, the TGF-β concentration also decreased as CYP2C19 activity decreased (804 pg/mL initially, versus 468 and 589 pg/mL at test 2 and 3 respectively). No changes in BMI were observed, but measurements were taken at intervals of 7 days which may be too short to detect any significant changes in weight.

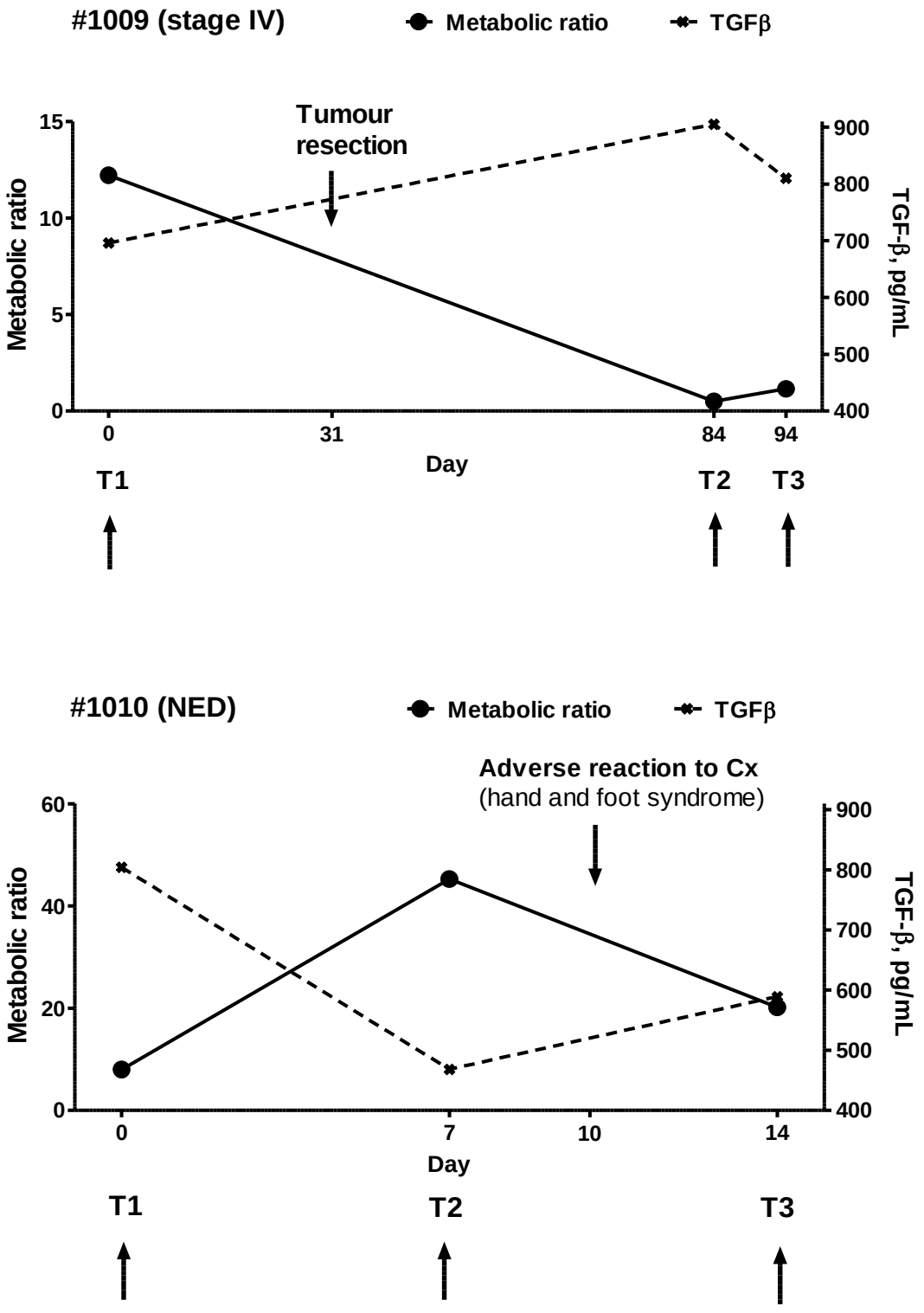

Figure 4.15. ***Changes in CYP2C19 function (metabolic ratio) relative to clinical events and circulating concentrations of TGF-β in two patients (#1009 and #1010).***

4.3 Discussion

The main aim of this study was to confirm the presence of CYP2C19 genotype-phenotype discordance observed previously in an independent population of patients. The majority of the patients in this study were of Caucasian origin, with the exception of one Indian and one Filipino. CYP2C19 genotype was determined in the subjects and all of the patients were genotypic extensive metabolisers. The genotype distribution of *CYP2C19* variants conformed to the Hardy-Weinberg equilibrium and the prevalence of the loss-of-function variant was as expected for a mainly Caucasian population. This confirms the previous reports of no association between the presence of *CYP2C19* variant alleles and the incidence of colorectal cancer (Tamer et al. 2006; Buyukdogan et al. 2009).

A number of individuals in this study were genotype-phenotype discordant, with the proportion of poor metabolisers higher in the patients with stage IV disease (27%) compared with those with no evaluable disease (14%). The proportion of poor metabolisers, however, was less than that observed previously in patients with advanced incurable disease (37%). Thus compromised metabolism also occurs in cancer patients who are being actively managed for the disease. This is an important finding as compromised drug metabolising activity could influence the response to certain chemotherapeutic agents. It will be important to determine whether compromised CYP2C19 activity also occurs in other patient populations. For example, patients with breast or haematological cancers are often treated with agents such as cyclophosphamide, which is activated by CYP2C19 (Timm et al. 2005; Xie et al. 2006; Helsby et al. 2010b).

Proguanil was used as an alternative probe substrate to evaluate CYP2C19 activity in this study. The relationship between proguanil metabolism to cycloguanil and CYP2C19 has been extensively studied in healthy populations (Ward et al. 1991; Coller et al. 1997; Hoskins et al. 1998; Herrlin et al. 2000; Satyanarayana et al. 2009). The antimode between extensive and poor metabolisers has previously been reported to be proguanil/cycloguanil = 10 (Ward et al. 1989b; Helsby et al. 1990b). A significant linear correlation between the metabolism of proguanil and omeprazole has been noted in healthy volunteers (Herrlin et al. 2000) and the antimode of proguanil (metabolic ratio > 10) was in agreement with the antimode of omeprazole (log hydroxylation index > 1). Individuals who were poor metabolisers of both drugs (log hydroxylation index > 1 and

proguanil/cycloguanil > 10) were homozygous variant for *CYP2C19* loss-of-function alleles (Herrlin et al. 2000). Hence individuals categorised as a poor metaboliser in this study will have similar low CYP2C19 activity as the poor metabolisers categorised in the previous study (chapter 3).

The median urinary metabolic ratio of proguanil in a healthy Caucasian population has been reported previously to be 1.4 and 2.5 in patients with *CYP2C19*1/*1* and *CYP2C19*1/*2* genotype respectively (Hoskins et al. 1998). In contrast, in the current study, the median metabolic ratio in the cancer patients (*wt/wt* or *wt/var*) was 6.15 with one patient having a metabolic ratio as high as 150. The median CYP2C19 metabolic ratio in the two disease categories (stage IV and no-evaluable disease) were 4.0 and 3.4 respectively. This may indicate that CYP2C19 function is decreased in many patients but not compromised to a large enough extent in some individuals to result in a change in phenotype category.

The decrease in proportion of CYP2C19 poor metabolisers (37%, 27% and 14%) across the disease categories (advanced incurable, stage IV and no-evaluable disease) suggested a possible role for the extent of tumour burden on the function of this enzyme. Relative tumour burden was measured using the RECIST criteria for the patients with stage IV disease. However, although there was no correlation between RECIST values and metabolic ratio, there was a trend to higher tumour burden in poor metaboliser than in extensive metaboliser category. Continued recruitment of patients into this preliminary study beyond the timeframe of this doctoral thesis may allow a suitable sample size to determine whether a relationship exists.

Although the majority of patients with stage IV disease had colorectal liver metastases, a direct effect on general liver function is not thought to be the cause of compromised CYP2C19 function. All patients were required to have normal liver and renal function for inclusion into the study. Moreover, one patient (#1009) was a discordant poor metaboliser despite the absence of liver metastases. In contrast, of the twelve patients with liver metastases, only three were phenotypic poor metabolisers.

The recruitment criteria excluded those subjects taking known CYP2C19 inducers or inhibitors and which could not be withdrawn for clinical reasons. However, the majority of the patients were on active chemotherapy regimens which includes drugs such as 5-fluorouracil. This drug has a short half life (~10-20 min) and does not directly inhibit CYP

enzymes (Afsar et al. 1996). However, chronic administration of 5-fluorouracil has been reported to down-regulate expression of multiple cytochrome P450 enzymes in rats (Afsar et al. 1996), perhaps due to a decreased rate of protein synthesis. In addition, some patients were prescribed with capecitabine which is converted to 5-fluorouracil selectively in the liver and tumour (Miwa et al. 1998). The half-life of this drug is short (less than 1 hour) (Schellens 2007), and was administered after the phenotype test, hence capecitabine was not expected to interfere directly with proguanil metabolism in the patients. One of the patients (#1020) was on the gemcitabine regimen which was administered intravenously weekly for 3 weeks. The plasma half-life of gemcitabine is approximately 1.5 hours (Saif 2005) and the phenotype test was conducted 7 days after the last chemotherapy administration, hence gemcitabine was also not expected to interfere with proguanil metabolism. Moreover, 5-fluorouracil containing therapies (5-FU/FA, XELOX, ECX and capecitabine) did not appear to associate with poor metabolic ratio in the patients. However, further studies to directly assess the effect of 5-fluorouracil on CYP2C19 expression should be considered. None of the other chemotherapeutics that the patients were on are known to be inducers or inhibitors of CYP2C19.

In the previous study (chapter 3), there was preliminary evidence for an association between increased genotype-phenotype discordance (poor metaboliser status) and low body mass index. However in the present study, the opposite was observed, i.e. better nutritional status was significantly associated with a lower CYP2C19 activity in the cancer patients with no-evaluable disease. There is increasing evidence that obesity is associated with non-alcoholic steatohepatitis (Marchesini et al. 2008; Buechler et al. 2011) and that obesity may affect metabolism by the cytochrome P450 enzymes (Kotlyar et al. 1999). Interestingly, there was a direct relationship where TNF-α concentration was lower in the cancer patients who were overweight or obese (BMI > 25) in this study. This is in contrast to reported findings in where increased TNF-α concentration was associated with obesity (Yudkin et al. 1999; Zahorska-Markiewicz et al. 2000; Hui et al. 2004; Olszanecka-Glinianowicz et al. 2004). One possible reason may be the small sample size in this preliminary analysis and continued recruitment may further elucidate this relationship. Literature has also reported that plasma circulating concentrations of cytokines such as TNF-α, did not reflect the intrahepatic responses during the systemic inflammatory response (Andrejko et al. 1996; Kox et al. 2000). Hence, the circulating inflammatory cytokines concentrations that were measured in the cancer patients in this study may not accurately predict the effect of intrahepatic inflammation on CYP2C19

activity. Liver inflammation due to disease may also influence the genotype-phenotype discordance and should be investigated further.

Differences in inflammatory markers were observed in the cancer patients with stage IV and no-evaluable disease. The relatively small sample size of the patients recruited to date limits the statistical power of the relationship and also limits comparison with the data in chapter 3 for patients with advanced incurable cancer. However, C-reactive protein and IL-6 were highest in patients with advanced incurable cancer and lowest in those with no-evaluable disease. In contrast, TGF-β concentration was lowest in patients with advanced incurable cancer and higher in those with no-evaluable disease. This cancer-associated inflammation may be one mechanism by which CYP2C19 is down-regulated in these patients. The concentration of C-reactive protein was significantly higher in the patients with genotype-phenotype discordance (poor metaboliser) and a non-significant positive correlation was observed between increasing C-reactive protein concentration and low CYP2C19 activity. Further recruitment of patients to the study is ongoing and this may help elucidate whether this is a true relationship. TNF-α was found to negatively correlate with CYP2C19 activity in the no-evaluable disease category. In contrast, previous reports have suggested that high TNF-α results in low CYP2C19 activity in patients with congestive heart failure (Frye et al. 2002). However in that study, the sample size was small (n = 16) as well, hence additional studies are required to confirm such a relationship.

The most striking relationship was that observed between the levels of TGF-β and CYP2C19 activity. Significantly lower TGF-β concentrations were associated with poor metaboliser status and in addition, CYP2C19 metabolic ratio was negatively correlated with the TGF-β concentrations in the patients, particularly in patients with stage IV disease. This was an unexpected finding as TGF-β has previously been shown to down-regulate *CYP2C19* mRNA expression in human hepatocyte culture (Aitken et al. 2007).

CYP2C19 metabolic ratio was determined on three separate occasions in the patients. The ratio remained stable in many patients but some individuals had significant changes in phenotype over the three separate tests, i.e. change in metaboliser category from extensive to poor metaboliser and vice versa. It has been reported that multiple phenotyping test with omeprazole showed a wide range of variability in the metabolic ratio; however those patients were extensive metabolisers and did not convert to a poor metaboliser over their repeated phenotyping (Balian et al. 1995; Kim et al. 2004).

Tumour resection clearly influenced the metabolic ratio and phenotype in patient #1009. One patient had a severe adverse event with capecitabine whilst enrolled in the study (patient #1010). The inflammation that accompanies hand-and-foot syndrome is postulated to influence the change in the metabolic ratio. Notably, this variability in metabolic ratio appears to correlate with changes in TGF-β, particularly in two patients (#1009 and #1010).

An inverse correlation between plasma nitric oxide levels and TGF-β, as well as TNF-α levels, has been reported (Fiorenza et al. 2005). TGF-β also down-regulates nitric oxide synthase (NOS) expression (Perrella et al. 1994). Nitric oxide can inhibit CYP activity (Wink et al. 1993) and it is postulated that low TGF-β concentrations in patients may result in higher levels of nitric oxide which may inhibit CYP2C19.

The ability of reactive nitrogen species such as nitric oxide to influence CYP2C19 function and expression is investigated in the following chapter.

CHAPTER 5

Effects of nitric oxide on CYP2C19 activity

5.1 Introduction

The inflammatory process has been proposed to down-regulate drug metabolising activity in cancer patients (Slaviero et al. 2003), and previous chapters (chapter 3 and 4) of this thesis have attempted to address whether there is any association between elevated inflammatory cytokines and compromised CYP2C19 activity in cancer patients. However, inflammatory cytokines are also known to induce nitric oxide synthase (NOS) enzymes and increase the formation of nitric oxide (NO) in human hepatocytes (Nussler et al. 1992). A schematic diagram of how nitric oxide synthase generates nitric oxide *in vivo* is shown in Figure 5.1 (Alderton et al. 2001).

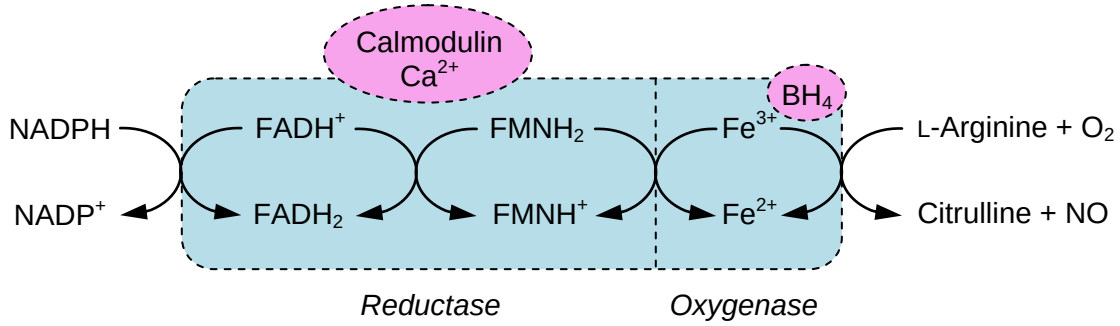

Figure 5.1. ***The overall reaction catalysed by nitric oxide synthase (NOS) and its cofactors.*** Blue indicates the bidomain structure of NOS: the N-terminal oxygenase domain contains binding sites for haem, BH_4 and L-arginine. The C-terminal reductase domain contains binding sites for FAD, FMN and NADPH. Electron flow through the reductase domain requires the presence of bound Ca^{2+} and calmodulin. Electrons donated by NADPH to the reductase domain proceeds to the oxygenase domain via FAD and FMN which then interact with haem and BH_4 to catalyse the reaction of oxygen with L-arginine to generate citrulline and NO. Diagram adapted from Alderton et al. (2001).

Nitric oxide has been proposed as a mediator of the down-regulation of CYP enzymes in acute inflammatory events such as infection and sepsis (Morgan 1997; Morgan et al. 2002; Aitken et al. 2008). However, it is not known whether elevated nitric oxide levels may account for decreased drug metabolising capacity in cancer patients.

Nitric oxide (NO) is a short lived, readily diffusible free radical with important roles in multiple physiological processes, including effects on vascular tone, neurotransmission and host defence against infection (Bredt et al. 1994; Pacher et al. 2007). Nitric oxide can also generate reactive nitrogen species, including peroxynitrite ($ONOO^-$) (Lim et al. 2008) which causes cellular damage due to interactions with macromolecules such as lipids, DNA and proteins (Pacher et al. 2007). A simplified schematic diagram illustrating

the possible pathways of nitric oxide in circulating blood is shown in Figure 5.2 (Kelm 1999).

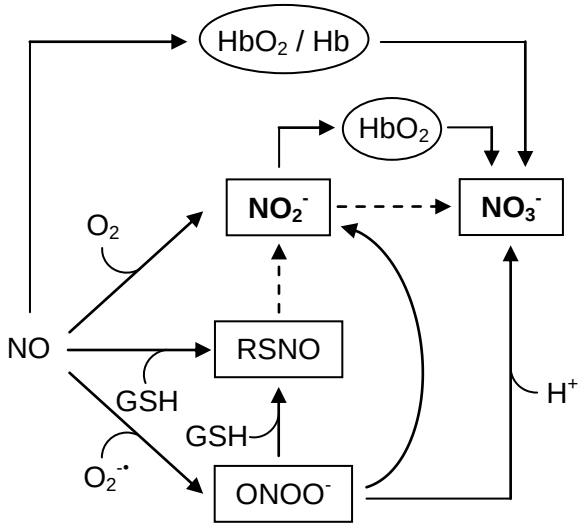

Figure 5.2. ***A simplified scheme of the metabolic pathways of nitric oxide in circulating blood.*** RSNO = *S*-nitrothiols, GSH = glutathione, $O_2^{-\bullet}$ = superoxide, Hb = haemoglobin, HbO_2 = oxyhaemoglobin, NO_2^- = nitrite, NO_3^- = nitrate. Broken arrow indicates decomposition. In plasma, NO may react with oxygen to form nitrite or with superoxide forming peroxynitrite which then decompose into nitrites and nitrates. It can also react with oxyhaemoglobin or haemoglobin forming nitrate and nitrosylhaemoglobin respectively as well as reacting with thiols forming nitrosothiols. Diagram adapted from Kelm (1999).

Nitric oxide can form iron-nitrosyl complexes with hemoproteins and can also irreversibly modify proteins. It has been known for some time that nitric oxide (NO) can inhibit CYP activity (Wink et al. 1993) and that this effect can be independent of inflammatory cytokines (Takemura et al. 1999). Several mechanisms to elucidate the role of nitric oxide in the inhibition of CYP activity have been reported. One possible mechanism is the direct inhibition of the haem moiety within the CYP enzyme (Wink et al. 1993; Masaki 1999). Oxygen is required for CYP activity; therefore nitric oxide may compete with oxygen for the haem moiety thus reversibly inhibiting the CYP activity (Wink et al. 1993). Another possible mechanism is the nitration of specific tyrosine residues on the enzyme which irreversibly inactivate enzyme activity (Roberts et al. 1998). Nitric oxide can also oxidise cytochrome P450 protein thiols, which forms dissociable complexes with CYP isozymes and irreversibly inactivates its catalytic functions (Minamiyama et al. 1997; Takemura et al. 1999). It has also been reported that nitric oxide can lead to increased degradation of CYP bound haem (i.e. loss of the haem prosthetic group) (Kim et al. 1995) resulting in loss of activity. Degradation of CYP protein following exposure to nitric oxide may also involve ubiquitination and the proteasomal degradation pathway (Ferrari et al. 2001; Lee et al. 2008; Lee et al. 2009a). In addition, nitric oxide have been

reported to down-regulate gene expression of a number of CYP isozymes (Hara et al. 2000; Ferrari et al. 2001; Hara et al. 2002; Lee et al. 2008; Lee et al. 2009a). Importantly, differential sensitivity of individual CYP isoforms to nitric oxide-induced down-regulation have been reported (Vernia et al. 2001). Due to the low and rapid loss of CYP2C19 expression in cultured human hepatocytes, little is known about the effect of nitric oxide on CYP2C19 expression and function.

Nitric oxide may also contribute to the inverse relationship between plasma TGF-β concentrations and CYP2C19 activity in cancer patients (chapter 4) since it has been reported that TGF-β can down-regulate nitric oxide synthase (NOS) expression (Perrella et al. 1994). In addition, TGF-β was negatively associated with plasma nitric oxide levels in patients with tuberculosis (Fiorenza et al. 2005).

The experiments described in this chapter attempt to determine whether nitric oxide can affect metabolising activity and/or the expression of CYP2C19. Two *in vitro* systems were used: (i) human liver microsomes and CYP2C19 supersomes^{TM} and (ii) the HCT116.*CYP2C19* cell line which has been transfected with *CYP2C19.* The effect of nitric oxide donors on the ability of these *in vitro* systems to metabolise omeprazole as a measure of CYP2C19 function was determined.

The nitric oxide donors used in this study were sodium nitroprusside (SP), *S*-nitroso-*N*-penicillamine (SNAP) and 2,2′-(hydroxynitrosohydrazino)bis-ethanamine (NOC-18). The chemical formulae of these molecules and their respective chemical breakdown to nitric oxide are shown in Figure 5.3.

SP: $[Fe(CN)_5NO]^{2-} \longrightarrow [Fe(CN)_6]^{4-} + NO$

SNAP: $ONSC(CH_3)_2CH(NHAc)COOH \longrightarrow {}^{\bullet}SC(CH_3)_2CH(NHAc)COOH + NO$

NOC-18: $(CH_2)_2NH_2N[N(O)NO]^-(CH_2)_2NH_3 \longrightarrow NH_2(CH_2)_2NH(CH_2)_2NH_3^+ + 2NO$

Figure 5.3. ***The chemical formulae of the nitric oxide donors used in this study*** **(Glidewell et al. 1987; Hrabie et al. 1993; Singh et al. 1996).**

The reported half-life of SP in aqueous solution is 2 minutes (Schulz 1984), whilst the half-life of SNAP in aqueous solution is 5 hours (Ignarro et al. 1981). NOC-18 in contrast, has a relatively long half-life of 20 hours (Mooradian et al. 1995). Hence the different nitric oxide donors produce the NO molecule at different rates.

Nitric oxide itself has a very short biological half life of < 1 s (Pacher et al. 2007), but is generally considered to be toxic due to reaction with reactive oxygen species to form peroxynitrite ($ONOO^-$) (Pacher et al. 2007). Circulating concentrations of the relatively stable oxidation products of nitric oxide, nitrite and nitrate, can be readily determined using the Griess reaction or by fluorometric detection using diaminonaphthalene (Bryan et al. 2007). Hence, in addition to the studies to determine the ability of nitric oxide to affect CYP2C19 function *in vitro,* the relationship between circulating nitrite/nitrate concentrations and CYP2C19 drug metabolising activity was determined using plasma samples from the cancer patients (chapter 3 and 4).

The hypothesis is that nitric oxide may be the inflammatory mediator responsible for the decreased expression/function of CYP2C19.

The aim of this chapter is to a) determine the ability of nitric oxide to inhibit CYP2C19 function, b) determine the *in vitro* conditions required to alter CYP2C19 activity, c) investigate the role of protein degradation in the decrease of CYP2C19 activity and d) determine the relationship between nitric oxide and CYP2C19 activity in cancer patients.

5.2 Experimental design

5.2.1 Determination of the ability of nitric oxide to inhibit CYP2C19 function

5.2.1.1 Preparation of reagents

Stock solutions of the nitric oxide donors and NADPH were made fresh daily prior to each experiment and stored in the dark until use. Sodium nitroprusside (SP; 2 mM), S-nitroso *N*-acetylpenicillamine (SNAP; 1 mM) and 2,2′-(hydroxynitrosohydrazino)bis-ethanamine (NOC-18; 1 mM) were dissolved in phosphate buffer (67 mM, pH 7.4). NADPH (10 mM) was also prepared in phosphate buffer. Omeprazole (1 mM) was prepared in alkalinised methanol and the internal standard, phenacetin (2.5 mg/mL), was prepared in methanol as described in section 2.3.1.

5.2.1.2 Experimental conditions for exposure of pooled human liver microsomes and CYP2C19 supersomes™ to nitric oxide

The ability of nitric oxide donors SP, SNAP and NOC-18 to inhibit human liver microsomal CYP2C19 or supersome activity directly were determined using omeprazole as a selective CYP2C19 probe substrate. Pooled human liver microsomes (0.5 mg/mL; BD Gentest™, New Jersey, USA) or CYP2C19 supersomes™ (2 pmol; BD Gentest™ Supersomes™, New Jersey, USA) were incubated in triplicate with omeprazole (10 µM) in the presence or absence of SP (0-500 µM), SNAP (0-500 µM) or NOC-18 (0-500 µM) in phosphate buffer (67 mM, pH 7.4). NADPH (1 mM) was added to the mixture to activate the reaction. The final incubation volume was 250 µL and the reaction was incubated at 37°C in a shaking heating block for up to 60 minutes.

The reaction was terminated by the addition of ice-cold acetonitrile (1:1 v/v; 250 µL) followed by the addition of the internal standard (3 µL). The supernatant was collected following centrifugation (10,000 *g*, 10 min) and evaporated under vacuum to approximately 120 µL in volume. Subsequently the sample was analysed as described in section 2.3.4.

5.2.1.3 Experimental conditions for exposure of cells to nitric oxide

Following determination of cell density, cells in 50 mL Falcon tubes were subjected to centrifugation (200 *g*, 5 minutes), the culture medium was then aspirated and discarded. Fresh culture medium was added to resuspend the cell pellet so that the final volume contained 1×10^6 cells per 350 µL. Cells were seeded in triplicate at a concentration of 1×10^6 cells/well in a 12-well plate and were incubated at 5% CO_2 at 37°C for up to 24 hours.

To determine the direct effect of nitric oxide donors on the activity of CYP2C19 in HCT116.*CYP2C19* cells, SP (0-100 mM); SNAP (0-500 µM), NOC-18 (0-500 µM) were co-incubated with omeprazole (10 µM) in tissue culture media (50 µL). Following addition of the compounds to the wells (to a final volume of 400 µL), the tissue culture plate was returned to the incubator (5% CO_2 at 37°C) for 1 hour. The media from each individual well was transferred into a microfuge tube containing ice-cold acetonitrile (1:1 v/v; 800 µL) to terminate the reaction. This was followed by the addition of the internal standard (phenacetin, 7.2 µL). The supernatant was collected following centrifugation (10,000 *g*, 10 min) and evaporated under vacuum to approximately 120 µL in volume. Subsequently the sample was analysed as described in section 2.3.4.

Further experiments to determine the effect of varying concentrations of nitric oxide and different exposure times and washout conditions on the function and expression of CYP2C19 in HCT116 cells were undertaken. Cells (10^6) were added to 12-well tissue culture plates and incubated for either 2 hours (logarithmic growth phase) or 24 hours (confluency) prior to addition of the nitric oxide donors. Following exposure to the relevant nitric oxide donor compound at a range of concentrations for varying times, the media was then removed and replenished with fresh media to allow for a washout period of between 1 and 24h. At this time, the media was removed and replenished with media containing omeprazole (10 µM) and returned to the incubator (5% CO_2 at 37°C) for 1 hour. The CYP2C19 activity was then determined as described above and in section 2.3.4. CYP2C19 activity was normalised to the control activity (without treatment) and to the cell number.

To determine CYP2C19 protein expression and also to quantify *CYP2C19* gene expression changes in HCT116.*CYP2C19* cells following exposure to the nitric oxide donors, parallel plates were prepared for each experimental treatment. Cell lysates were

prepared from each well as described in section 2.9.1 and 2.9.2 for CYP2C19 protein immunoblot analysis and parallel samples were lysed as described in section 2.10.1 for preparation of RNA for real-time PCR analysis. Details of the immunoblot analysis are described in section 2.9 with β-actin as the internal control and real-time PCR was performed as described in section 2.10 with *GAPDH* as the reference gene. Relative gene expression is reported using the $2^{-\Delta\Delta C_T}$ method (Livak et al. 2001) as described in section 2.10.4.

5.2.1.4 Data analysis

Statistical analyses were determined using SigmaPlot (Version 11, Systat Software Inc, Germany) and GraphPad Prism (Version 5.02, GraphPad Software Inc, USA) and the mean ± standard deviation was reported. Unpaired t-test was used to compare variable across two groups. Two-sided *P*-values < 0.05 were considered statistically significant.

5.3 Results

5.3.1 Characterisation of *CYP2C19* expression in HCT116.*CYP2C19* cell-line

To confirm whether *CYP2C19* was expressed in the cell-line, real-time PCR was used to determine *CYP2C19* mRNA expression with *GAPDH* as the reference gene as described in section 2.10. An initial experiment was carried out to investigate the appropriate number of cells required to detect *CYP2C19* and *GAPDH* mRNA expression in the cell-line.

CYP2C19 and *GAPDH* expression were both detectable in the HCT116.*CYP2C19* cell-line. The mean threshold cycle (C_T) for both the gene of interest and the reference gene were linear between 10^3-10^6 cells (Figure 5.4). All further experiments using real-time PCR to quantify *CYP2C19* gene expression were conducted using 10^6 cells.

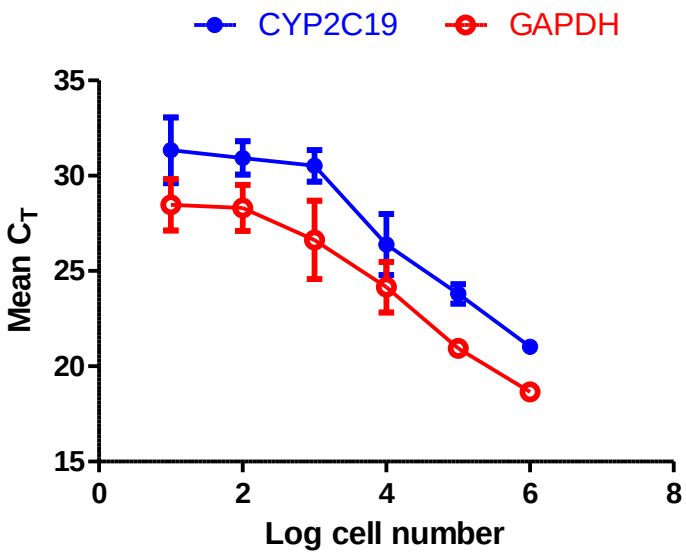

Figure 5.4. ***The optimal number of HCT116.CYP2C19 cells required to detect CYP2C19 and GAPDH expression in real-time PCR.***

Comparison of *CYP2C19* expression in HCT116 cells overexpressing *CYP2C19* and the wildtype cell-line was undertaken. It was observed that there was negligible *CYP2C19* mRNA expression in the HCT116 wildtype cell-line (mean C_T values of greater than 30 cycles). The *CYP2C19* overexpressing cell-line had acceptable expression of the gene (mean C_T = 21.0 ± 0.3) (Table 5.1). Expression of the internal control gene, *GAPDH,* was similar in both cell-lines.

Cell-line	Mean *CYP2C19* C_T	Mean *GAPDH* C_T
HCT116.wildtype	34.6 ± 0.4	20.1 ± 0.1
HCT116.*CYP2C19*	21.0 ± 0.3	18.7 ± 0.3

Table 5.1. ***The mean threshold cycle (C_T) of CYP2C19 and GAPDH mRNA expression in wild type or HCT116.CYP2C19 cell-line at 10^6 cells.*** Mean ± SD of triplicate determinations are reported.

Further characterisation of the HCT116.*CYP2C19* cells was undertaken to confirm CYP2C19 protein expression. Immunoblotting was carried out as described in section 2.9. A typical protein concentration of total cell lysate prepared from HCT116.*CYP2C19* cell-line (10^6 cells/mL) was 2.7 mg/mL. Protein samples (10 µg/well) were transferred to PVDF membranes and probed with a commercially available antibody. CYP2C19 protein was detected in CYP2C19 supersomes™ and human liver microsomes (Lane 1 and 2; Figure 5.5). However the antibody was unable to detect immunoreactive CYP2C19 protein in the HCT116.*CYP2C19* cell lysate (Lane 3; Figure 5.5).

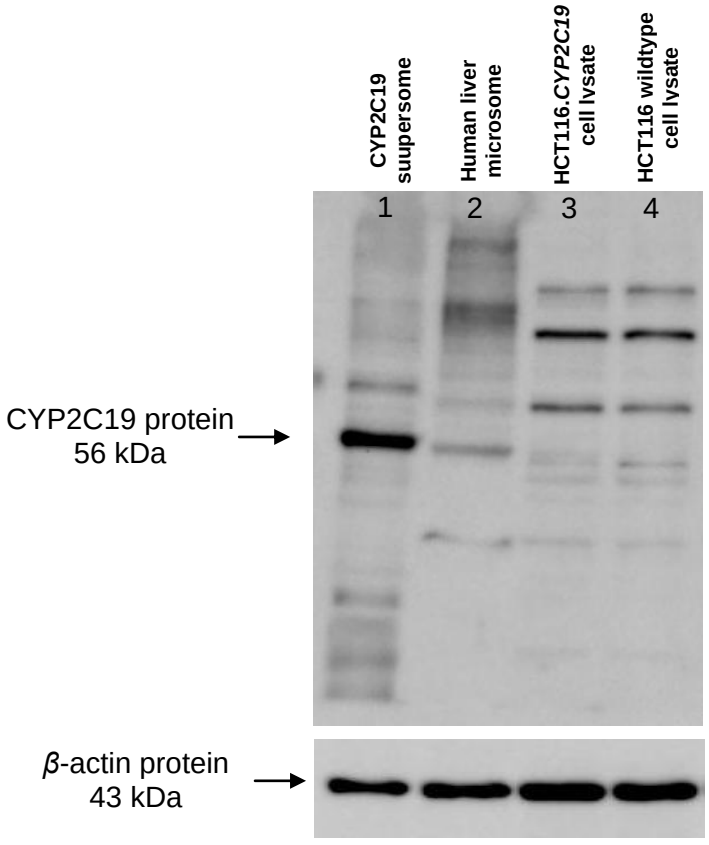

Figure 5.5. ***CYP2C19 immunoreactive protein was not detected in the HCT116.*CYP2C19 *cell-line*.** Lane 1) CYP2C19 supersomes™, 2) human liver microsomes, 3) HCT116.*CYP2C19* cell lysate and 4) HCT116.wildtype cell lysate

There were numerous immunoreactive bands with higher and lower molecular weight detected in both the wildtype and CYP2C19 overexpressing cell lysates, as well as supersomes and microsomes (Figure 5.5). This is suggestive of non-specific binding of the antibody at the dilution used (1:5000 v/v). 10 µg of total protein was loaded into each well and even loading was confirmed by immunoblotting for *β*- actin. However, although similar amounts of total protein were loaded in each lane, the cell lysates consist of total cellular proteins whereas the supersomes and microsomes were a semi-purified sub-cellular preparation. Relatively low levels of the endoplasmic reticulum-associated CYP enzymes compared with cytoplasmic cellular proteins may thus preclude detection of immunoreactive CYP2C19 in the cell lysates. However, even following preparation of a microsomal fraction from *HCT116.CYP2C19* cells (section 2.9.2), CYP2C19 immunoreactive protein could not be visualised (data not shown).

Various methods were also undertaken to increase the sensitivity of the immunoreactive detection for this protein including alternative cell lysis methods such as freeze-thaw cell lysis (Rudolph et al. 1999) and directly heating of cell extracts in sample buffer, as well as variation of antibody dilutions. However, it was not possible to specifically detect immunoreactive CYP2C19 protein in HCT116.*CYP2C19* cells using this western blotting method.

In contrast to the lack of detection of an immunoreactive protein, the functional activity of CYP2C19 could be selectively detected in the HCT116.*CYP2C19* cells. Following incubation of the cell-line with omeprazole for 1 h with quantification of the 5'hydroxy omeprazole metabolite as described in section 5.2.1.3, HCT116.*CYP2C19* cells had detectable omeprazole 5'hydroxylase activity (1.6 ± 0.4 pmol/min/10^6 cells). This activity was specific to the CYP2C19 overexpressing cells as there was no detectable activity in the wildtype (WT) cells (Table 5.2).

In summary, although immunoreactive CYP2C19 protein could not be detected, *CYP2C19* mRNA and CYP2C19 function was detected and quantifiable in the HCT116.*CYP2C19* cell-line. This was specific as no gene expression or functional enzyme was detected in the wildtype cell-line. Hence, the cell-line system was considered suitable for further experiments to elucidate the role of nitric oxide on CYP2C19 activity and expression.

The CYP2C19 activity in HCT116.*CYP2C19* cell-line compared with typical *in vitro* systems (human liver microsomes and CYP2C19 supersomes™) is shown in Table 5.2.

Protein	CYP2C19 activity (omeprazole hydroxylase activity)
Pooled human liver microsome	36.4 ± 8.1 pmol/min/mg
CYP2C19 supersomes™	2.3 ± 0.3 pmol/min/pmol P450
HCT116.*CYP2C19* cell-line	1.6 ± 0.4 pmol/min/10^6 cells[a]
HCT116 wildtype cell-line	Not detected

Table 5.2. ***CYP2C19 activity determined in the different in vitro systems used in this study.*** [a] 10^6 cells = 2.7 mg/mL protein following cell lysis.

5.3.2 The effect of nitric oxide on CYP2C19 activity

5.3.2.1 The direct inhibition of CYP2C19 activity by nitric oxide

The ability of nitric oxide to inhibit CYP2C19 activity was investigated by co-incubation of nitric oxide donors and omeprazole with pooled human liver microsomes, CYP2C19 supersomes™ or HCT116.*CYP2C19* cells (Figure 5.6).

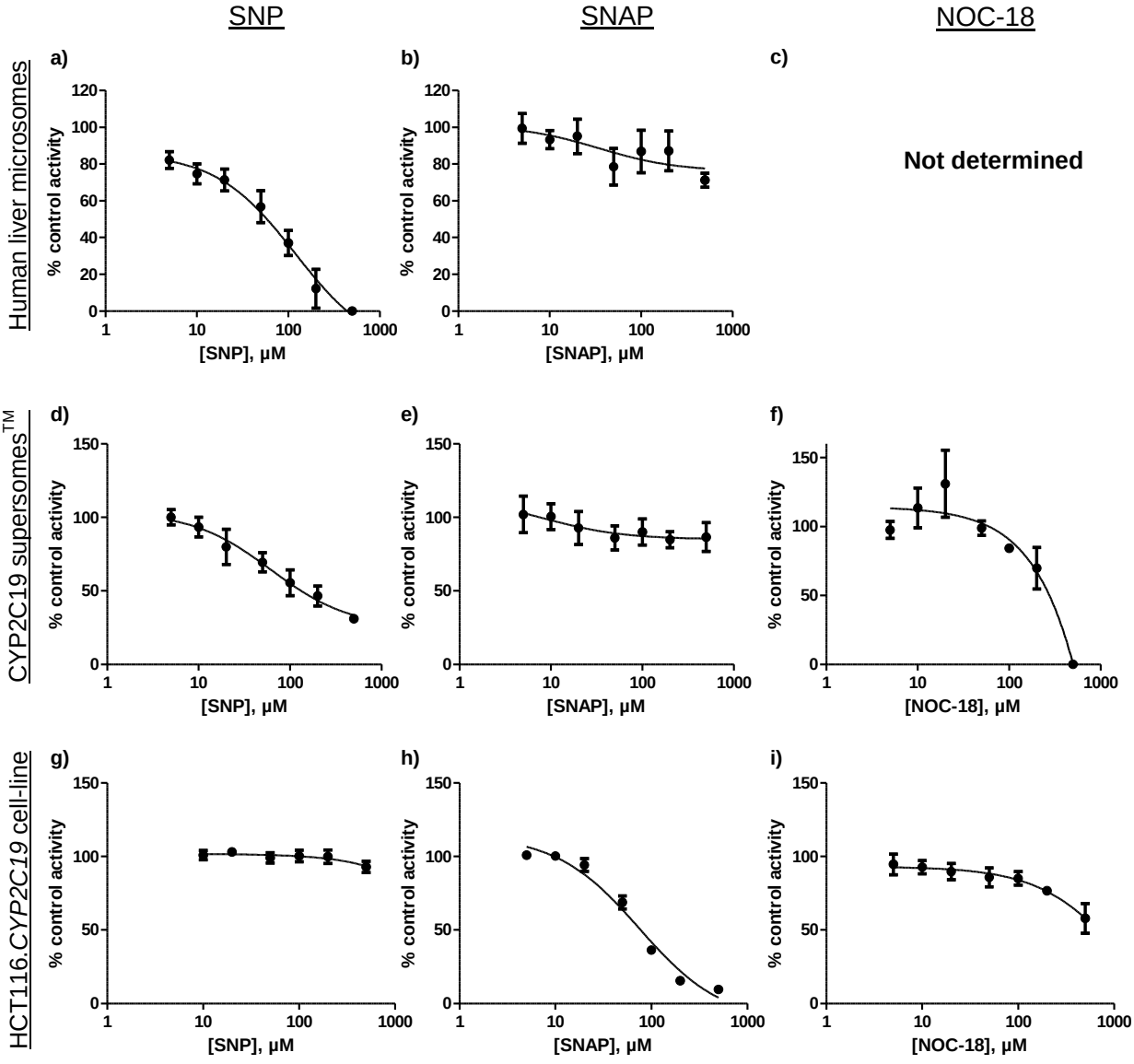

Figure 5.6. ***The inhibitory effect of nitric oxide donors (SP, SNAP and NOC-18) on CYP2C19 activity in human liver microsomes (a-c); CYP2C19 supersomes™ (d-f) and in HCT116.CYP2C19 cells (g-i) of triplicate determinations.*** CYP2C19 activity was measured following co-incubation of nitric oxide donors with omeprazole for 20 minutes (microsomes/supersomes) or 1 hour (HCT116.*CYP2C19* cells).

The nitric oxide donor SP was an inhibitor of CYP2C19 activity in human liver microsomes with an IC_{50} = 54.1 µM (Figure 5.6a). In contrast, SNAP did not extensively inhibit human liver microsomal CYP2C19 activity at concentrations up to 500 µM (Figure 5.6b). At the highest concentration tested (500 µM), SNAP inhibited 5'hydroxy omeprazole formation by only 20%. The effect of NOC-18 on microsomal activity was not determined.

SP and NOC-18 inhibited CYP2C19 catalysed formation of 5'hydroxy omeprazole in CYP2C19 supersomes™ with IC_{50} values of 137.7 µM and 267.9 µM respectively (Figure 5.6d and Figure 5.6f). Similar to that observed in human liver microsomes, SNAP also did not extensively inhibit CYP2C19 supersome activity, with approximately 15% inhibition at the highest concentration tested (500 µM; Figure 5.6e).

Following co-incubation of the nitric oxide donors and omeprazole (10 µM) for 1 hour, the CYP2C19 activity of HCT116.*CYP2C19* was also inhibited. Complete inhibition of CYP2C19 activity was seen at lower concentrations of SNAP compared with microsomes/supersomes (IC_{50} = 74.0 µM; Figure 5.6h). NOC-18 also inhibited CYP2C19 activity in the cell-line but in contrast to SNAP, a higher concentration of the nitric oxide donor was required than for supersomes (Figure 5.6i). At the maximum concentration of NOC-18 tested (500 µM), approximately 40% inhibition was observed. Unlike human liver microsomes and CYP2C19 supersomes™, SP did not inhibit CYP2C19 activity in the HCT116.*CYP2C19* cell-line at the maximum concentration tested (Figure 5.6g). Complete inhibition of CYP2C19 activity by SP was only observed at 100 mM (IC_{50} = 5688.5 µM; Figure 5.7).

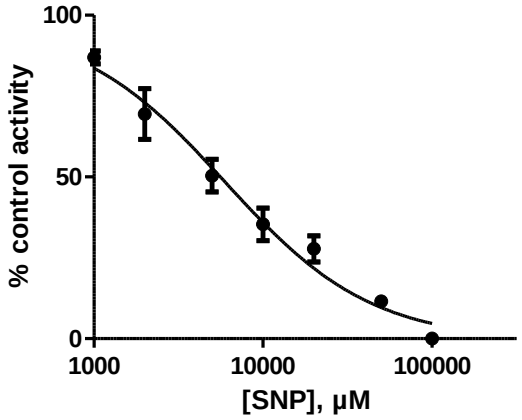

Figure 5.7. ***The inhibition of CYP2C19 activity in HCT116.CYP2C19 cell-line at high (> 1 mM) concentrations of SP of triplicate determinations.***

Comparison of the IC_{50} values of the different nitric oxide donors following co-incubation with omeprazole in the three *in vitro* systems is shown in Table 5.3.

	IC_{50} values (μM)		
	SP	**SNAP**	**NOC-18**
Pooled human liver microsomes	54.1	> 500 (20% inhibition)[b]	ND
CYP2C19 supersomes™	137.7	> 500 (15% inhibition)[b]	267.9
HCT116.*CYP2C19* cell-line	No inhibition[a]	74.0	≥ 500 (42% inhibition)[b]

Table 5.3. ***The IC_{50} values of the nitric oxide donors in the inhibition of CYP2C19 activity.*** [a] complete inhibition was only observed at 10 mM SP with IC_{50} of 5688.5 μM (Figure 5.7). [b] percentage inhibition observed at 500 μM.

The concentrations of SP and SNAP required to inhibit CYP2C19 activity by 50% in human liver microsomes and CYP2C19 supersomes™ were broadly similar. The IC_{50} value of NOC-18 in supersomes was similar to that observed in the cell-line. In contrast, CYP2C19 activity in the cell-line was more sensitive to the effects of low concentrations of SNAP than in supersomes (74 μM versus 500 μM respectively). There was no inhibitory effect of SP (at concentrations < 1 mM) on CYP2C19 activity in the cell-line and inhibition was only observed at very high concentrations (> 10 mM).

However, relative amounts of nitric oxide (NO) released over the period of incubation will differ for each donor compound. SP releases NO in aqueous solution with a $t_{1/2}$ of 2 minutes whereas SNAP has a $t_{1/2}$ of 5 hours. Therefore lower amounts of nitric oxide will be generated by SNAP over the one hour incubation compared with SP at equimolar concentration of NO donor. Although NOC-18 has a long $t_{1/2}$ in aqueous solution (20 hours) compared with SP and SNAP, equimolar concentrations of this donor release twice as much NO (Figure 5.3) and thus CYP2C19 function may be inhibited at apparently lower concentration of NOC-18 than SNAP.

The data described so far were based on known concentrations of the nitric oxide donors. Although the amount of nitric oxide produced by each nitric oxide donor was not directly measured in the study, a hypothetical concentration value can be estimated using the following formula (Wagner 1976),

$$C_t = C_0 \times e^{-k \times t}$$

where C_t is concentration after time (t), C_0 is initial concentration at t = 0, k is the elimination rate constant based on half-life ($t_{1/2}$) and $k = 0.693 \div t_{1/2}$ (Wagner 1976). The inhibitory effect of the calculated amounts of nitric oxide on CYP2C19 activity in microsomes/supersomes and HCT116.*CYP2C19* cells are shown in Figure 5.8.

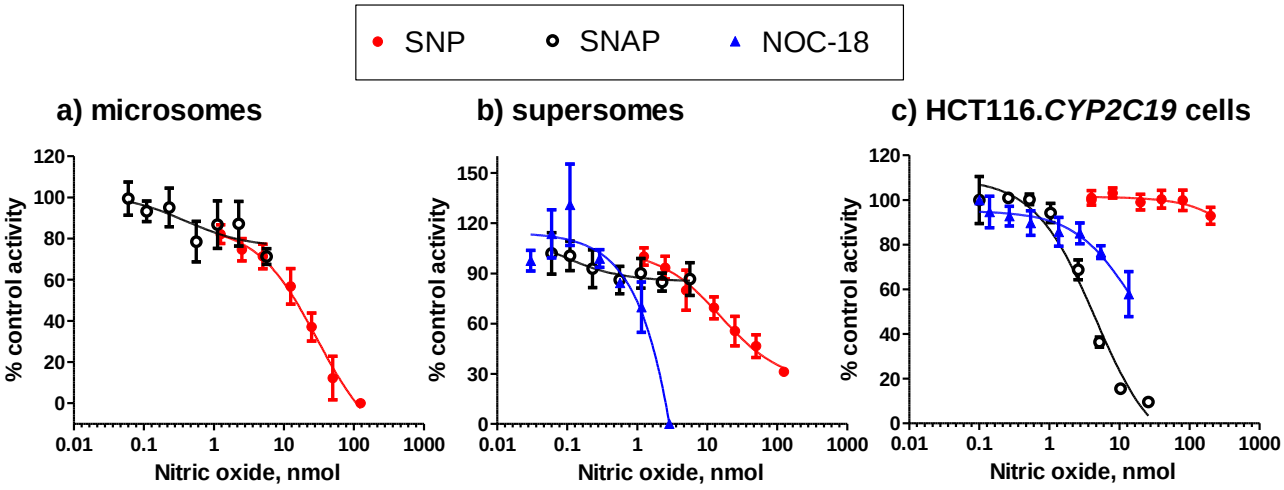

Figure 5.8. ***The inhibitory effect of relative amounts of nitric oxide formed from nitric oxide donors on CYP2C19 activity in a) human liver microsomes, b) CYP2C19 supersomes and c) HCT116.CYP2C19 cells of triplicate determinations.*** Closed red circle = SP, open black circle = SNAP and closed blue triangle = NOC-18.

The IC_{50} values of the estimated amount of nitric oxide released from SP, SNAP and NOC-18 in the *in vitro* systems is shown in Table 5.4.

	IC_{50} values (nmol of NO released from NO donors)		
	SP	**SNAP**	**NOC-18**
Pooled human liver microsomes	13.8	> 5.6 (20% inhibition)[b]	ND
CYP2C19 supersomes™	33.8	> 5.6 (15% inhibition)[b]	1.5
HCT116.*CYP2C19* cell-line	No inhibition[a]	3.8	≥ 13.6 (42% inhibition)[b]

Table 5.4. ***The IC_{50} values of the nitric oxide released from the nitric oxide donors in the inhibition of CYP2C19 activity.*** [a] complete inhibition was only observed at 10 mM SP with IC_{50} of 2275.1 nmol. [b] percentage inhibition observed at highest concentration.

Approximately 13.8 and 33.8 nmol of nitric oxide generated from SP were required to decrease CYP2C19 activity by 50% in microsomes and supersomes respectively. In contrast, CYP2C19 activity in microsomes and supersomes was decreased by 20% and 15% respectively at 5.6 nmol of nitric oxide released from SNAP. In addition, the IC_{50} for nitric oxide generated from NOC-18 was 1.5 nmol in supersomes. 3.8 and 13.6 nmol of nitric oxide generated from SNAP and NOC-18 were required to decrease CYP2C19 activity in HCT116.*CYP2C19* cells by 50% and 42% respectively (Table 5.4).

Although a similar NO-donor concentration range was used for each compound, it is clear that due to the different rates of NO-generation from each donor, higher concentrations of SNAP should be used to determine the IC_{50} value for inhibition of CYP2C19 activity in microsomes and supersomes.

The calculation of nitric oxide formation was based on the assumption that its formation from NO donors in aqueous solution is linear (Wagner 1976). However, the decomposition of SP to nitric oxide has been reported to be non-linear (Feelisch et al. 1987), whereas SNAP releases nitric oxide in a linear fashion over a wide concentration range (Feelisch 1988).

Denitrosation of SNAP is not spontaneous and may be metabolically activated by components of the plasma cell membrane, as well as the inner mitochondrial and endoplasmic reticulum membranes (Kowaluk et al. 1990). Thus activation of this donor by the cell may account for the relative increase in sensitivity to this compound compared with microsomes/supersomes.

SNAP also differs from both SP and NOC-18 in that, in solution, it can generate not only nitric oxide but also considerable quantities of NO^- and NO^+ (Arnelle et al. 1995). SNAP is also sensitive to the presence of thiols, which increase the hydrolysis of the compound. Hence in tissue culture, this donor may generate greater amounts of nitric oxide, including additional reactive nitrogen species. In addition, the presence of relatively high levels of oxygen in the tissue incubator (95% O_2, 5% CO_2) compared with atmospheric oxygen (21%) (Machta et al. 1970), may also increase generation of reactive nitrogen species such as nitrogen dioxide ($NO_2^{\bullet}$) (Lim et al. 2008).

It is also important to note that the relative NO concentration calculated is based on the reported $t_{1/2}$ in aqueous solution. However, the half-life of the nitric oxide donors in a biological system, i.e. cells incubated in culture media differs to that reported in aqueous solution, i.e. in phosphate buffer. For example, the reported half-life of NOC-18 was 20 hours in aqueous solution (Mooradian et al. 1995; Keefer et al. 1996) but was 78 minutes culture medium at pH 7.4 and 37°C (Shimaoka et al. 1995). Importantly, it has been reported that significantly lower levels of NO was formed following incubation in tissue culture media compared with aqueous solutions (Keynes et al. 2003). Compounds in media may act as radical scavengers or suppress radical generation. In particular, nitric oxide can react with compounds such as glutathione (GSH) and amino acids, such as cysteine, tyrosine and tryptophan (Lim et al. 2008). Hence, presence of these compounds and other antioxidants in tissue culture media may alter the apparent IC_{50} of a nitric oxide donor compound.

Therefore the relative differences in sensitivity of HCT116.*CYP2C19* cells to the NO donors compared with microsomes/ supersomes may be the result of (a) cellular activation of the NO donors, (b) altered kinetics of NO formation in complex tissue culture media compared with aqueous solution or (c) scavenging/sequestration of NO by compounds in the tissue culture media.

The nitric oxide donors, SNAP and NOC-18, releases nitric oxide in solution; however, the use of these donors may be complicated by the concomitant release of their respective by-products (Figure 5.3). SP releases cyanide which can oxidise haem (Schulz 1984), hence inhibition of CYP2C19 activity in microsomes and supersomes may also be attributed to the presence of cyanide as well as NO for this donor compound.

To verify that the release of nitric oxide and not the by-product of SNAP, *N*-acetylpenicillamine (NAP), was the cause of decreased CYP2C19 activity in the cell-line, NAP (0-200 µM) was prepared in phosphate buffer (67 mM, pH 7.4) and then co-incubated with omeprazole (10 µM) in the HCT116.*CYP2C19* cell-line for 1 hour. NAP did not inhibit CYP2C19 activity in the cell-line (Figure 5.9).

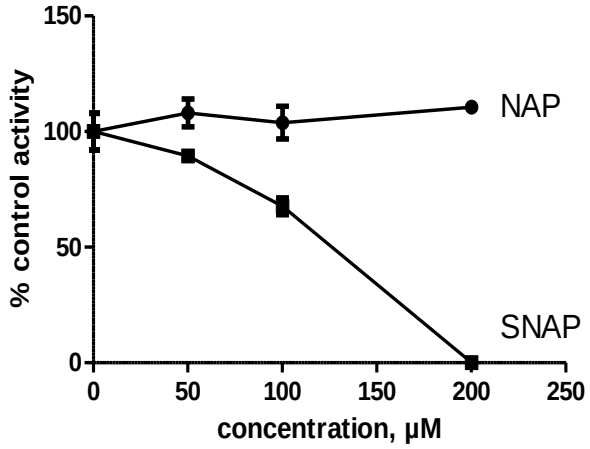

Figure 5.9. ***The inhibitory effect of SNAP and NAP on CYP2C19 activity in HCT116.CYP2C19 cell-line of triplicate determinations.*** NAP did not inhibit CYP2C19 activity.

This confirms that the inhibitory effect that SNAP exerts on omeprazole metabolism in HCT116.*CYP2C19* cells was mediated by nitric oxide.

5.3.2.2 Effect of nitric oxide exposure on cellular CYP2C19 activity with or without washout period

To investigate whether the ability of nitric oxide to decrease CYP2C19 activity was a result of direct inhibition of the enzyme, HCT116.*CYP2C19* cells were incubated with the nitric oxide donor SNAP (100 µM) for 1 hour followed by a replacement of fresh media for either a 1 hour or 24 hour washout period, after which time the omeprazole 5'hydroxylase activity was determined.

After a one hour washout, cells exposed to SNAP had significantly increased CYP2C19 activity compared with the respective control (*P* = 0.003; Figure 5.10a). This same stimulation of activity was observed in a second independent experiment although it was not significant (*P* > 0.05; Figure 5.10b). Similarly, following a 24 hour washout period, stimulation of CYP2C19 activity was observed, with activity significantly different from its respective control (*P* = 0.03; Figure 5.10a). However, this stimulation at 24 hours was not observed in a second independent experiment.

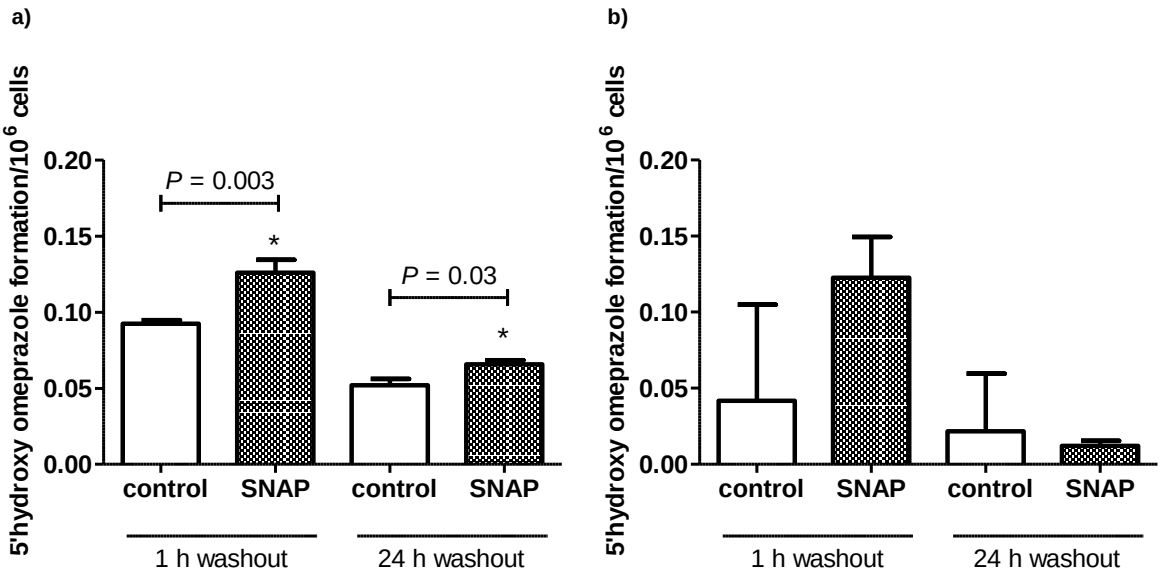

Figure 5.10. ***CYP2C19 activity after incubation with SNAP (100 µM) with or without a washout period.*** 5'hydroxy omeprazole formation (peak area ratio units per hour). a) and b) are data from two independent experiments of triplicate determinations. CYP2C19 activity recovered following washout.

Importantly, it appears that the inhibitory effect of SNAP on cellular CYP2C19 activity observed previously (Figure 5.6) is reversible, as activity was not suppressed following a washout period. Acute exposure of cells to 100 µM SNAP for one hour followed by a washout period appeared to stimulate CYP2C19 activity.

In summary, nitric oxide does directly inhibit CYP2C19 activity in both microsomes/supersomes and cell-line system. This direct inhibition by nitric oxide is reversible following a 1 h or 24 h washout.

5.3.2.3 The effect of chronic exposure of nitric oxide on cellular CYP2C19 activity

Following exposure of HCT116.*CYP2C19* cells to SNAP (100 µM or 500 µM) for 1 hour or 24 hours, the culture media was aspirated and the CYP2C19 activity was determined by incubation with omeprazole in fresh media. CYP2C19 activity was significantly decreased after 24 hours exposure to SNAP compared with respective controls ($P < 0.05$, Figure 5.11a-b). Exposure of cells to high concentration of SNAP (500 µM) for 1 hour also led to a significant decrease in CYP2C19 activity ($P = 0.02$; Figure 5.11b).

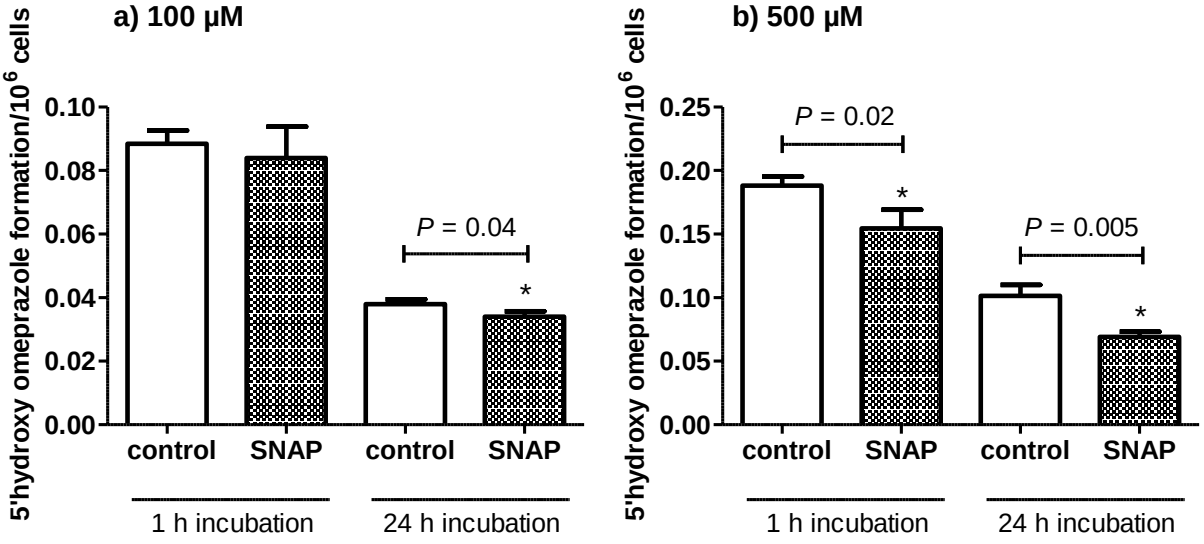

Figure 5.11. ***The inhibitory effect of 24 hour exposure to SNAP at a) 100 µM or b) 500 µM on CYP2C19 activity in HCT116.CYP2C19 cells of triplicate determinations.*** CYP2C19 activity significantly decreased following longer exposure (100 µM, 24 h; $P = 0.04$) and at a higher SNAP concentration (500 µM, 1 h; $P = 0.02$)

To investigate further the effect of the chronic exposure to nitric oxide, cells were exposed to increasing concentrations (0-1000 µM) of SNAP or NOC-18 for 24 hours. There was a concentration dependent decrease in CYP2C19 activity as the concentration of each nitric oxide donor increased (Figure 5.12).

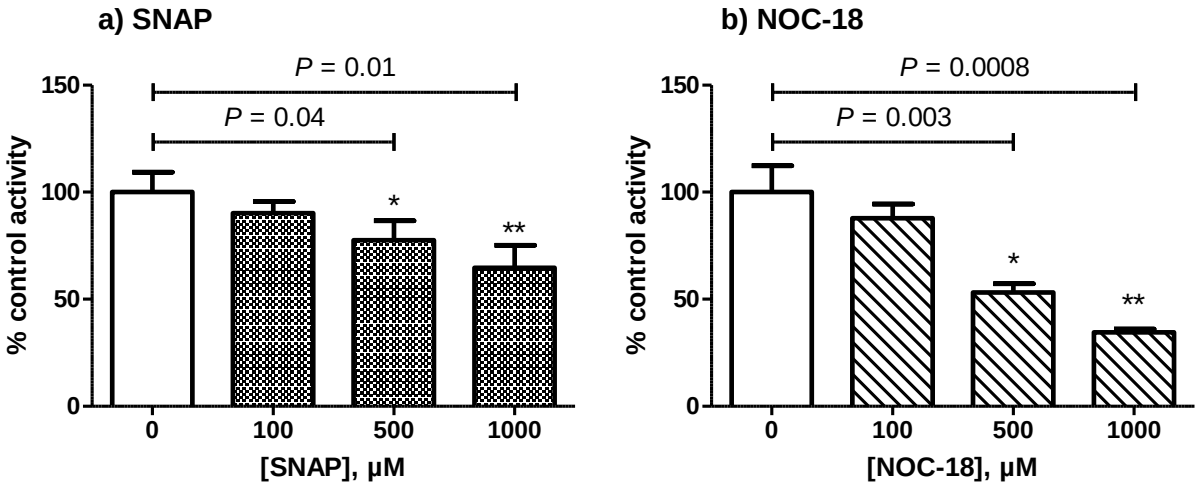

Figure 5.12. ***The effect of 24 hour exposure to nitric oxide donor (SNAP and NOC-18) on CYP2C19 activity in HCT116.CYP2C19 cells.*** a) 24h SNAP incubation and b) 24h NOC-18 incubation. % control activity was normalised to cell number. A concentration dependent decrease in CYP2C19 activity was observed as SNAP/NOC-18 increased.

At concentrations greater than 500 µM both the nitric oxide donors, SNAP and NOC-18 significantly decreased CYP2C19 activity (78% and 53% of control activity respectively; $P < 0.05$). At the highest concentration tested (1000 µM), SNAP and NOC-18 also decreased CYP2C19 activity (65% and 35% of control activity respectively; $P < 0.05$).

For these experiments, cells were plated at 10^6 cells/well, and the cell number increased 2-3 fold by 24 hours at which point they reach confluency, i.e. cells are in logarithmic growth phase during the 24 hours period in these experiments. Subsequent experiments were conducted on confluent cells, i.e. following 24 hour growth.

HCT116.*CYP2C19* cells were plated for 24 hours (confluency) prior to exposure to nitric oxide donors (SNAP and NOC-18; 0-1000 µM) for 5 hours followed by the replacement of the media with fresh media containing omeprazole. The effect of exposure to SNAP or NOC-18 for 5 hours on CYP2C19 activity in the confluent cells is shown in Figure 5.13.

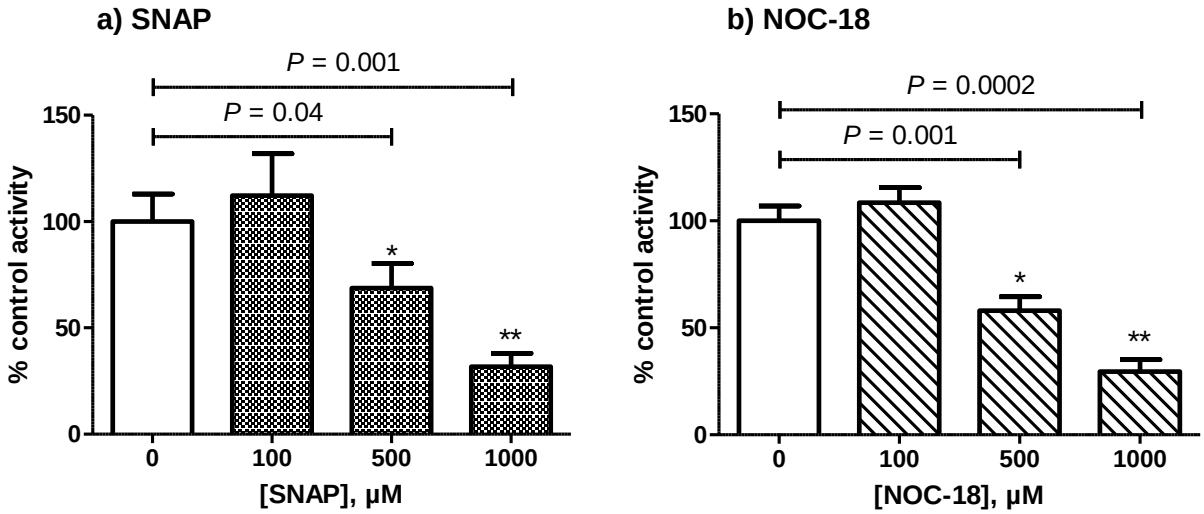

Figure 5.13. ***The effect of exposure to nitric oxide donors (SNAP and NOC-18) on confluent cells.*** a) 5h SNAP incubation and b) 5h NOC-18 incubation. % control activity was normalised to cell number. A concentration dependent decrease in CYP2C19 activity was observed as SNAP/NOC-18 increased.

Similar to the effects observed in cells undergoing logarithmic cell growth (Figure 5.12), there was also a concentration-dependent decrease in CYP2C19 activity. Both of the nitric oxide donors, SNAP and NOC-18, significantly decreased CYP2C19 activity (69% and 58% of control activity respectively at 500 µM; $P < 0.05$). At the highest concentration tested (1000 µM), there was a significant decrease in CYP2C19 activity by SNAP and NOC-18 (32% and 30% of control activity respectively; $P < 0.05$).

In summary, as the concentration of the nitric oxide donors SNAP and NOC-18 increase, there was an associated decrease in CYP2C19 activity in HCT116.*CYP2C19* cells at both logarithmic growth phase (Figure 5.12) and confluency (Figure 5.13). Whether this change in activity is due to the decreased expression or increased degradation of CYP2C19 protein following exposure to nitric oxide is unknown. CYP2C19 protein expression could not be detected in the cells due to the lack of detection of immunoreactive CYP2C19 protein as discussed earlier (section 5.3.1). An alternative method to elucidate the potential role of nitric oxide in changes of cellular CYP2C19 protein expression is by the use of a proteasome inhibitor. The cellular proteasome system can degrade damaged and structurally altered proteins and MG132 is a widely used inhibitor of the proteasome (Lee et al. 1998). If MG132 prevents the degradation of CYP2C19 protein following nitric oxide exposure, no change in CYP2C19 activity would be expected.

5.3.3 The effect of proteasome inhibition on nitric oxide-induced changes in cellular CYP2C19 function

The proteasome inhibitor, MG132 (10 µM), was co-incubated with or without the nitric oxide donors (SNAP and NOC-18; 1000 µM) for 1 hour followed by the determination of CYP2C19 activity in HCT116.*CYP2C19* cells grown to confluency.

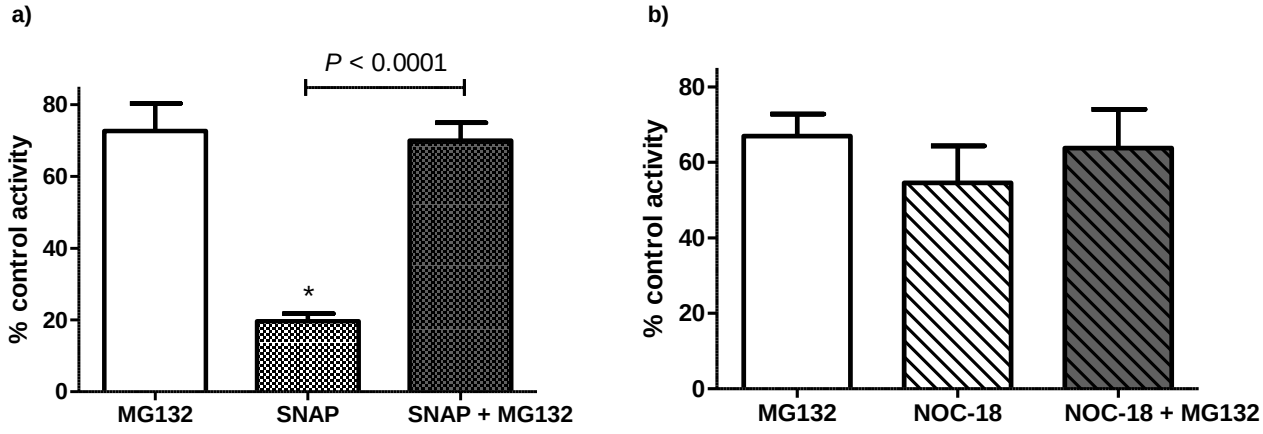

Figure 5.14. ***The effect of the proteasome inhibitor, MG132, on NO-mediated changes in CYP2C19 activity in HCT116.CYP2C19 cells.*** Confluent cells were incubated with 1000 µM) NO-donors (a: SNAP or b: NOC-18) for 5 hours prior to determination of CYP2C19 activity. % control activity was normalised to cell number and the activity in the absence of NO-donor and MG132. Co-incubation of SNAP with MG132 prevented the decrease in CYP2C19 activity ($P < 0.0001$). Co-incubation of NOC-18 with MG132 also prevented the decrease but was insignificant.

As expected, SNAP significantly decreased CYP2C19 function to 20% of control activity ($P = 0.0012$). Co-incubation of SNAP with MG132 for 5 hours prevented this decrease and this effect was significant ($P < 0.0001$; Figure 5.14a). NOC-18 also decreased cellular CYP2C19 activity by 45%, but to a lesser extent than SNAP (Figure 5.14). NOC-18 (1000 µM) did not decrease cellular CYP2C19 activity as effectively as in the previous experiment (55% versus 30% of respective control activity; Figure 5.14b versus Figure 5.13b respectively). MG132 co-incubation with NOC-18 appeared to prevent this decrease, but this effect was not significant ($P = 0.3239$; Figure 5.14b).

It is postulated from this preliminary experiment that MG132 may potentially inhibit the proteasomal degradation of nitric oxide-exposed CYP2C19 protein, thus preventing the decrease of CYP2C19 activity following exposure to nitric oxide. However, it was observed that MG132 also decreased CYP2C19 activity by approximately 30% compared with control activity (Figure 5.14). This may indicate that MG132 is either

cytotoxic to HCT116.*CYP2C19* cells at the concentration used (although activity was normalised to cell number for all these experiments) or MG132 may have a direct inhibitory effect on CYP2C19. Further experiments to clarify the effect of MG132 on CYP2C19 are required.

To determine whether the protective effect of MG132 on cellular CYP2C19 activity following exposure to nitric oxide is a result of changes in gene expression, parallel experimental plates were prepared. The cells were lysed and *CYP2C19* mRNA expression was determined as described in section 2.10. Although CYP2C19 activity was decreased, there was no change in *CYP2C19* mRNA expression following co-incubation of SNAP or NOC-18 (1000 µM) with MG132 (10 µM) for 5 hours in confluent HCT116.*CYP2C19* cells (Table 5.5). However, the data is preliminary and there is considerable variability in the mRNA expression in this experiment and further optimisation and investigation is required.

5 h SNAP	***CYP2C19* expression relative to control**
Control	1.00 (0.24 – 4.20)
MG132	1.38 (0.34 – 5.62)
SNAP	0.52 (0.32 – 0.86)
SNAP + MG132	1.64 (0.33 – 8.09)
5 h NOC-18	***CYP2C19* expression relative to control**
Control	1.00 (0.60 – 1.66)
MG132	1.09 (0.82 – 1.44)
NOC-18	1.62 (1.44 – 1.83)
NOC-18 + MG132	2.13 (1.47 – 3.10)

Table 5.5. ***CYP2C19* mRNA expression in the presence or absence of nitric oxide donors (SNAP or NOC-18) or proteasome inhibitor (MG132) in HCT116.CYP2C19 cells.** Mean and standard deviation range was reported.

In summary, in addition to the direct reversible effects of nitric oxide on CYP2C19 activity observed following co-incubation of nitric oxide donors with omeprazole, nitric oxide also appears to have additional longer lasting effects on CYP2C19 functional activity. Whether the decrease in CYP2C19 function is due to changes in protein or gene expression is not yet clear. However, the protective effect of the proteasomal inhibitor MG132 may indicate a role for increased degradation of the nitric oxide-exposed CYP2C19 protein as a possible mechanism for the longer lasting effects of nitric oxide exposure on cellular functional activity.

5.3.4 Nitric oxide levels in cancer patients

In the previous chapter (chapter 4), it was observed that plasma TGF-β concentrations in the cancer patients were negatively associated with CYP2C19 metabolic ratio, i.e increased TGF-β was correlated with increased CYP2C19 activity. TGF-β has been reported to down-regulate the expression of the enzyme, nitric oxide synthase, which generates nitric oxide (Perrella et al. 1994). In this chapter, nitric oxide has been shown to decrease CYP2C19 activity in *in vitro* systems. Therefore it was hypothesised that nitric oxide may affect CYP2C19 activity in the cancer patients resulting in a genotype-phenotype discordance in some individuals.

Nitric oxide has a short biological half-life of seconds (Pacher et al. 2007) and is almost completely converted into nitrite and nitrate *in vivo* as shown in Figure 5.15 (Feelisch 1991). Nitrites and nitrates are stable in frozen plasma for at least one year (Moshage et al. 1995).

$$2\,NO + O_2 \rightarrow 2\,NO_2$$
$$2\,NO_2 \rightleftharpoons N_2O_4$$
$$NO_2 + NO \rightleftharpoons N_2O_3$$
$$N_2O_3 + H_2O \rightarrow 2\,NO_2^- + 2H^+$$
$$N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+$$

Figure 5.15. ***The reaction sequence of nitric oxide with oxygen to form nitrite and nitrate.*** Adapted from Feelish (1991).

It is possible to measure the levels of nitrite and nitrate using fluorometric detection; hence the concentrations of nitrite/nitrate were determined in the patients' plasma as described in section 2.11. The levels of nitrite/nitrate determined in the cancer patients were correlated with their CYP2C19 activity as well as the levels of inflammatory cytokines that were measured previously (chapter 3 and 4; Figure 5.16).

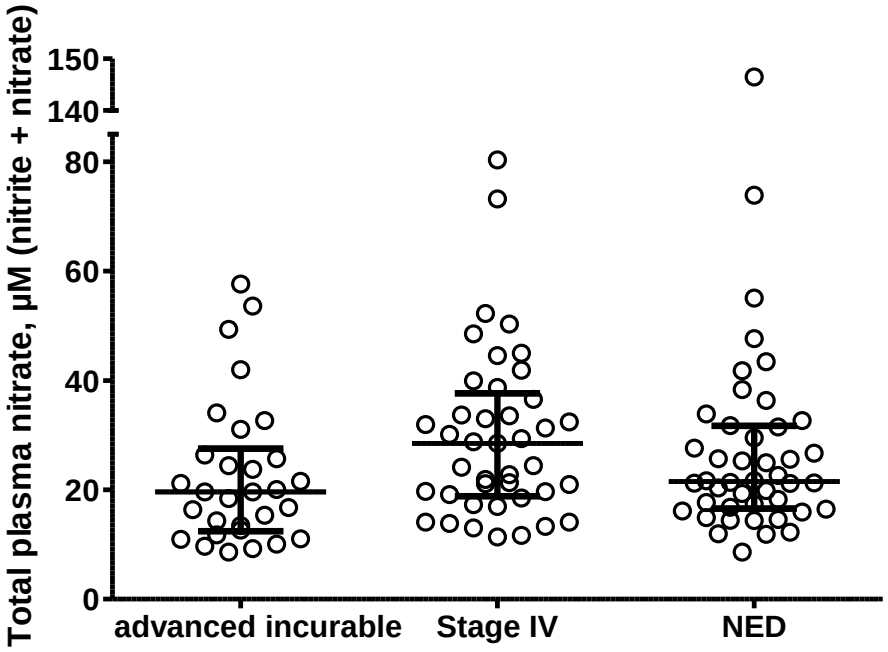

Figure 5.16. ***The total plasma nitrate concentrations in different disease categories.*** Median and interquartile range are shown.

The median total plasma nitrate concentration in the patients with advanced incurable cancer was 19.6 µM (IQR 12.4-27.5) compared with 28.5 µM (IQR 18.8-37.6) and 21.5 µM (IQR 16.5-31.7) in patients with stage IV and no-evaluable disease respectively (Figure 5.16). There were variable concentrations of total plasma nitrate in patients in each disease category but the median total nitrate concentrations were similar between the different disease categories. However, it was observed that the total plasma nitrate concentrations were considerably lower compared with the reported mean values of 116.5 ± 22.6 µM and 190.7 ± 24.4 µM in healthy controls and in patients with gastric cancer respectively (Bakan et al. 2002). In addition, millimolar values (42.8 ± 5.8 mM) have been reported in patients with sepsis (Novotny et al. 2007).

The relationship between nitrate concentrations and the inflammatory markers were investigated in the patients (Table 5.6). Although TGF-β has previously been reported to down-regulate nitric oxide synthase expression, there was no association between the total nitrate levels and TGF-β concentrations in the patients (P = 0.96; Table 5.6).

	Advanced incurable		Stage IV		No-evaluable disease		All patients	
	R_S	*P-value*	R_S	*P-value*	R_S	*P-value*	R_S	*P-value*
IL-1α	0.0238	*0.90*	0.0179	*0.91*	-0.3185	*0.04*	-0.1950	*0.04*
IL-1β	0.0913	*0.63*	0.3156	*0.05*	0.3025	*0.05*	0.2966	*0.002*
IL-6	0.3304	*0.075*	0.1299	*0.43*	-0.0606	*0.70*	0.0057	*0.95*
TNF-α	0.1003	*0.60*	0.2062	*0.21*	-0.0221	*0.89*	0.0813	*0.40*
TGF-β	-0.0111	*0.95*	-0.1022	*0.54*	-0.2399	*0.13*	-0.0051	*0.96*
CRP	-0.3973	*0.03*	0.0763	*0.68*	0.0166	*0.92*	-0.1080	*0.28*
IFN-γ	ND	*ND*	0.1982	*0.23*	-0.2147	*0.17*	ND	*ND*

Table 5.6. ***The relationship between total plasma nitrate concentrations and the inflammatory markers measured in the cancer patients of different disease categories.*** The Spearman rank value (R_S) and *P*-values are reported.

Although nitric oxide has been demonstrated in this chapter to decrease CYP2C19 activity *in vitro*, the total plasma nitrate concentrations in the patients did not correlate with CYP2C19 metabolic activity (Figure 5.17).

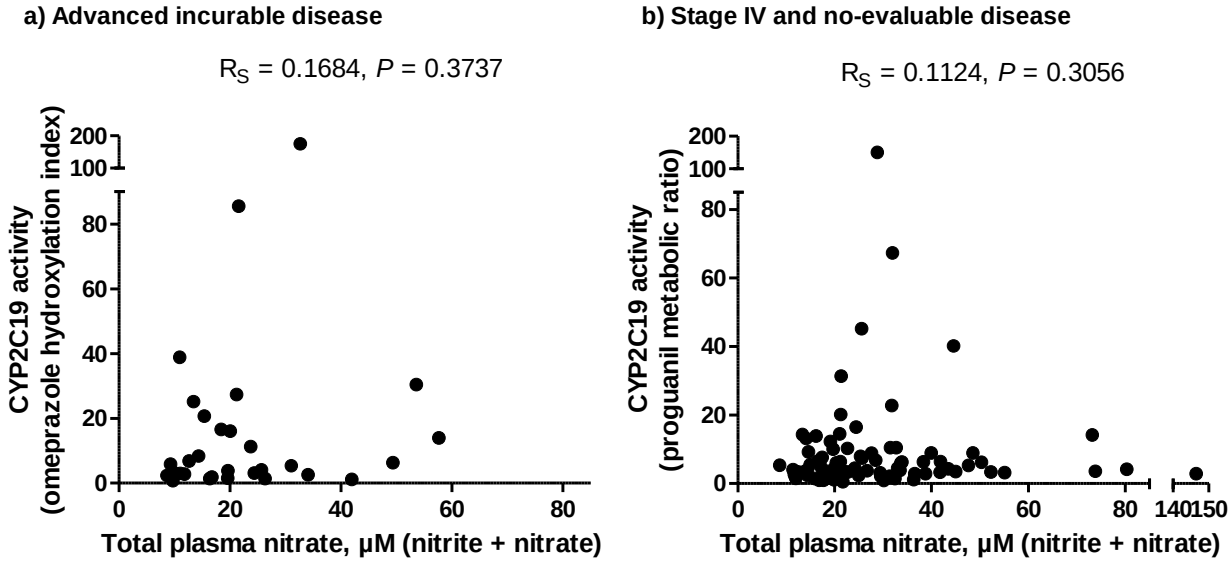

Figure 5.17. ***The lack of relationship between total plasma nitrate concentrations and CYP2C19 activity in the cancer patients with a) advanced incurable disease and b) stage IV or no-evaluable disease.*** The *P*-values from Spearman's rank correlation test are shown on the graphs.

5.4 Discussion

Nitric oxide (NO) is released in solution by the donor compounds SP, SNAP, and NOC-18. These compounds were shown to decrease the functional activity of CYP2C19 directly following a one hour co-incubation with omeprazole in the microsome/supersome system and also in HCT116.*CYP2C19* cells. This confirms that CYP2C19, like many other rat and human CYP isoforms (Wink et al. 1993; Masaki 1999; Vernia et al. 2001), can be directly inhibited by nitric oxide.

It is assumed that this direct inhibition of CYP2C19 activity is due to competition of nitric oxide with oxygen for the haem moiety of the enzyme thus reversibly inhibiting the CYP activity, as previously proposed (Wink et al. 1993). The avid binding of NO to haem is not unexpected since, for example, ferrous iron in hemoglobin binds NO with 10,000-fold greater affinity than O_2 (Sharma et al. 1987). Attempts were made to normalise the data by calculation of relative exposure to NO generated by each donor over the time of incubation. However, such calculations require assumptions that may not hold true particularly in complex biological media and cellular incubations. Direct measurement of the nitric oxide formed should be undertaken in any future experiments. It should be noted that a nitric oxide concentration of 1 μM is considered “typical of an inflamed tissue” (Lim et al. 2008).

The relative differences in effect of the different donors on CYP2C19 function may relate to the different rate of NO release by each compound. SP rapidly releases NO in aqueous solution ($t_{1/2}$ = 2 minutes) (Schulz 1984), SNAP $t_{1/2}$ is 5 hours (Ignarro et al. 1981) and NOC-18 has a $t_{1/2}$ of 20 hours (Mooradian et al. 1995). The rate of NO formation was also increased by exposure to light or thiols for SP and SNAP and acidic pH for NOC-18 (Feelisch 1998). In addition, NOC-18 releases two molecules of NO whereas the other compounds have stoichometric formation (Figure 5.3).

The reversible nature of this direct inhibition by nitric oxide was demonstrated following exposure to a nitric oxide donor with a period of washout, whereby cellular CYP2C19 activity was no longer decreased. In addition to the direct reversible inhibition of activity by nitric oxide, chronic exposure to nitric oxide appears to result in significant loss of activity in HCT116.*CYP2C19* cells during both logarithmic and confluent growth phase.

However, relatively high concentrations of nitric oxide donors (≥ 500 μM) are required for this effect.

A number of other mechanisms whereby nitric oxide may inactivate CYP enzymes and lead to the loss of catalytic function have been proposed (see section 5.1). These include irreversible modification of proteins and increased degradation of the protein. Whilst no attempt to determine mechanisms of CYP modification have been undertaken in this chapter, preliminary studies to determine whether exposure of CYP2C19 to nitric oxide leads to irreversible changes in function and/or changes in expression were carried out.

These preliminary studies suggest that nitric oxide may have longer term effects on CYP2C19 activity and further work to determine whether this results from changes in protein degradation or decreased gene expression, in addition to a direct inhibitory affect are required.

Elevated cytokines are known to induce nitric oxide synthase (NOS) and result in increased levels of nitric oxide in hepatocyte culture (Nussler et al. 1992). It has been reported that TGF-β can down-regulate nitric oxide synthase (NOS) expression (Perrella et al. 1994) and that TGF-β was negatively associated with plasma nitric oxide levels (Fiorenza et al. 2005). An association between TGF-β and CYP2C19 phenotype (activity) was observed in the cancer patients (chapter 4). However, there was no relationship between circulating plasma concentrations of nitrate/nitrite and CYP2C19 activity in the patients. Because nitric oxide decomposes rapidly (half-life of 2-5 s), a common method of estimating NO levels is to measure the by-products, nitrite and nitrate. The assay used measures total levels of nitrate following conversion of any remaining nitrites to nitrate by the addition of nitrate reductase to the sample. The levels of total plasma nitrate in the patient samples was relatively low to that reported in other patient populations with diseases as diverse as tuberculosis and sepsis as well as gastric cancer (Bakan et al. 2002; Fiorenza et al. 2005; Novotny et al. 2007). The plasma samples had been stored for up to 24 months prior to analysis and hence may not reflect the actual amount of nitric oxide circulating in the patients at the time of the CYP2C19 phenotype test. However, the half-life of nitrite and nitrate in whole blood is approximately 110 seconds and 5-8 hours respectively (Kelm 1999), which highlights the importance of rapid sample processing. Nitrite and nitrates are stable in frozen plasma for at least one year (Moshage et al. 1995). Future studies to investigate the association

of circulating NO and CYP2C19 activity should consider rapid sample analysis for detection of nitrates.

The lack of association between CYP2C19 phenotype and total plasma nitrate levels may, however, relate to the concentration of nitric oxide formed within the hepatocytes by nitric oxide synthase adjacent to the CYP enzymes in the endoplasmic reticulum. This intracellular NO may not be reflected in the circulating concentrations. It has been reported that the NO status in blood does not accurately reflect the corresponding status in mammalian tissues (Bryan et al. 2005). If this mechanism occurs and the effects of NO are direct and reversible, then nitric oxide scavengers or NOS inhibitors may be used to ameliorate any detrimental effects that nitric oxide may have on liver drug metabolising capacity.

To test whether a “circulatory inflammatory mediator” such as NO in the cancer patients was responsible for CYP2C19 inactivation and loss of function would require incubating patients’ serum with human hepatocytes, microsomal preparation or purified CYP2C19 enzyme (CYP2C19 supersome^{TM}). However, there were insufficient volumes of patients’ serum remaining from our clinical studies to attempt such a model. In addition, the stability of NO or other unknown factors may limit this approach. Nevertheless, circulating serum factors following inflammation have been reported previously to alter rabbit and human CYP isoforms in hepatocytes (El-Kadi et al. 1997; Bleau et al. 2000; El-Kadi et al. 2000). As CYP2C19 is poorly expressed in human hepatocyte culture, cell-lines transfected with *CYP2C19* may provide a tool in which to investigate further whether circulating mediators from cancer patients can influence CYP2C19 activity.

CHAPTER 6

Protein binding of CYP2C19 substrates

6.1 Introduction

It has long been recognised that drug disposition may be influenced by drug-binding proteins found in the plasma since only free (unbound) drug is considered to be available for activity at receptors or able to bind to the catalytic site of drug metabolising enzymes (Goldstein 1949; Meyer et al. 1968; Vallner 1977). If a drug is highly protein-bound, the small free (unbound) fraction may be influenced by changes in the concentration and profile of the plasma proteins. Relatively small changes in free drug available for hepatic clearance could potentially alter the amount of metabolite formed. Changes in free fraction of drug are considered to be therapeutically irrelevant for drugs at steady state due to the equilibration of drug distribution to the tissue compartment following multiple dosing (Wagner et al. 1965; Perrier et al. 1973). In contrast, changes in free fraction of drug available for hepatic metabolism may result in apparent changes in metabolic clearance of a drug when measured following a single dose.

In theory, the plasma drug/metabolite ratio, used to categorise subjects as extensive or poor metabolisers following a single dose of a probe drug, could be influenced by changes in drug protein binding. For example, increased drug-protein binding could lead to lower concentrations of substrate available for hepatic metabolism and result in a decrease in metabolite formation. Extraction of plasma using organic solvents measures the total (both free and bound) drug and metabolite levels. Hence, following solvent extraction of plasma, an apparent relative increase in the amount of drug relative to metabolite would be observed. This would result in an increased drug/metabolite ratio and an individual could potentially be misclassified as a “poor metaboliser”. In contrast, if drug-protein binding decreases there would be a relative increase in the drug available for hepatic drug metabolism. This could be associated with an apparent decrease in the drug to metabolite ratio would result in a decrease in the drug/metabolite ratio and an “ultrarapid” phenotype would be recorded.

The major drug binding proteins in plasma are considered to be albumin, alpha$_1$-acid glycoprotein (orosomucoid) and lipoprotein (Goldstein 1949; Meyer et al. 1968; Grandison et al. 2000). These proteins are all highly influenced by inflammation; the hepatic synthesis of these proteins is altered, during what is termed the “acute phase response” (Kushner 1982; Baumann et al. 1994). Alpha$_1$-acid glycoprotein is a positive acute phase protein which is elevated during inflammation (Gabay et al. 1999; Fournier

et al. 2000). Conversely, albumin concentrations are decreased during inflammation and this is a negative acute phase response protein (Moshage et al. 1987). The concentrations of alpha$_1$-acid glycoprotein and albumin can alter by up to 2-fold during the acute phase response (Mariani et al. 1976; Uslu et al. 2003). Inflammatory cytokines such as IL-6 are thought to be responsible for the induction of the hepatic acute phase response (Castell et al. 1990).

A number of other proteins are also positively induced during the acute phase response, including C-reactive protein (Gabay et al. 1999). C-reactive protein is involved in the opsonisation of pathogens and material from damaged cells and also activates the classical complement pathway (Rabinovitch et al. 1986).

Consistent with the above, the highly inflammatory status associated with cancer results in elevation of positive acute phase response proteins such as C-reactive protein (Bolayırlı et al. 2007), as well as a decrease in negative acute phase response proteins such as albumin (Mariani et al. 1976; Fearon et al. 1998) which is clinically observed as hypoalbuminemia. The lipoprotein status may also be altered in cancer patients due to either the acute phase response (Khovidhunkit et al. 2004) or due to changes in the nutritional status and or cachexia as part of the acute phase response (Esper et al. 2005). For example, the levels of high-density lipoprotein decrease and conversely low-density lipoprotein increase in patients with colorectal adenoma (Bayerdörffer et al. 1993). This appears to be regulated by induction of an acute phase response via IL-6 and TNF-α (Haas et al. 2010). In highly inflammatory conditions, such as cancer, there is also an increase in the plasma concentrations of alpha$_1$-acid glycoprotein (Uslu et al. 2003). The inflammatory cytokines, IL-1 and IL-6 have been shown to modulate alpha$_1$-acid glycoprotein gene expression in human hepatocytes (Lee et al. 2001).

The studies described so far in this thesis, as well as studies by other research groups (Rivory et al. 2002; Slaviero et al. 2003), have attempted to correlate altered probe drug metabolism (drug/metabolite ratio) with inflammation using biomarkers of the acute phase response such as C-reactive protein and albumin. However, theoretically these biomarkers of acute phase response may also influence the free fraction (drug-protein binding) of the probe drug, which could potentially lead to apparent changes in the metabolic ratio (drug/metabolite). For example, the CYP2C19 probe substrate omeprazole is reported to be highly protein bound with less than 5% of free drug in the plasma (Regårdh et al. 1985) and the major plasma binding protein for this drug is

albumin (Regårdh et al. 1985). Proguanil is an alternative probe substrate for CYP2C19 (Desta et al. 2002). In contrast to omeprazole, proguanil is only 75% plasma protein bound (GlaxoSmithKline 2008). Proguanil is a basic drug (pK_a = 10.4) (Findlay 1951) and therefore it is assumed that the major protein responsible for its protein binding will be $alpha_1$-acid glycoprotein (Paxton 1983; Kremer et al. 1988).

Hence, variable levels of albumin and $alpha_1$-acid glycoprotein in cancer patients with inflammation may lead to variable sequestration of omeprazole or proguanil by plasma proteins. This could potentially result in a "false" CYP2C19 metaboliser status. Variable drug-protein binding could be a cause of the high incidence of the genotype-phenotype discordance observed in the two independent studies in the cancer patients reported in this thesis (chapter 3 and chapter 4).

In addition to albumin and $alpha_1$-acid glycoprotein, there are more than 289 other proteins found in plasma (Table 6.1) (Anderson et al. 2002). A number of these proteins are also associated with the acute phase response. For example, globulins are also reported to be involved in drug-protein binding of methadone (Olsen 1973; Piafsky 1980). Hence any number of these plasma proteins could also influence drug-protein binding. Although the main plasma proteins assumed to be responsible for drug-protein binding of omeprazole and proguanil are albumin and $alpha_1$-acid glycoprotein respectively, little is known about the relative contribution of other plasma proteins. Of particular interest are the lipoproteins, which are altered in cancer (Bayerdörffer et al. 1993). The acute phase response protein, C-reactive protein, was reported to correlate with decreased CYP3A4 metabolism in cancer patients (Rivory et al. 2002) and has some correlation with decreased CYP2C19 activity (chapter 4). However, the ability of C-reactive protein, and other acute phase response proteins, to bind the probe drugs is not known.

5'Nucleotidase	Collagen III c-terminal propeptide	Haptoglobin beta chain	Pancreatic zymogen granule membrane protein GP-2
Acid labile subunit of IGFBP	Collagen III n-terminal propeptide	Haptoglobin beta chain, cleaved	Paraoxonase parathyroid hormone
Acid phosphatase, tartrate-resistant	Collagen IV 7S n-terminal propeptide	Haptoglobin-related gene product	Parathyroid hormone-related protein
Acid phosphatase, prostatic	Complement C1 inhibitor	Hemoglobin, alpha	PASP
Actin beta (from platelets)	Complement C1q, A	Hemoglobin, beta	Pepsinogen A
Actin gamma (from platelets)	Complement C1q, B	Hemopexin (beta-1B-glycoprotein)	Plasma hyaluronan binding protein
Adenosine Deaminase	Complement C1q, C	Histidine-rich alpha-2-glycoprotein	Plasma kallikrein
Adiponectin	Complement C1r	ICAM-1, soluble	Plasma serine protease inhibitor
Alanine aminotransferase (ALT)	Complement C1s	Ig kappa light chain	Plasminogen – Glu
Albumin	Complement C2	Ig lambda light chain	Plasminogen – Lys
Aldolase (muscle type)	Complement C3A anaphylotoxin	IgA1	Platelet factor 4
Alkaline phosphatase (bone)	Complement C3B, alpha	IgA2	Pre-alpha trypsin inhibitor, H3
Alpha 1,3-fucosyltransferase (FUT6)	Complement C3B, beta	IgD	Pregnancy-associated plasma protein-A
Alpha-1-acid glycoprotein	Complement C4, anaphylotoxin	IgE	Pregnancy-associated plasma protein-A2
Alpha-1-B glycoprotein	Complement C4, alpha	IGFBP-3	Pregnancy-specific beta-1-glycoprotein 3
Alpha-1-microglobulin	Complement C4, beta	IgG1	Prolactin
Alpha-2-antiplasmin	Complement C4, gamma	IgG2	Prolyl hydroxylase, alpha
Alpha-2-HS glycoprotein	Complement C4-binding protein, alpha	IgG3	Prolyl hydroxylase, beta
Alpha-2-macroglobulin	Complement C4-binding protein, beta	IgG4	Prostaglandin-H2 D-isomerase
Alpha-fetoprotein	Complement C5A anaphylotoxin	IgJ-chain	Prostate specific antigen
Amylase (pancreatic)	Complement C5B, alpha	IgM	Protein C, H
Angiostatin	Complement C5B, beta	Inhibin (activin), beta A	Protein C, L
Angiotensin converting enzyme (ACE)	Complement C6	Inhibin (activin), beta B	Protein S
Angiotensinogen	Complement C7	Inhibin (activin), beta C	Protein Z
Antithrombin III (AT3)	Complement C8, alpha	Inhibin (activin), beta E	P-selectin, soluble
Apolipoprotein A-I	Complement C8, beta	Inhibin, alpha	Rantes
Apolipoprotein A-II	Complement C8, gamma	Insulin C-peptide	Renin
Apolipoprotein A-IV	Complement C9	Insulin, A chain	Retinol binding protein
Apolipoprotein B-100	Complement factor B	Insulin, B chain	S100 protein
Apolipoprotein B-48	Complement factor B – Bb fragment	Insulin-like growth factor IA	Secretogranin V
Apolipoprotein C-I	Complement factor D	Insulin-like growth factor II	Serum amyloid A
Apolipoprotein C-II	Complement factor H	Inter-alpha trypsin inhibitor, H1	Serum amyloid P
Apolipoprotein C-III	Complement factor I	Inter-alpha trypsin inhibitor, H2	Sex hormone binding globulin
Apolipoprotein C-IV	Connective tissue activating peptide III	Inter-alpha trypsin inhibitor, H4	Tetranectin
Apolipoprotein D	Corticotropin releasing hormone (CRH)	Inter-alpha trypsin inhibitor L	Thyroglobulin
Apolipoprotein E	C-reactive protein	Interferon alpha	Thyroid stimulating hormone
Apolipoprotein F	Creatine kinase, B	Interferon beta	Thyrotropin-releasing hormone
Apolipoprotein H	Creatine kinase, M	Interferon gamma	Thyroxin binding globulin
Apolipoprotein J (clusterin)	CRHBP	Interleukin-1 beta	Tissue factor
Apolipoprotein(a)	Cystatin C	Interleukin 10	Tissue inhibitor of metalloproteinases-1
Aspartate aminotransferase (AST)	Elastase (neutrophil)	Interleukin-12, alpha	Tissue inhibitor of metalloproteinases-2
Beta thromboglobulin	Eosinophil granule major basic protein	Interleukin-12, beta	Tissue plasminogen activator
Beta-2-microglobulin	E-selectin, soluble	Interleukin-1 receptor antagonist	Tissue plasminogen activator inhibitor
CA 125	Ferritin, H	Interleukin-2	TNF-alpha
CA 19-9	Ferritin, L	Interleukin-4	TNF-binding protein 1
CA 72-4	Fibrin fragment D-dimer	Interleukin-5	TNF-binding protein 2
CA 27.29/15-3 (MUC1 mucin antigens)	Fibrinogen extended gamma chain	Interleukin-6	Transcobalamin
Calreticulin	Fibrinogen, alpha	Interleukin-8	Transcortin
Carboxypeptidase N, regulatory	Fibrinogen, beta	IP-10, small inducible cytokine B10	Transferrin
Carboxypeptidase N, catalytic	Fibrinogen, gamma	Isocitrate dehydrogenase	Transferrin (asialo-, tau-, beta-2-)
Carcinoembryonic antigen	Fibronectin	Kininogen	Transferrin receptor (soluble)
Cathepsin D	Fibulin-1	Ksp37	Transthyretin
CD5 antigen-like protein	Ficolin 1	Laminin, alpha	Triacylglycerol lipase (pancreatic)
Ceruloplasmin	Ficolin 2	Laminin, beta	Troponin I (cardiac)
Cholinesterase plasma	Ficolin 3	Laminin, gamma	Troponin I (skeletal)
Chorionic gonadotropin beta (hCG)	Follicle stimulating hormone	LDH (heart)	Troponin T (cardiac)
Chromogranin A	G-6-PD	Lecithin-cholesterol acyltransferase	Tryptase, beta-2
Chromogranin B (secretogranins I)	Galactoglycoprotein (leukosialin)	Leucine-rich alpha-2-glycoprotein	Tyrosine hydroxylase
Coagulation factor II (prothrombin)	Gamma-glutamyl transferase alpha	LHRH	Urokinase (high MW kidney type) A
Coagulation factor IX	Gc-globulin	Lipase	Urokinase (high MW kidney type) B
Coagulation factor V	GCSF	Luteinizing hormone (LH), beta	VCAM-1, soluble
Coagulation factor VII, H	Gelsolin	Mannose-binding protein	Vitronectin
Coagulation factor VII, L	GHRH	Matrix metalloproteinase-2	Von Willebrand factor
Coagulation factor VIII	Glutamate carboxypeptidase II	M-CSF	Zn-alpha-2-glycoprotien
Coagulation factor X	Glutathione peroxidase	Melastatin	
Coagulation factor XI	Glutathione S-transferase	MIP-1 alpha	
Coagulation factor XII	Glycoprotein hormones alpha chain	MIP-1 beta	
Coagulation factor XIII A	GMCSF	MSE 55	
Coagulation factor XIII B	Growth hormone	Myelin basic protein	
Collagen I c-terminal propeptide	Growth hormone binding protein	N-acetyl-B-D-glucosaminidase, alpha	
Collagen I c-terminal telopeptide (ICTP)	Haptoglobin alpha-1	N-acetyl-B-D-glucosaminidase, beta	
Collagen I n-terminal propeptide	Haptoglobin alpha-2-chain		
Collagen I n-terminal telopeptide (NTx)			

Table 6.1. ***A list of 289 plasma (and serum) proteins documented in literature.*** Adapted from Anderson et al. 2002.

The hypothesis is that changes in protein binding of probe drugs may alter metabolic ratio.

In order to clarify the validity of the use of probe drug metabolic ratios (drug/metabolite) to categorise CYP2C19 hepatic function in patients with potentially a highly variable plasma protein profile, the following studies were undertaken:

a) Confirmation of the literature values for the drug-protein binding of omeprazole and proguanil in normal healthy plasma.
b) Determination of the relative ability of albumin, alpha$_1$-acid glycoprotein, C-reactive protein, low-density and high-density lipoprotein to bind omeprazole and proguanil.
c) Assessment of the variability in the total plasma protein concentration of individual patients.
d) Measurement of the unbound and total drug concentrations in individual patients who had received the probe drugs.
e) Comparison of the measured unbound drug in the patients with the predicted values based on spiked normal plasma.
f) Calculation of an individualised hepatic clearance (CL_H) based on the measured fraction unbound in each patient.

The data collected from these studies was then used to investigate whether variable drug-protein binding was a possible cause of the high incidence of genotype-phenotype discordance observed in the cancer patients.

6.1.1 Study design

6.1.1.1 Chemicals and reagents

Human serum albumin and alpha$_1$-acid glycoprotein were purchased from Sigma-Aldrich, St. Louis, Missouri, United States. C-reactive protein was obtained from AbD Serotec, Oxford, United Kingdom. High-density and low-density lipoproteins were supplied by Applichem, Darmstadt, Germany. Tween-20 was purchased from BDH Chemicals, VWR International Limited, Poole, England. Human plasma was obtained from the NZ Blood Service, Auckland, New Zealand.

6.1.1.2 Preparation of solutions

Human serum was prepared from whole blood by incubating at 4°C for 1 h followed by centrifugation at 1500 *g* at 4°C for 10 min as described in section 2.4.2. Low density lipoprotein (LDL, 44.3 g/L) and high density lipoprotein (HDL, 13.78 g/L) were supplied by AppliChem in liquid form with no preservatives following purification from human serum. C-reactive protein (CRP, 1 g/L) was supplied in liquid form in Tris buffered saline (pH 8.0). Stock solutions of human serum albumin (HSA, 40 g/L) and alpha$_1$-acid glycoprotein (AAG, 0.7 g/L) were prepared in phosphate buffer (67 mM, pH 7.4). All stock solutions were further diluted in phosphate buffer to the desired final concentration listed in Table 6.2. A stock solution of omeprazole (250 µg/mL) was prepared in alkalinised methanol (0.1% TEA) and proguanil (30 µg/mL) was prepared in ethanol. Patients' plasma samples were prepared from whole blood collected 2 or 3 hours post-dose of omeprazole and proguanil respectively as described in chapter 3 and 4.

6.1.1.3 Incubation conditions

The binding of the CYP2C19 substrates, omeprazole and proguanil, to selected plasma proteins was studied *in vitro* using standard ultrafiltration methods as described below. Omeprazole (500 ng) and proguanil (150 ng) were incubated with 500 µL plasma, serum or the individual plasma protein solutions for 30 minutes at 37°C with gentle agitation prior to ultrafiltration. All determinations were carried out in triplicate. The concentrations

of omeprazole and proguanil used in these incubations were based on the C_{max} concentrations achieved following the standard oral dose (omeprazole 20 mg and proguanil 200 mg) used in the determination of the metabolic ratio for these CYP2C19 probes (Watkins et al. 1990; Setiabudy et al. 1995; Shimizu et al. 2006). The concentrations of albumin, $alpha_1$-acid glycoprotein, low-density (LDL) and high-density lipoprotein (HDL) used were based on typical values observed in healthy individuals (Gill 2000; Kyle 2008). The C-reactive protein concentration used was based on the upper value observed previously (chapter 3) in the advanced incurable cancer patients (Table 6.2).

Protein	Final incubation concentration
Albumin	40000 mg/L
$Alpha_1$-acid glycoprotein (AAG)	700 mg/L
C-reactive protein (CRP)	100 mg/L
Low-density lipoprotein (LDL)	1000 mg/L
High-density lipoprotein (HDL)	500 mg/L

Table 6.2. ***The final concentration of each plasma protein used per triplicate incubation.*** The concentrations of albumin, AAG, CRP, LDL and HDL were based on typical values observed in healthy individuals. CRP concentration was based on the upper value observed previously in advanced incurable cancer patients (chapter 3).

6.1.1.4 Ultrafiltration procedure

Free (unbound drug) was separated from protein bound drug by ultrafiltration using Microcon® centrifugal filter devices with an Ultracel YM membrane (molecular weight cut-off at 10 kDa; Millipore, USA).

To improve recovery of proteins, the Microcon® centrifugal filter devices were pre-treated overnight with a passivation solution (5% Tween-20® in MilliQ water; 500 µL) as recommended by the manufacturer in order to block non-specific binding sites in the filter. The devices were rinsed thoroughly with water and were washed twice with MilliQ water (500 µL) followed by centrifugation (14,000 *g* for 10 min). Any remaining water was eliminated by inverting the reservoir in the vial with further centrifugation (1000 *g* for 3 min). Samples (500 µL) were then pipetted into the reservoir and free and protein-bound drug were separated by centrifuging the unit for 30 minutes at 14,000 *g*. The

retentate (protein-bound drug) was then collected by inverting the filter into a new vial followed by centrifugation (3 min at 1000 *g*).

The filtrate (unbound drug) was evaporated to approximately 100 µL under vacuum. The final volume was measured and the sample was analysed by the HPLC using the appropriate analysis method (section 2.3.4 and 2.7.3).

6.1.1.5 Calibration curve

In order to quantify the amount of omeprazole and proguanil in the filtrate (unbound drug), calibration curves were prepared using authentic standards by sequential injection (5-100 µL) of a 0.1 µg/mL and 5 µg/mL drug stock solution in phosphate buffer directly onto the HPLC. The calibration curves used and linearity parameters are shown below (Figure 6.1 and Figure 6.2).

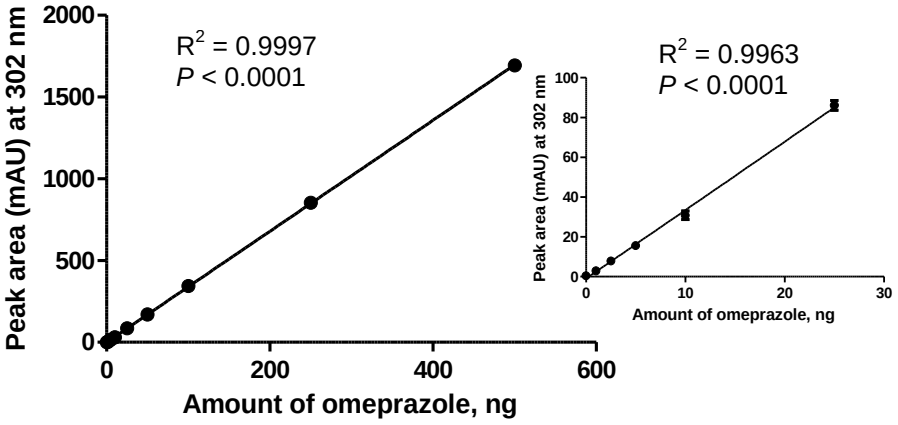

Figure 6.1. A typical c*alibration curve of omeprazole in phosphate buffer of triplicate determinations.* Slope = 3.395, R^2 = 0.9997, *P* < 0.0001. Inset shows the linearity of the calibration curve at lower concentrations.

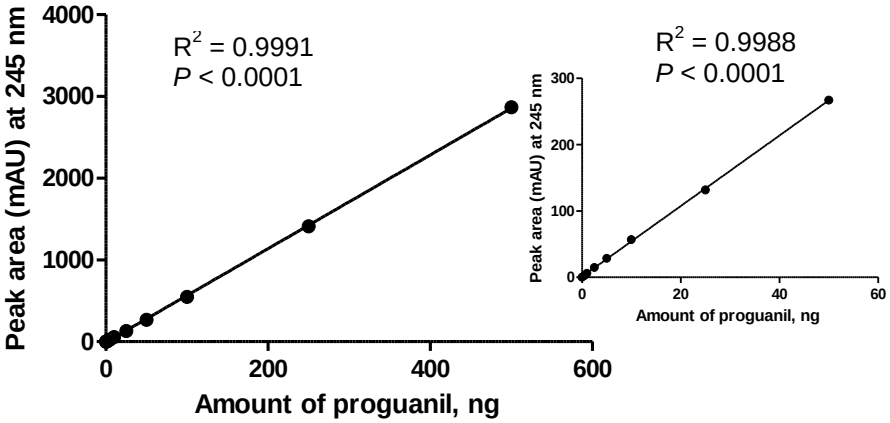

Figure 6.2. A typical c*alibration curve of proguanil in phosphate buffer of triplicate determinations.* Slope = 5.728, R^2 = 0.9991, *P* < 0.0001. Inset shows the linearity of the calibration curve at lower concentrations.

6.1.1.6 Inter-individual variability in total plasma protein concentration

The patients' plasma samples were serially diluted with 0.1 M NaOH to 1:10, 1:20, 1:40 v/v. The total plasma protein concentration in each patient was determined using the BioRad DC assay kit (BioRad Laboratories, CA, USA) as described in section 2.9.3.

6.1.1.7 Data analysis

The amounts of omeprazole or proguanil in the filtrate (ng) were determined from the calibration curves (Figure 6.1 and Figure 6.2). The values were adjusted according to the final volume of the concentrated filtrate measured prior to HPLC analysis.

The percentage of unbound drug was determined as the amount of drug present in the filtrate divided by the known amount of drug added to the plasma protein mixture. This was then normalised to the amount of unbound drug that was spiked in phosphate buffer to account for any non-specific binding.

Statistical analyses were determined using SigmaPlot (Version 11, Systat Software Inc, Germany) and GraphPad Prism (Version 5.02, GraphPad Software Inc, USA) and the mean ± standard deviation was reported. Associations between variables were assessed using Spearman's rank correlation, and Mann-Whitney rank-sum tests were

used to compare variables across groups. Unpaired t-test was used to compare variable across two groups when sample size is too small for analysis by Mann-Whitney rank-sum test. Two-sided *P*-values < 0.05 were considered statistically significant. Dunn's multiple comparison post-test was applied where possible.

6.2 Results

6.2.1 Protein binding of omeprazole

To determine plasma protein binding of omeprazole in normal healthy plasma, the amount (ng) of unbound omeprazole detected following ultrafiltration of spiked normal healthy plasma was determined from the calibration curve (Figure 6.1) and was converted to concentration (ng/mL).

The fraction unbound (f_U) of omeprazole in normal healthy human plasma was 4.23% ± 3.60%. These values were in agreement with values reported from the literature (Regårdh et al. 1985). The amount of binding in serum was higher than in plasma with 1.14 ± 0.004% fraction unbound, although the difference was not statistically significant (*P* = 0.2120).

The concentrations of unbound omeprazole were then measured in the plasma samples from individual patients (n = 26) with advanced incurable cancer (Table 6.3). These samples were from the patients who received a single oral dose of omeprazole (20 mg) as probe drug previously reported in chapter 3. Only patients with a homozygous or heterozygous wildtype (*CYP2C19*1/*1* or *CYP2C19*1/*2*) genotype were included in this part of the study. The concentrations of unbound omeprazole were compared with the total concentration of drug in each individual patient that had been previously measured (chapter 3). The fraction unbound of drug in the patients' plasma was calculated as the unbound drug concentration divided by the total (solvent extracted) drug concentration.

Following a single oral dose of omeprazole, the mean measured unbound concentration of omeprazole in the 2 hours post-dose plasma of these patients was 5.60 ± 4.29 ng/mL. However, there was a wide inter-patient variability with values ranging from 0.72 to 15.37 ng/mL. In these same plasma samples, the total (solvent extracted) omeprazole

concentration was also variable (593.77 ± 466.73 ng/mL) and ranged from 63.43 to 1748.99 ng/mL (Table 6.3).

The predicted unbound concentration of omeprazole was calculated for each individual patient (Table 6.3). This was based on the measured total omeprazole concentration and the expected fraction unbound (f_U) of 4.23% determined in normal healthy plasma. The measured unbound omeprazole concentrations in the patients were considerably lower than the predicted values. For example, patient #402 had a total extracted omeprazole concentration of 236.29 ng/mL. The predicted unbound omeprazole in this patient was 10 ng/mL but the actual measured unbound concentration was more than 5-fold lower at 1.73 ng/mL. The mean measured fraction unbound of omeprazole in these patients was 0.98 ± 0.32% which was 4-fold lower than the 4.23 ± 3.60% measured in normal healthy plasma following spiking with omeprazole at a final concentration of 1000 ng/mL (Table 6.3).

Patient #	Measured[a] unbound OMP, ng/mL	Total extracted[b] OMP, ng/mL	Predicted[c] unbound OMP, ng/mL	Measured fraction unbound[d] (f_U), %	Total extracted[e] 5'OH-OMP, ng/mL	Metabolic ratio (OMP/5'OH OMP)
401	11.16	935.64	39.58	1.192	137.17	6.82
402	1.73	236.29	10.00	0.731	125.88	1.88
403	15.37	1219.38	51.58	1.260	31.34	38.91
405	5.52	690.60	29.21	0.799	178.72	3.86
406	4.66	274.22	11.60	1.701	10.00	27.42
407	5.89	824.52	34.88	0.714	27.04	30.49
409	10.88	1159.90	49.06	0.938	13.56	85.54
410	2.94	234.68	9.93	1.255	83.65	2.81
412	5.44	404.48	17.11	1.345	131.32	3.08
413	13.39	1748.99	73.98	0.765	10.00	174.90
415	12.00	1432.06	60.58	0.838	102.35	13.99
417	6.26	887.50	37.54	0.705	165.22	5.37
418	0.74	98.72	4.18	0.75	63.29	1.56
419	3.53	219.35	9.28	1.611	152.64	1.44
424	0.72	63.43	2.68	1.135	45.00	1.41
426	8.18	1105.53	46.76	0.740	132.06	8.37
427	0.74	144.27	6.10	0.510	123.29	1.17
428	0.79	113.53	4.80	0.696	10.00	11.35
429	4.42	528.54	22.36	0.836	130.05	4.06
430	1.32	138.13	5.84	0.959	44.33	3.12
431	2.11	266.59	11.28	0.790	12.88	20.70
432	9.66	634.24	26.83	1.523	200.69	3.16
435	1.95	274.51	11.61	0.711	103.45	2.65
436	6.94	917.32	38.80	0.757	57.09	16.07
437	2.36	216.44	9.16	1.088	89.22	2.43
438	7.04	669.24	28.31	1.052	113.89	5.88
Mean ± sd	**5.60 ± 4.29**	**593.77 ± 466.73**	**25.12 ± 19.74**	**0.98 ± 0.32**	**88.24 ± 58.16**	**18.40 ± 36.65**

Table 6.3. ***Measured versus predicted plasma protein binding of omeprazole in advanced incurable cancer patients following an oral dose of omeprazole (20 mg).***
[a] lowest arbitrary value assumed to be the same as the lowest amount (1 ng) injected for calibration (Figure 6.1). [b] total solvent extracted drug as in methods section 2.3.3. [c] based on f_u = 4.23% previously determined in healthy plasma. [d] percentage of measured unbound omeprazole over total extracted omeprazole in patients. [e] total solvent extracted metabolite as in methods section 2.3.3.

It is not known what factors may cause the difference between the fraction unbound predicted from spiking normal healthy plasma with drug and the measured fraction unbound in the cancer patients' plasma following drug administration. One proposed factor is cancer-associated inflammation which may influence the levels of various acute phase response proteins in the patients' plasma and thus alter drug-protein binding.

It is known that albumin is the major binding protein for omeprazole (Regårdh et al. 1985). Albumin is a negatively regulated acute phase response protein (Castell et al. 1990) and hypoalbuminemia is common in cancer (Mariani et al. 1976). Therefore variation in the acute phase response in the cancer patients may lead to variable levels of unbound omeprazole. However, the role of other acute phase response proteins such as alpha$_1$-acid glycoprotein, C-reactive protein, low-density lipoprotein and high-density lipoprotein in omeprazole binding is not known.

The relative contribution of these acute phase response proteins compared with albumin for omeprazole binding was determined by addition of 1000 ng/mL of omeprazole to solutions of these proteins at concentrations reported in normal healthy plasma (Table 6.2). Following ultrafiltration, the fraction unbound was calculated for each individual protein (Figure 6.3).

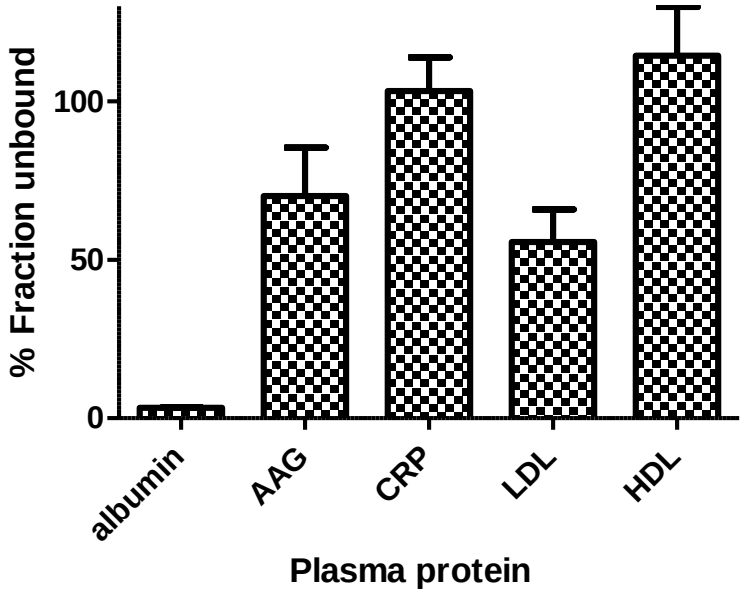

Figure 6.3. ***The percentage fraction unbound of omeprazole in various plasma protein solutions.***
AAG = alpha$_1$-acid glycoprotein; CRP = C-reactive protein; LDL = low-density lipoprotein; HDL = high-density lipoprotein. Protein concentrations were based on normal concentrations observed in healthy state with the exception of CRP, which was measured at a concentration observed during inflammation. Level of binding to omeprazole: albumin > LDL > AAG, CRP and HDL had negligible binding ability.

Of the acute phase response proteins studied, albumin had the greatest ability to bind omeprazole, with 3.26 ± 0.14% fraction unbound (Figure 6.3). Low-density lipoprotein and alpha$_1$-acid glycoprotein could also bind omeprazole, but with a lower relative contribution (55.58 ± 10.39% and 70.16 ± 15.23 % fraction unbound, respectively). In contrast, C-reactive protein and high-density lipoprotein at concentrations likely to be observed in plasma did not appreciably bind omeprazole (Figure 6.3).

Since albumin was clearly the major drug binding protein, the relationship between albumin concentration and the fraction unbound of omeprazole in each individual patient was then investigated. The mean albumin concentration in these cancer patients was 44.81 ± 6.61 g/L. Two patients, #410 and #436, were clinically hypoalbuminemic (< 35 g/L) with albumin levels of 25.48 g/L and 34.89 g/L respectively. However, there was no direct correlation between albumin concentration in the patients and the measured unbound plasma omeprazole concentrations in the patients (Spearman r = -0.004788, *P* = 0.9815) (data not shown).

Hypothetically, if albumin concentrations increase, then there should be less free drug available for hepatic clearance and therefore a lower amount of metabolite will be formed leading to a higher apparent metabolic ratio (drug/metabolite). However there was no direct relationship between albumin concentrations and the omeprazole metabolic ratio (Spearman r = -0.07591, *P* = 0.7125) (Figure 6.4).

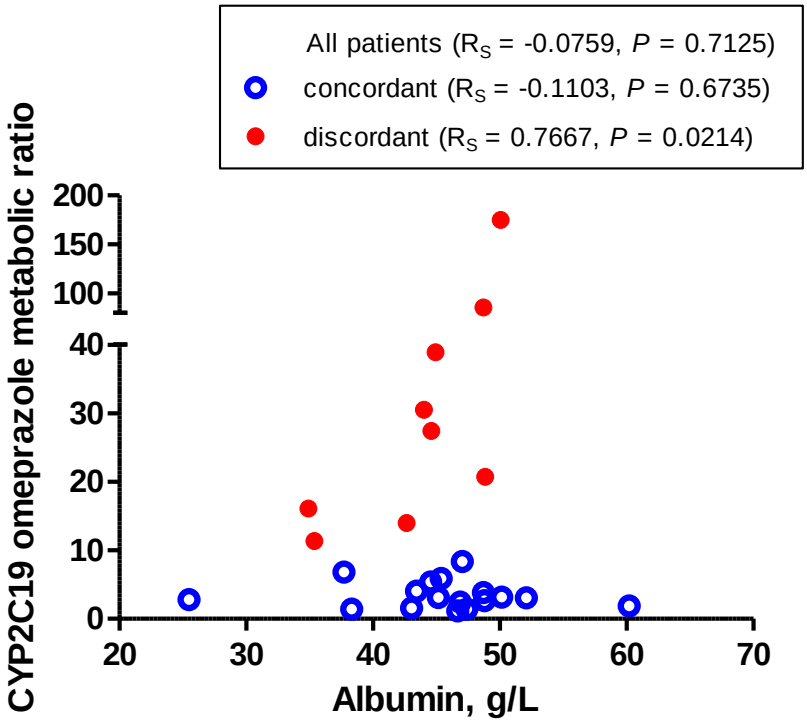

Figure 6.4. ***The lack of relationship between CYP2C19 omeprazole metabolic ratio and albumin concentrations in patients with advanced incurable cancer.*** The metabolic ratio was determined in chapter 3 as drug/metabolite and reported in Table 6.3. The *P*-values from Spearman's rank correlation test are shown on the graph.

Hence, the inter-individual variability in plasma albumin concentrations did not appear to influence the metabolic ratio. However, sub-group analysis of the patients who were genotype-phenotype discordant (metabolic ratio > 10) indicated a significant positive relationship (Spearman r = 0.7667, *P* = 0.0214). In contrast, in the patients who were concordant (metabolic ratio < 10), there was no correlation (Spearman r = -0.1103, *P* = 0.6735) (Figure 6.4).

In summary, variability in albumin concentrations did not influence the fraction unbound of the probe drug omeprazole. However, there was a relationship between increasing albumin concentrations and the drug/metabolite ratio, but only in those individuals who were genotype-phenotype discordant.

C-reactive protein, which did not have any significant ability to bind omeprazole (Figure 6.3), also had no direct correlation with the measured unbound omeprazole concentration (Spearman r = -0.04854, *P* = 0.8178) (data not shown).

Additional acute phase response proteins that could potentially influence the levels of unbound omeprazole were low-density lipoprotein and alpha$_1$-acid glycoprotein (Figure 6.3). Moreover, there may also be other plasma proteins (Table 6.1) involved in omeprazole binding which have not been investigated in this study, such as transferrin and globulins. However, individual determinations of these proteins were not carried out due to insufficient plasma sample. Therefore, instead of measuring the concentrations of each binding protein independently, total plasma protein concentrations were determined for each individual patient's plasma sample.

Total plasma protein concentrations were variable in the cancer patients with a mean of 63.54 ± 9.92 g/L, but it was similar to the reported total plasma protein levels observed in normal healthy individuals (70-77 g/L) (Wadsworth et al. 1953; Kanabrocki 1961). Surprisingly, there was a significant inverse (positive) correlation (Spearman r = 0.4196, *P* = 0.0329) between the total plasma protein concentration and unbound omeprazole in the patients (Figure 6.5).

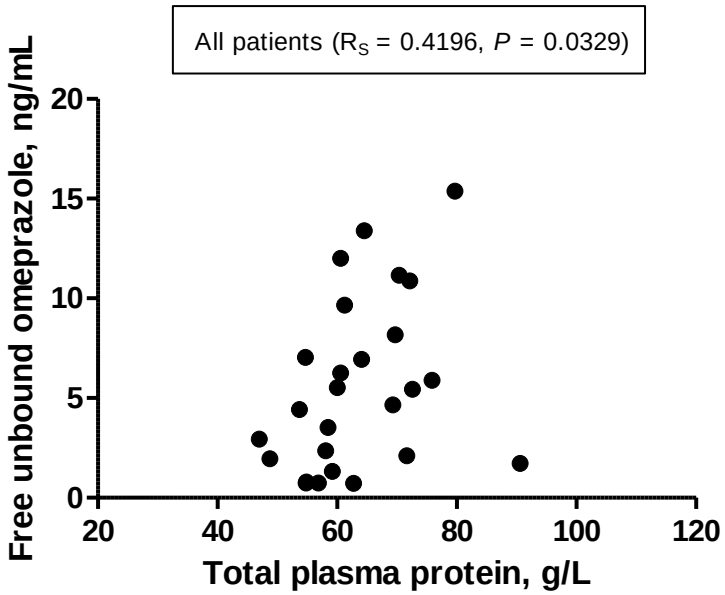

Figure 6.5. ***The positive relationship between concentrations of total plasma protein and unbound omeprazole in patients with advanced incurable cancer***. Spearman r = 0.4196, *P* = 0.0329.

Thus, as total plasma protein concentrations increased, there was an increase in free (unbound) drug. This suggests that increased drug-binding proteins are not influencing the fraction unbound of omeprazole.

Moreover, total plasma protein concentration also correlated with the CYP2C19 omeprazole metabolic ratio in the patients. There was a significant positive relationship so that high total plasma protein concentrations were associated with a "poor" metabolic ratio (Spearman r = 0.4858, *P* = 0.0119) (Figure 6.6). This was particularly the case for the discordant individuals (metabolic ratio > 10) although not significant (Spearman r = 0.6833, *P* = 0.0503).

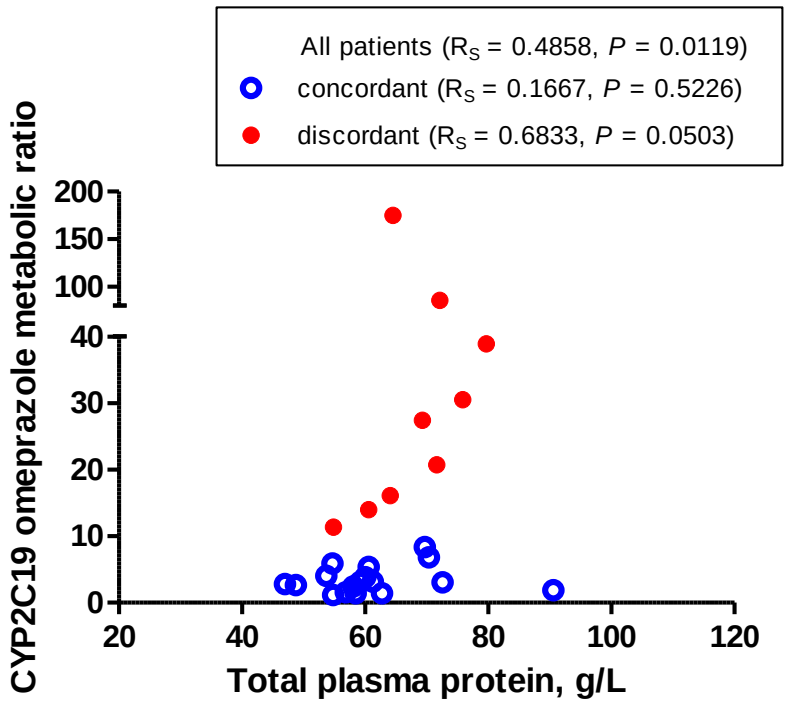

Figure 6.6. ***The relationship between the CYP2C19 omeprazole metabolic ratio and total plasma protein concentration in patients with advanced incurable cancer.*** The *P*-values from Spearman's rank correlation test are shown on the graph. A significant positive relationship between total plasma protein and CYP2C19 metabolic ratio was only observed in patients with genotype-phenotype discordance (R_S = 0.6833, *P* = 0.05).

Hence these results unexpectedly suggest that free unbound drug does not decrease as total plasma protein or albumin concentrations increase. The inverse relationship between the increase in plasma albumin and/or total plasma protein and poor metaboliser status based on the CYP2C19 metabolic ratio (drug/metabolite) suggests that total plasma protein concentration may be a biomarker of global inflammatory (acute phase response) status, which has been postulated to be a cause of "poor" metabolism. If this is true, then a negative correlation between the formation of the 5'hydroxy omeprazole metabolite versus total plasma protein concentrations would be expected in the cancer patients. The concentration of total (solvent extracted) 5'hydroxy omeprazole was determined as described in section 2.3.3 (Table 6.3). There was no significant

negative correlation between the concentrations of the 5'hydroxy omeprazole metabolite and total plasma protein (Spearman r = -0.1755, *P* = 0.3911) (Figure 6.7).

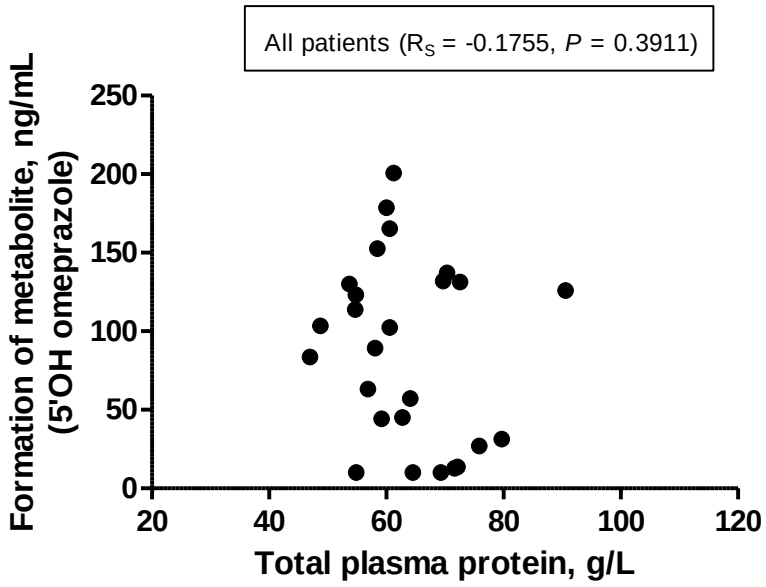

Figure 6.7. ***The lack of relationship between 5'hydroxy omeprazole metabolite and total plasma protein concentration in patients with advanced incurable cancer.*** Spearman r = -0.1755, *P* = 0.3911.

In addition, the 5'hydroxy omeprazole metabolite concentration did not correlate with the albumin concentrations in the patients (Spearman r = 0.1037, *P* = 0.6142) (data not shown).

In summary, increasing albumin or total plasma proteins in the patients did not result in a decrease in unbound fraction of drug. Thus the change in the metabolic ratios in these patients is unlikely to be due to direct changes in fraction unbound of omeprazole.

6.2.2 Protein binding of proguanil

Proguanil, the other CYP2C19 substrate used as a probe drug in the clinical studies in this thesis, has plasma binding properties that differ from omeprazole. The amount of unbound proguanil in normal healthy plasma was determined in a similar manner to that described for omeprazole (section 6.2.1) and the concentration of unbound proguanil detected following ultrafiltration of spiked normal healthy plasma was determined from the calibration curve (Figure 6.2).

The fraction unbound (f_U) of proguanil in normal healthy plasma was 21.56 ± 1.56%. This value was in agreement with the literature value of 25% (GlaxoSmithKline 2008). The amount of binding in serum was significantly higher than in plasma with 10.49 ± 0.11% fraction unbound (P = 0.003).

The concentrations of unbound proguanil were then measured in the 3 hours post-dose plasma samples from individual patients (n = 29) with either stage IV or no-evaluable disease post-surgery (Table 6.4 and Table 6.5 respectively). These patients had received a single oral dose of proguanil (200 mg) as previously reported in chapter 4. Each patient was administered a single dose of the drug on three separate occasions more than 7 days apart. No patient was a genotypic poor metaboliser, therefore all samples were included in this study. The concentrations of unbound proguanil were compared with the previously reported total (solvent extracted) concentrations of drug in each individual patient's sample (chapter 4). The fraction unbound of drug in the patients' plasma was calculated as the unbound drug concentration divided by the total (solvent extracted) drug concentration as measured in chapter 4.

Stage IV disease						
Patient #	**Measured unbound PG, ng/mL**	**Total extracted[a] PG, ng/mL**	**Predicted[b] unbound PG, ng/mL**	**Measured fraction unbound[c] (f_U), %**	**Total extracted[d] CG, ng/mL**	**Metabolic ratio**
1009-1	13.28	167.71	36.16	7.92	13.72	12.22
1001-1	14.90	136.10	29.34	10.95	33.90	4.01
1001-2	18.75	196.10	42.28	9.56	46.92	4.18
1001-3	12.29	159.88	34.47	7.69	42.70	3.74
1002-1	12.63	126.11	27.19	10.02	40.35	3.13
1002-2	19.40	139.41	30.06	13.92	39.80	3.50
1002-3	10.39	142.57	30.74	7.29	43.23	3.30
1003-1	16.39	296.10	63.84	5.53	44.07	6.72
1003-2	16.34	234.79	50.62	6.96	16.41	14.31
1003-3	13.31	207.99	44.84	6.40	15.86	13.12
1004-1	9.08	135.57	29.23	6.70	8.23	16.47
1004-2	13.70	90.98	19.62	15.05	6.28	14.48
1004-3	8.53	40.13	8.65	21.26	1.00	40.13
1005-1	13.93	150.57	32.46	9.25	1.00	150.57
1005-2	9.56	67.35	14.52	14.20	1.00	67.35
1005-3	1.70	31.30	6.75	5.42	1.00	31.30
1006-1	18.88	116.32	25.08	16.23	11.62	10.01
1006-2	21.64	194.66	41.97	11.12	21.87	8.90
1006-3	18.74	175.98	37.94	10.65	19.71	8.93
1008-1	12.80	107.37	23.15	11.92	23.98	4.48
1008-2	14.71	125.74	27.11	11.70	38.51	3.27
1008-3	7.82	83.31	17.96	9.38	19.93	4.18
1013-1	9.76	55.79	12.03	17.50	9.08	6.15
1013-2	13.16	87.07	18.77	15.11	14.73	5.91
1013-3	14.65	76.22	16.43	19.22	11.26	6.77
1020-1	41.22	195.26	42.10	21.11	91.91	2.12
1020-2	29.27	181.01	39.03	16.17	126.09	1.44
1020-3	30.81	138.70	29.90	22.21	48.18	2.88
1021-1	11.90	138.86	29.94	8.57	42.51	3.27
1021-2	21.68	241.97	52.17	8.96	37.87	6.39
1021-3	16.25	134.16	28.92	12.12	43.04	3.12
1022-1	24.00	198.70	42.84	12.08	59.37	3.35
1022-2	21.77	176.03	37.95	12.37	39.23	4.49
1022-3	40.43	227.49	49.05	17.77	16.09	14.14
1027-1	12.12	100.93	21.76	12.01	114.44	0.88
1027-2	14.95	132.85	28.64	11.25	92.92	1.43
1027-3	16.76	95.27	20.54	17.60	21.10	4.51
1028-1	19.28	104.56	22.54	18.44	27.18	3.85
1028-2	26.17	139.15	30.00	18.80	58.32	2.39
1028-3	27.58	100.85	21.74	27.35	35.23	2.86
1029-1	17.70	48.10	10.37	36.79	18.21	2.64
Mean ± sd	**17.27 ± 8.07**	**139.00 ± 58.68**	**29.97 ± 12.65**	**13.53 ± 6.33**	**34.09 ± 29.25**	**12.36 ± 25.23**

Table 6.4. ***Measured versus predicted plasma protein binding of proguanil in patients with stage IV disease following an oral dose of proguanil (200 mg).***
[a] total solvent extracted drug as in section 2.7.2. [b] based on f_U = 21.56% previously determined in healthy plasma. [c] percentage of measured unbound proguanil over total solvent extracted proguanil in patients. [d] total solvent extracted metabolite as in section 2.7.2.

No-evaluable disease						
Patient #	**Measured unbound PG, ng/mL**	**Total extracted[a] PG, ng/mL**	**Predicted[b] unbound PG, ng/mL**	**Measured fraction unbound[c] (f_U), %**	**Total extracted[d] CG, ng/mL**	**Metabolic ratio**
1009-2	5.57	23.71	5.11	23.50	46.96	0.50
1009-3	12.25	70.47	15.19	17.39	61.09	1.15
1007-1	20.38	143.64	30.97	14.19	61.97	2.32
1007-2	15.67	134.20	28.93	11.68	41.79	3.21
1007-3	17.96	171.39	36.95	10.48	50.31	3.41
1010-1	5.83	48.70	10.50	11.98	6.11	7.97
1010-2	4.82	45.26	9.76	10.66	1.00	45.26
1010-3	1.87	20.17	4.35	9.26	1.00	20.17
1011-1	13.38	139.85	30.15	9.57	6.15	22.74
1011-2	10.80	86.37	18.62	12.51	8.43	10.24
1011-3	13.43	83.80	18.07	16.02	8.00	10.47
1012-1	16.51	154.74	33.36	10.67	47.80	3.24
1012-2	20.64	164.12	35.38	12.58	57.58	2.85
1012-3	20.75	155.98	33.63	13.30	24.83	6.28
1014-1	5.99	92.38	19.92	6.48	25.65	3.60
1014-2	6.81	92.99	20.05	7.32	8.90	10.45
1014-3	8.44	96.45	20.79	8.75	10.89	8.85
1015-1	11.43	133.92	28.87	8.53	21.17	6.32
1015-2	8.51	112.02	24.15	7.60	26.26	4.27
1015-3	6.16	90.20	19.45	6.83	17.22	5.24
1016-1	11.12	85.13	18.35	13.07	15.97	5.33
1016-2	15.23	143.21	30.88	10.64	22.20	6.45
1016-3	5.55	80.35	17.32	6.90	8.70	9.24
1017-1	11.09	128.73	27.75	8.62	17.43	7.38
1017-2	6.12	117.27	25.28	5.22	19.46	6.03
1017-3	11.43	120.80	26.04	9.46	18.98	6.37
1018-1	8.57	93.24	20.10	9.19	6.72	13.87
1018-2	9.41	82.00	17.68	11.48	10.78	7.61
1018-3	12.43	71.36	15.39	17.41	12.97	5.50
1019-1	9.88	133.14	28.71	7.42	125.61	1.06
1019-2	10.72	113.60	24.49	9.43	68.29	1.66
1019-3	12.33	57.25	12.34	21.53	22.97	2.49
1023-1	21.56	166.00	35.79	12.99	129.22	1.28
1023-2	19.93	140.83	30.36	14.15	97.37	1.45
1023-3	16.79	151.39	32.64	11.09	41.21	3.67
1024-1	21.48	136.58	29.45	15.73	146.55	0.93
1024-2	31.98	210.32	45.35	15.20	142.63	1.47
1024-3	25.63	148.95	32.11	17.21	75.66	1.97
1025-1	19.46	123.06	26.53	15.81	132.80	0.93
1025-2	22.34	129.05	27.82	17.31	88.70	1.45
1025-3	21.93	95.98	20.69	22.85	40.46	2.37
1026-1	17.49	156.74	33.79	11.16	73.25	2.14
1026-2	15.43	211.51	45.60	7.30	56.18	3.76
1026-3	26.81	159.44	34.38	16.81	42.83	3.72
Mean ± sd	**13.91 ± 6.79**	**116.28 ± 43.79**	**25.07 ± 9.44**	**12.21 ± 4.41**	**44.32 ± 41.04**	**6.29 ± 7.67**

Table 6.5. ***Measured versus predicted plasma protein binding of proguanil in patients with no-evaluable disease following an oral dose of proguanil (200 mg).***
[a] total solvent extracted drug as in method 2.7.2. [b] based on f_U = 21.56% previously determined in healthy plasma. [c] percentage of measured unbound proguanil over total solvent extracted proguanil in patients. [d] total solvent extracted metabolite as in method 2.3.4.

Following each single oral dose of proguanil, the measured unbound concentration of proguanil in the plasma sample of these patients was 15.53 ± 7.58 ng/mL and ranged from 1.70 to 41.22 ng/mL. In these same plasma samples, the total (solvent extracted) proguanil concentration was also variable (127.24 ± 52.46 ng/mL) and ranged from 20.17 to 296.10 ng/mL (Table 6.4). Sub-group analysis of stage IV and no-evaluable disease patients did not indicate any significant difference in unbound (17.27 ± 8.07 ng/mL and 13.91 ± 6.79 ng/mL respectively) and total proguanil concentrations (139.00 ± 58.68 and 116.28 ± 43.79 ng/mL respectively).

The predicted unbound concentration of proguanil was calculated for each individual patient (Table 6.4). This was based on the measured total proguanil concentration and the fraction unbound previously determined in normal healthy plasma. Similar to omeprazole, the measured unbound proguanil concentrations in the patient samples were lower than the expected values. For example, patient #1003-1 had a total extracted proguanil concentration of 296.10 ng/mL (Table 6.4). The predicted unbound proguanil concentration was 63.84 ng/mL, however the actual measured unbound concentration was more than 3-fold lower at 16.39 ng/mL. The mean measured fraction unbound of proguanil in the cancer patients was 12.84 ± 5.43% which was 1.7-fold lower than the 21.56 ± 1.56% that was measured in normal healthy plasma following spiking with proguanil at 300 ng/mL final concentration (Table 6.4).

In contrast to omeprazole (Table 6.3), six patients in this study had measured fraction unbound values similar to the predicted values. For example, #1019-3 had a measured f_U proguanil of 12.33 ng/mL versus a predicted f_U of 12.34 ng/mL. Similarly for #1020-1 (measured f_U of 41.22 ng/mL versus predicted f_U of 42.10 ng/mL) and #1025-3 (measured f_U of 21.93 ng/mL versus predicted f_U of 20.69 ng/mL). All of these patients had a metabolic ratio of 2.49, 2.12 and 2.37 respectively that was concordant with their genotype. These patients had either stage IV (#1020) or no-evaluable disease (#1019 and #1025).

It is not known which plasma protein is the major contributor to binding of proguanil. Because proguanil is a basic drug, it was expected to be bound to alpha$_1$-acid glycoprotein (Paxton 1983; Kremer et al. 1988). However, the major plasma protein involved in the binding of proguanil appeared to be high-density lipoprotein (44.52 ± 2.27% f_U), followed by low-density lipoprotein (39.37 ± 1.59% f_U) and albumin (51.82 ± 5.00% f_U). C-reactive protein and alpha$_1$-acid glycoprotein had a negligible role in the

binding of proguanil, with values not significantly (P > 0.05) different from control (87.44 ± 16.32 and 86.36 ± 9.40 % f_U respectively) (Figure 6.8).

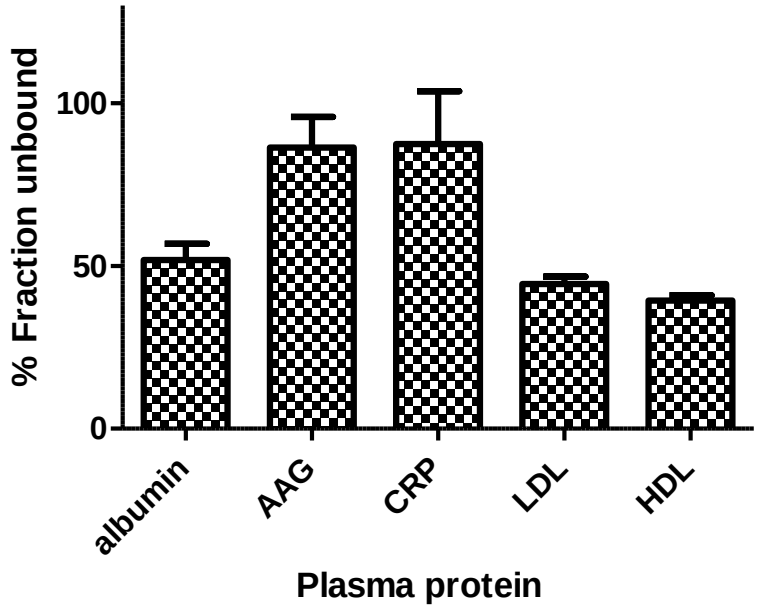

Figure 6.8. ***The percentage fraction unbound of proguanil in various proteins found in plasma.***
AAG = alpha$_1$-acid glycoprotein; CRP = C-reactive protein; LDL = low-density lipoprotein; HDL = high-density lipoprotein. Protein concentrations were based on normal concentrations observed in healthy state with the exception of CRP concentration observed during inflammation. Level of binding to proguanil: HDL > LDL > albumin, AAG and CRP had negligible binding ability.

Since albumin did appear to have an important role in the plasma protein binding of proguanil, the relationship between albumin concentrations and the fraction unbound of proguanil in each individual patient was investigated. Albumin concentrations were determined by a clinical biochemistry test carried out by the local laboratory (LabPlus). The test was not carried out for each phenotype test and hence only albumin values adjacent to the CYP2C19 metabolic ratio test value was reported in Table 6.4 and Table 6.5. The mean albumin concentration in the patients was 41.50 ± 3.00 g/L. Two patients (#1016-1 and 1025-1) were clinically hypoalbuminemic (31 g/L and 34g/L respectively). These albumin concentrations were significantly different from those observed in the patients with advanced incurable cancer (P = 0.0001). However, there was a narrower range of albumin values in these patients (31-48 g/L) compared to the patients with advanced incurable cancer (25.48-60.22 g/L). The albumin concentrations between the groups of patients with stage IV disease or no-evaluable disease were similar (41.74 ± 2.98 g/L and 41.26 ± 3.04 g/L respectively; P = 0.5741).

There was no relationship between albumin concentrations and the measured unbound proguanil concentration in the patients (Spearman r = -0.0684, *P* = 0.5740) (Figure 6.9).

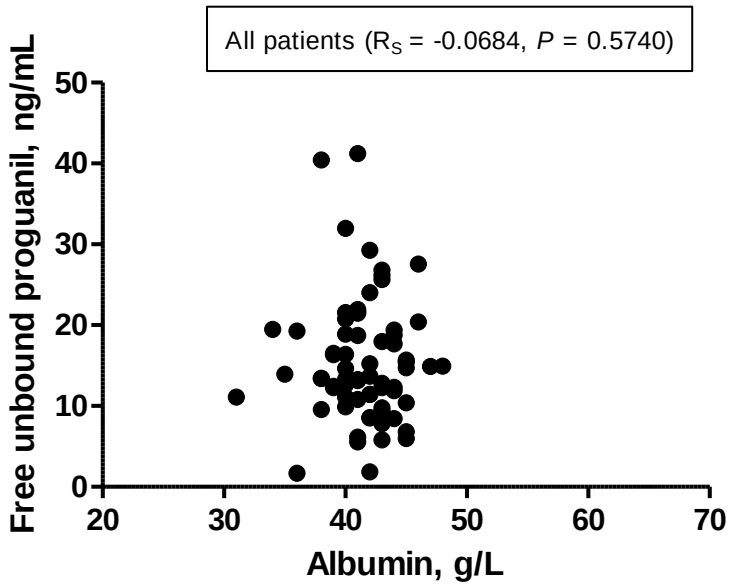

Figure 6.9. ***The lack of relationship between concentrations of albumin and unbound proguanil in cancer patients with stage IV or no-evaluable disease.*** Spearman r = -0.0684, *P* = 0.5740.

However, there was a significant negative correlation between the CYP2C19 proguanil metabolic ratio and albumin concentrations in these patients (Spearman r = -0.2531, *P* = 0.0345) (Figure 6.10). In particular, there was a negative correlation between albumin concentrations and the CYP2C19 proguanil metabolic ratio in the discordant patients (Spearman r = -0.2985) although not significant (*P* = 0.2446).

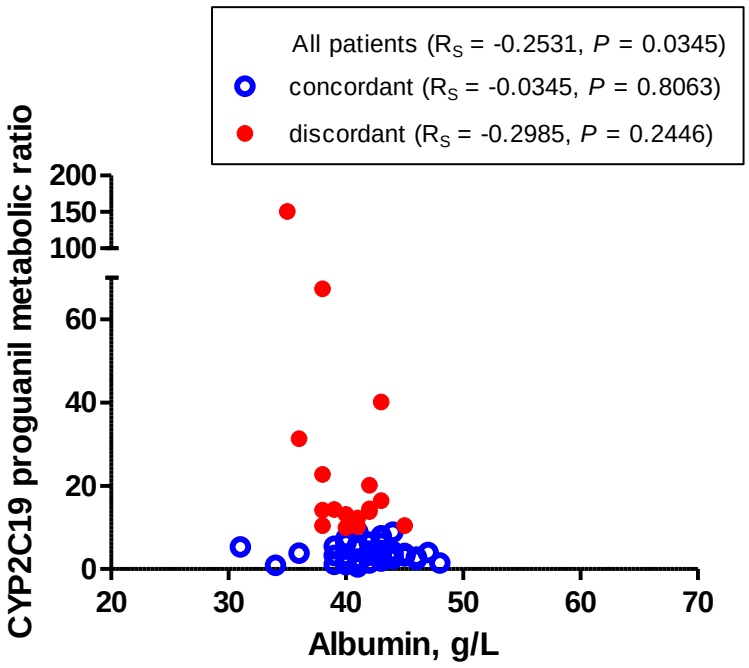

Figure 6.10. ***The relationship between the CYP2C19 proguanil metabolic ratio and albumin concentrations in cancer patients with stage IV or no-evaluable disease.*** The *P*-values from Spearman's rank correlation test are shown on the graph. A negative relationship between albumin concentration and CYP2C19 metabolic ratio was only observed in patients with genotype-phenotype discordance, although not significant (R_S = -0.2985, *P* = 0.2446).

C-reactive protein did not bind proguanil (Figure 6.8). However, interestingly, elevated CRP was associated with an increase in the measured concentration of unbound proguanil in the patients (Spearman r = 0.2558, *P* = 0.0268; Figure 6.11). Moreover, as previously noted (chapter 4), there was a significant positive correlation between CRP concentrations with the CYP2C19 proguanil metabolic ratio in the patients.

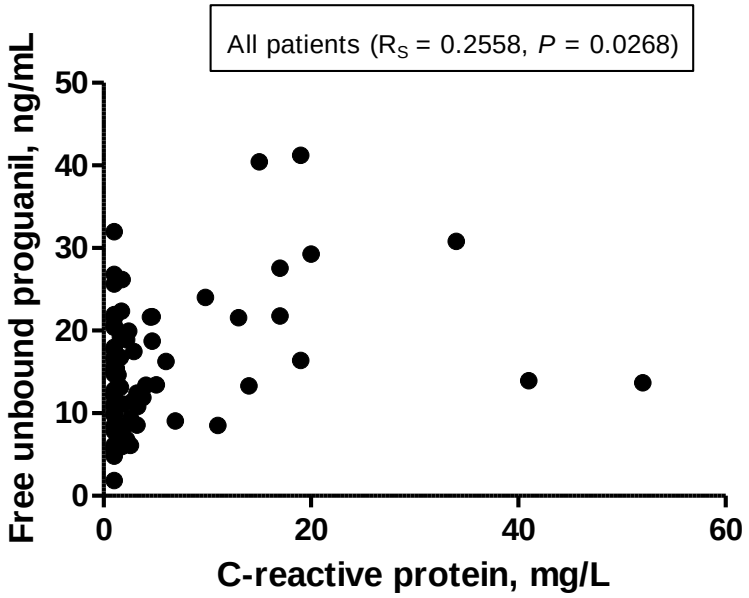

Figure 6.11. ***The significant positive relationship between the concentrations of CRP and unbound proguanil in cancer patients with stage IV or no-evaluable disease.*** Spearman r = 0.2558, *P* = 0.0268.

High-density and low-density lipoproteins were also capable of binding proguanil (Figure 6.8). However, there was insufficient plasma sample to measure each individual protein; therefore the total plasma protein concentration was determined in each individual patient's plasma sample.

The total plasma protein concentrations were variable in the patients with a mean of 65.19 ± 18.34 g/L. This was similar to the values reported for patients with advanced incurable cancer (63.54 ± 9.92 g/L; *P* = 0.6940). However, the mean total plasma protein concentration of the patients with stage IV disease was 69.31 ± 16.30 g/L which was significantly higher than the mean total plasma concentration in patients with no-evaluable disease (61.36 ± 19.45 g/L) (*P* = 0.0326).

There was no relationship between the concentrations of unbound proguanil and total plasma protein in the patients (Spearman r = -0.1400, *P* = 0.2012) (Figure 6.12).

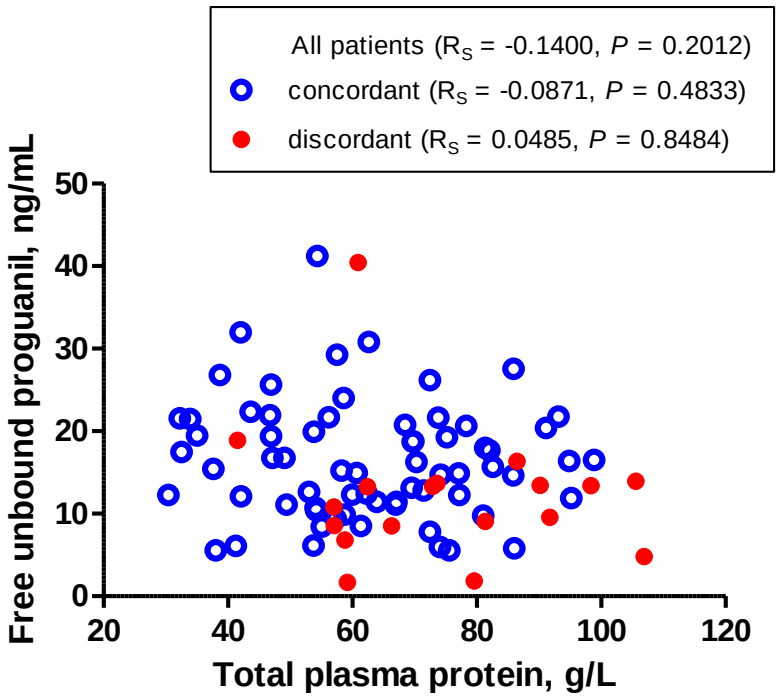

Figure 6.12. ***The lack of relationship between the concentrations of total plasma protein and unbound proguanil in cancer patients with stage IV or no-evaluable disease.*** The *P*-values from Spearman's rank correlation test are shown on the graph.

However, an increase in the CYP2C19 proguanil metabolic ratio was positively associated with increased total plasma protein concentrations in the patients (Spearman r = 0.3261, *P* = 0.0023) (Figure 6.13). This was particularly the case for the genotype-phenotype discordant (metabolic ratio > 10) individuals (Spearman r = 0.6904, *P* = 0.0015).

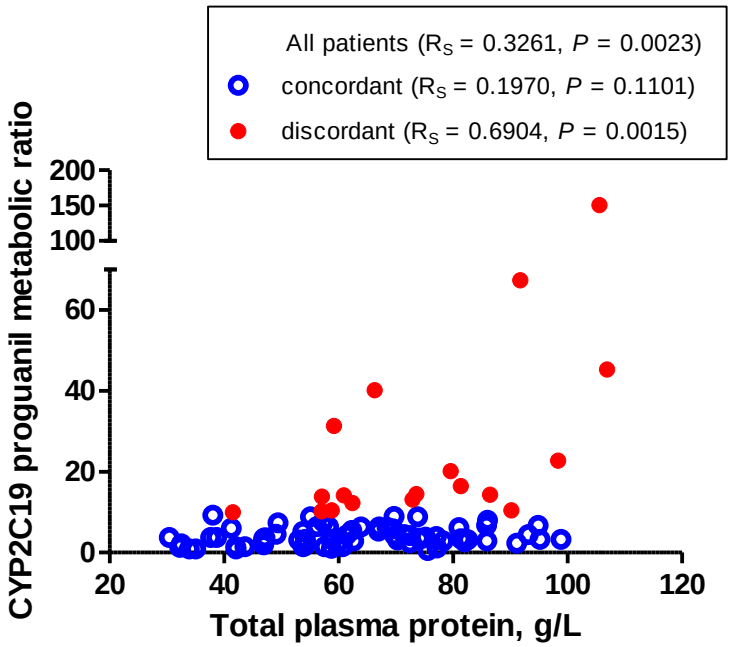

Figure 6.13. ***The relationship between the CYP2C19 proguanil metabolic ratio and total plasma protein concentration in cancer patients with stage IV or no-evaluable disease.*** The *P*-values from Spearman's rank correlation test are shown on the graph. A significant positive relationship between total plasma protein and CYP2C19 metabolic ratio was only observed in patients with genotype-phenotype discordance (R_S = 0.6904, *P* = 0.0015).

As observed with omeprazole, the inverse association between the total plasma protein and poor metaboliser status suggests that the total plasma protein concentration may be a global biomarker of inflammation (acute phase response). If this was the case, the cycloguanil metabolite concentration would be expected to decrease as total plasma protein concentrations increase. There was indeed a significant negative correlation between the concentrations of the total (solvent extracted) cycloguanil metabolite and total plasma protein in the patients (Spearman r = -0.3584, *P* = 0.0008) (Figure 6.14). There was a negative correlation in discordant individuals although not significant (Spearman r = -0.4194, *P* = 0.0832). In contrast, the negative correlation is significant in concordant individuals (Spearman r = -0.2686, *P* = 0.0280).

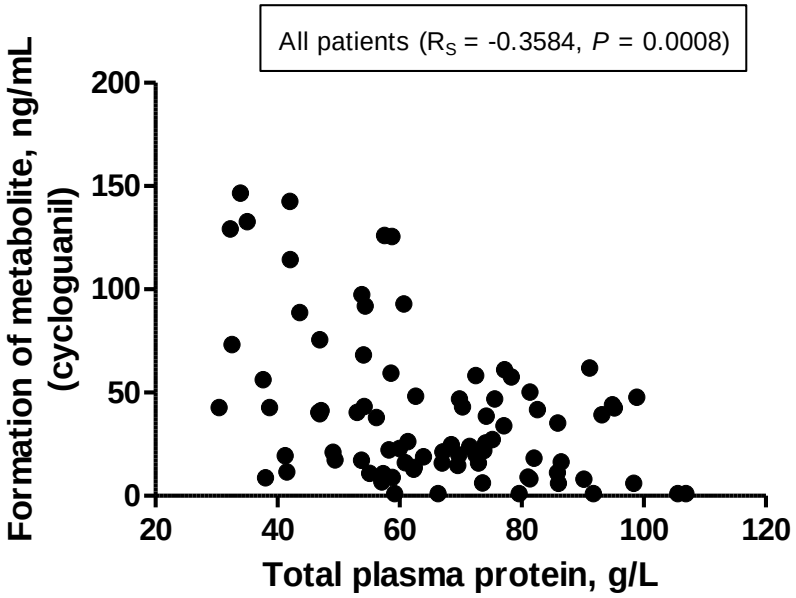

Figure 6.14. ***The negative relationship between the concentration of the cycloguanil metabolite and total plasma protein in cancer patients with stage IV or no-evaluable disease.*** Spearman r = -0.3584, *P* = 0.0008.

However, the cycloguanil metabolite formation did not correlate with the albumin concentrations in the patients (Spearman r = -0.1010, *P* = 0.6092) (data not shown).

In summary, there was no association with the measured fraction unbound of proguanil and the total plasma protein concentration. Hence, the discordant metabolic ratio in some individuals was unlikely to be due to an altered fraction unbound, resulting in a "false" metabolic ratio. Moreover, the positive correlation with C-reactive protein (which did not bind proguanil) and also the decreased formation of the cycloguanil metabolite as total plasma proteins increase suggests a role of inflammation in this relationship. Indeed, either C-reactive protein or total plasma proteins may be a biomarker of a global inflammatory status in these patients.

6.2.3 Individualised hepatic clearance of omeprazole and proguanil

The drug/metabolite ratio is an empirical *in vivo* measure of the activity of an enzyme to catalyse the formation of the metabolite. The activity of the enzyme can be defined in terms of the intrinsic clearance of the unbound drug from the blood by an organ (CL_{int}). However, the steady state concentration of parent drug (C_P) and metabolite (C_M) in the blood is determined by both renal excretion as well as hepatic metabolism (Figure 6.15).

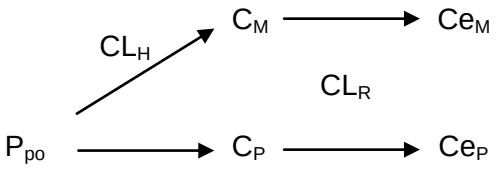

Figure 6.15. ***Relationship between amount of drug and metabolite in blood with respect to hepatic and renal clearance of the drug.*** P = parent drug, M = metabolite, po = *per os* (oral administration); CL_H = hepatic clearance, CL_R = renal clearance; C = steady state concentration in blood, Ce = concentration excreted in urine. Following oral administration, the parent drug undergoes hepatic metabolism and reaches steady state concentration which further undergoes renal clearance and is excreted in the urine.

The value of the drug/metabolite ratio as an index of enzyme activity (CL_{int}) can be obscured by changes in renal clearance (CL_R), hepatic blood-flow (Q) and the unbound drug concentration fraction (f_U). This chapter of the thesis has attempted to determine the possible effect of drug-protein binding on the use of proguanil or omeprazole drug/metabolite ratio as an index of CYP2C19 activity. However, as noted above, several factors may influence this metabolic ratio.

Variability in renal clearance of drug or metabolite could be an additional cause of the discordant metabolic ratio in some patients. However, no patient in the two studies (chapter 3 and chapter 4) had renal failure and an inclusion criteria for the study was that all patients were required to have creatinine levels less than 0.12 mmol/L. It is also important to note that for omeprazole renal clearance (CL_R) is negligible (Regårdh et al. 1985) and therefore can be discounted. The renal clearance (CL_R) of proguanil was 5.0 ± 1.9 ml/min and for the metabolite cycloguanil, the CL_R was 4.1 ± 1.8 ml/min (Wattanagoon et al. 1987). Hence, any variability in the renal clearance should influence the amount of proguanil (P) and cycloguanil (M) equally and therefore the drug/metabolite ratio should still relate to hepatic clearance.

An additional factor which could also theoretically influence the concentrations of metabolite (C_M) is secondary metabolism. The metabolites of omeprazole are 5'hydroxy omeprazole, omeprazole sulphone and 5-*O*-desmethyl omeprazole. The major metabolic route is 5'hydroxy formation which is catalysed by CYP2C19. The CL_{int} via this route was approximately 4-7-fold higher than formation of the sulphone, which is catalysed by CYP3A4 (Andersson et al. 1993a; Äbelö et al. 2000). Importantly the 5'hydroxy metabolite is a terminal metabolite and hence the concentration of metabolite (C_M) is not influenced by further metabolism. The major metabolic route for the hepatic clearance of proguanil is cycloguanil, which is also a terminal metabolite . A minor metabolite 4-chlorophenylbiguanide (CPB) is also formed from proguanil and is associated with CYP2C19 activity (Brøsen et al. 1993; Herrlin et al. 2000). Therefore secondary metabolism of the metabolites of these two probe drugs are unlikely to influence the metabolic ratio significantly as both metabolites of interest are terminal products.

Thus, theoretically, the drug/metabolite ratio as an index of CYP2C19 enzyme activity using either omeprazole or proguanil as probe drugs, should only be potentially influenced by drug-protein binding (f_U) and liver blood-flow (*Q*) in addition to intrinsic activity of the enzyme (CL_{int}).

Liver blood-flow (*Q*) can influence the hepatic extraction ratio (E) of a drug as illustrated in equation 1 (Rane et al. 1977).

$$E = \frac{CL_H}{Q} \quad (1)$$

The expected hepatic clearance (CL_H) of omeprazole and proguanil were calculated for a nominal healthy person with a fully functional CYP2C19 enzyme (*CYP2C19*1/*1*). The relationship between the hepatic clearance (CL_H), hepatic blood-flow (*Q*), intrinsic clearance (CL_{int}) and fraction unbound in blood ($f_{U,\ blood}$) was calculated from equation 2 (Rowland et al. 1973).

$$CL_H = Q \times \left(\frac{f_{U,\ blood} \times CL_{int}}{Q + (f_{U,\ blood} \times CL_{int})}\right) \quad (2)$$

The fraction unbound in blood ($f_{U,blood}$) was calculated for a nominal healthy individual using the measured plasma protein binding values determined following spiking normal healthy human plasma (section 6.2.1 and section 6.2.2) and the reported blood-to-

plasma ratio (R_B) for omeprazole of 0.69 (Tallman et al. 2004) and 2.7 for proguanil (Helsby et al. 1993) using equation 3 (Burton et al. 2006).

$$f_{U,\ blood} = \frac{f_U\ (plasma)}{R_B} \quad (3)$$

The intrinsic clearance (CL_{int}) of omeprazole and proguanil were calculated using equation 4 (Rane et al. 1977) and the mean of the reported V_{max} and K_m values listed in Table 6.6

$$CL_{int} = \frac{V_{max}}{K_m} \quad (4)$$

Drug	Reference	V_{max} (nmol/min/mg)	K_m (µM)	CL_{int} (mL/min/mg)
Omeprazole	(Chiba et al. 1993)	0.088 ± 0.070	6.0 ± 2.4	
	(Andersson et al. 1993a)	0.099 ± 0.068	8.6 ± 5.6	
	mean	**0.0935**	**7.3**	**0.0128**
Proguanil	(Funck-Brentano et al. 1997)	0.01 ± 0.0131	197 ± 55	
	(Helsby et al. 1990c)	0.005 ± 0.003	25.9 ± 3.9	
	(Rasmussen et al. 1998)	0.02	92	
	(Coller et al. 1999a)	0.018 ± 0.012	102 ± 123	
	mean	**0.0133**	**104**	**0.000128**

Table 6.6. ***The reported in vitro V_{max} and K_m and the calculated intrinsic clearance values of omeprazole and proguanil metabolism in human liver microsome.***

Since the amount of microsomal protein per gram of liver was reported to be 32 mg/g (Barter et al. 2007) and the average weight of the liver in a 70 kg human was assumed to be 1800 g (Davies et al. 1993), the predicted intrinsic clearance (CL_{int}) in a healthy person with functional CYP2C19 for omeprazole is 737.75 mL/min (44.26 L/h) and for proguanil is 7.35 mL/min (0.441 L/h) using the equations stated above.

Accounting for unbound drug fraction in blood ($f_{U,\ blood}$) using equation 3 and assuming that the hepatic blood-flow and intrinsic clearance are constant, the hepatic clearance (CL_H) for this nominal healthy (*CYP2C19*1/*1*) individual with normal protein binding was calculated from equation 2, to be 43.86 mL/min for omeprazole and 0.58 mL/min for proguanil.

The hepatic extraction ratio (E) of a drug is illustrated in equation 5 (Rane et al. 1977).

$$E = \frac{CL_H}{Q} \quad (5)$$

Assuming a liver blood-flow (*Q*) of 1450 mL/min (Davies et al. 1993), both omeprazole and proguanil were considered to be capacity-limited drugs since they have low hepatic extraction ratios (E = 0.03 and 0.0004 respectively). Therefore unbound clearance of these capacity-limited drugs could be influenced by liver size and enzyme activity but not liver blood-flow (Le Couteur et al. 1998).

Metabolite formation was individualised for each patient (Table 6.7-Table 6.9) based on the measured fraction unbound of the parent drug. These values were compared with the expected hepatic clearance of omeprazole and proguanil calculated for a nominal healthy person with a fully functional CYP2C19 enzyme (*CYP2C19*1/*1*) and “normal” plasma protein concentrations (43.86 mL/min and 0.58 mL/min respectively).

Advanced incurable disease, probe drug: omeprazole				
Patient #	**Genotype**	**Fraction unbound in blood ($f_{U, blood}$)**	**[a]Individualised CL_H, mL/min**	**Metabolic ratio (drug/metabolite)**
401	*1/*1	0.01728	12.64	6.82
402	*1/*1	0.01059	7.77	1.88
409	*1/*1	0.01360	9.96	85.54
410	*1/*1	0.01818	13.29	2.81
412	*1/*1	0.01950	14.24	3.08
417	*1/*1	0.01022	7.50	5.37
418	*1/*1	0.01086	7.97	1.56
419	*1/*1	0.02334	17.02	1.44
424	*1/*1	0.01645	12.04	1.41
427	*1/*1	0.00739	5.43	1.17
428	*1/*1	0.01008	7.40	11.35
431	*1/*1	0.01145	8.40	20.70
435	*1/*1	0.01031	7.57	2.65
436	*1/*1	0.01097	8.04	16.07
437	*1/*1	0.01577	11.54	2.43
Mean ± sd		**0.01373 ± 0.00446**	**10.05 ± 3.24**	**10.95 ± 21.46**

Table 6.7. ***Inter-individual variability in hepatic clearance (CL_H) of omeprazole in patients with advanced incurable cancer.*** [a] Based on the measured f_u in each individual patient (Table 6.3) and calculated using equation 3.

Stage IV disease, probe drug: proguanil				
Patient #	**Genotype**	**Fraction unbound in blood ($f_{U, blood}$)**	**[a]Individualised CL_H, mL/min**	**Metabolic ratio (drug/metabolite)**
1009-1	**1/*1*	0.02933	0.21	12.22
1002-1	**1/*1*	0.03709	0.27	3.13
1002-2	**1/*1*	0.05154	0.38	3.50
1002-3	**1/*1*	0.02700	0.20	3.30
1003-1	**1/*1*	0.02050	0.15	6.72
1003-2	**1/*1*	0.02578	0.19	14.31
1003-3	**1/*1*	0.02370	0.17	13.12
1004-1	**1/*1*	0.02480	0.18	16.47
1004-2	**1/*1*	0.05576	0.41	14.48
1004-3	**1/*1*	0.07873	0.58	40.13
1008-1	**1/*1*	0.04414	0.32	4.48
1008-2	**1/*1*	0.04333	0.32	3.27
1008-3	**1/*1*	0.03476	0.25	4.18
1020-1	**1/*1*	0.07819	0.57	2.12
1020-2	**1/*1*	0.05988	0.44	1.44
1020-3	**1/*1*	0.08226	0.60	2.88
1021-1	**1/*1*	0.03175	0.23	3.27
1021-2	**1/*1*	0.03318	0.24	6.39
1021-3	**1/*1*	0.04488	0.33	3.12
1027-1	**1/*1*	0.04447	0.33	0.88
1027-2	**1/*1*	0.04166	0.31	1.43
1027-3	**1/*1*	0.06517	0.48	4.51
1028-1	**1/*1*	0.06830	0.50	3.85
1028-2	**1/*1*	0.06964	0.51	2.39
1028-3	**1/*1*	0.10130	0.74	2.86
1006-1	**1/*17*	0.06013	0.44	10.01
1006-2	**1/*17*	0.04117	0.30	8.90
1006-3	**1/*17*	0.03944	0.29	8.93
1005-1	**17/*17*	0.03426	0.25	150.57
1005-2	**17/*17*	0.05258	0.38	67.35
1005-3	**17/*17*	0.02008	0.15	31.30
1013-1	**17/*17*	0.06482	0.47	6.15
1013-2	**17/*17*	0.05597	0.41	5.91
1013-3	**17/*17*	0.07117	0.52	6.77
1029-1	**17/*17*	0.13627	1.00	2.64
Mean ± sd		**0.05123 ± 0.02478**	**0.38 ± 0.18**	**13.51 ± 27.14**

Table 6.8. ***Inter-individual variability in hepatic clearance (CL_H) of proguanil in cancer patients with stage IV disease.*** [a] Based on the measured f_u in each individual patient (Table 6.4) and calculated using equation 3.

No-evaluable disease, probe drug: proguanil				
Patient #	**Genotype**	**Fraction unbound in blood ($f_{U, blood}$)**	**[a]Individualised CL_H, mL/min**	**Metabolic ratio (drug/metabolite)**
1009-2	**1/*1*	0.08705	0.64	0.50
1009-3	**1/*1*	0.06440	0.47	1.15
1010-1	**1/*1*	0.04437	0.32	7.97
1010-2	**1/*1*	0.03946	0.29	45.26
1010-3	**1/*1*	0.03429	0.25	20.17
1011-1	**1/*1*	0.03544	0.26	22.74
1011-2	**1/*1*	0.04632	0.34	10.24
1011-3	**1/*1*	0.05934	0.43	10.47
1012-1	**1/*1*	0.03952	0.29	3.24
1012-2	**1/*1*	0.04659	0.34	2.85
1012-3	**1/*1*	0.04926	0.36	6.28
1018-1	**1/*1*	0.03404	0.25	13.87
1018-2	**1/*1*	0.04252	0.31	7.61
1018-3	**1/*1*	0.06450	0.47	5.50
1019-1	**1/*1*	0.02749	0.20	1.06
1019-2	**1/*1*	0.03494	0.26	1.66
1019-3	**1/*1*	0.07975	0.58	2.49
1023-1	**1/*1*	0.04811	0.35	1.28
1023-2	**1/*1*	0.05241	0.38	1.45
1023-3	**1/*1*	0.04107	0.30	3.67
1025-1	**1/*1*	0.05855	0.43	0.93
1025-2	**1/*1*	0.06411	0.47	1.45
1025-3	**1/*1*	0.08463	0.62	2.37
1007-1	**1/*17*	0.05255	0.38	2.32
1007-2	**1/*17*	0.04326	0.32	3.21
1007-3	**1/*17*	0.03881	0.28	3.41
1014-1	**1/*17*	0.02402	0.18	3.60
1014-2	**1/*17*	0.02710	0.20	10.45
1014-3	**1/*17*	0.03242	0.24	8.85
1017-1	**1/*17*	0.03191	0.23	7.38
1017-2	**1/*17*	0.01932	0.14	6.03
1017-3	**1/*17*	0.03503	0.26	6.37
Mean ± sd		**0.04633 ± 0.01690**	**0.34 ± 0.12**	**7.06 ± 8.78**

Table 6.9. ***Inter-individual variability in hepatic clearance (CL_H) of proguanil in cancer patients with no-evaluable disease.*** [a] Based on the measured f_u in each individual patient (Table 6.5) and calculated using equation 3.

Compared with the predicted hepatic clearance of omeprazole (43.86 mL/min) in a nominal healthy (*CYP2C19*1/*1*) person, the mean hepatic clearance of omeprazole in the patients with *CYP2C19*1/*1* was more than 4-fold lower at 10.05 ± 3.24 mL/min. This was equivalent to the approximately 4-fold higher drug-protein binding in these patients.

Of the eleven *CYP2C19*1/*1* individuals who were genotype-phenotype concordant (metabolic ratio < 10), the mean metabolic ratio was 2.78 ± 1.78. The mean individualised hepatic clearance in these patients was 10.64 ± 3.59 mL/min. The reported values for omeprazole metabolic ratio in *CYP2C19*1/*1* genotype healthy individuals were 3.8 ± 1.25 (Ishizawa et al. 2005) and 1.75 (95% CI 1.66-1.88) (Uno et al. 2007). The four *CYP2C19*1/*1* individuals who were genotype-phenotype discordant (mean metabolic ratio of 33.41 ± 34.96) had a mean individualised hepatic clearance of 8.45 ± 1.09 mL/min. Importantly this individualised hepatic clearance (based on f_U) was not significantly different than that observed in the concordant subjects (*P* = 0.4727). Hence, in these discordant subjects, the high metabolic ratio (> 10) can only be accounted for by decreased enzyme activity (CL_{int}).

The hepatic clearance of proguanil in the patients was 0.36 ± 0.16 mL/min and was also lower than the predicted clearance (0.58 mL/min) in a nominal healthy individual. This decrease appears to be similar to the approximately 3-fold higher drug-protein binding in these patients. There was no difference in the mean clearance in patients with stage IV disease compared with patients with no-evaluable disease (0.37 ± 0.18 mL/min and 0.34 ± 0.12 mL/min respectively; *P* = 0.5891).

The reported median value for the proguanil metabolic ratio in *CYP2C19*1/*1* genotype healthy individuals are 1.4 (range 0.23-5.9) (Hoskins et al. 1998) and 4.0 (95% CI 2.01-5.50) (Herrlin et al. 2000). This previously reported data for homozygous wildtype subjects will include individuals with the *CYP2C19*17* variant allele. Of the concordant *CYP2C19*1/*1* (or *CYP2C19*17*) individuals with either stage IV or no-evaluable disease, the mean metabolic ratios were 4.12 ± 2.18 and 3.71 ± 2.53 respectively. There was no significant difference in the calculated hepatic clearances in these two groups of patients (0.41 ± 0.19 mL/min and 0.35 ± 0.13 mL/min respectively; *P* = 0.3666). Comparison of discordant individuals (metabolic ratio >10) with either stage IV or no-evaluable disease also did not demonstrate any significant difference in hepatic clearance (0.30 ± 0.15 mL/min and 0.29 ± 0.08 mL/min respectively; *P* = 0.5905).

Importantly the individualised hepatic clearance (based on f_U) in discordant patients was not significantly different than that observed in the concordant subjects (P = 0.0734 and P = 0.2012 for stage IV or no-evaluable disease groups respectively).

Therefore, since differences in the fraction unbound of drug have been individually accounted for, the high metabolic ratio in these subjects can only be due to decreased enzyme activity (intrinsic clearance, CL_{int}), i.e. compromised CYP2C19 activity. Hence the difference in metabolic ratio between concordant and discordant patients using both probe drugs was not due to changes in f_U since the predicted hepatic clearance was similar in both groups.

6.3 Discussion

The use of a probe drug to measure CYP2C19 activity (metabolic status) is a fundamental part of the work described in this thesis. The metabolic ratio of omeprazole and proguanil have been used extensively to phenotype healthy subjects and both probe drugs have been demonstrated to have an excellent correlation with *CYP2C19* genotype (Balian et al. 1995; Kortunay et al. 1997b; Hoskins et al. 1998; Xie et al. 1999b; Herrlin et al. 2000; Hoskins et al. 2003).

The discordance between genotype and phenotype observed in some cancer patients (Helsby et al. 2008) chapter 3 and 4) may be an important measure of compromised drug metabolism in these patients. However, although many studies have demonstrated a clear relationship between metabolic ratio and genotype, a number of other factors could also influence the amount of drug and metabolite in the plasma. These factors include renal clearance, secondary metabolism, liver blood flow and the amount of unbound drug, as well as the intrinsic activity of the enzyme. To ensure that the discordant CYP2C19 metabolic ratio measured in the cancer patients was a true reflection of decreased CYP2C19 activity and not due to changes in liver blood flow or free drug fraction, the studies described in this chapter were undertaken.

Initial experiments to measure the free drug fraction in normal healthy plasma confirmed the reported literature values for the two probe drugs (Regårdh et al. 1985; GlaxoSmithKline 2008). However, when the actual free fraction was determined in the patients' samples, it was found to be significantly lower. Previous studies of drug-protein

binding for drugs such as penbutolol and thio-TEPA in cancer patients have also shown this effect (Hagen et al. 1987; Aguirre et al. 1996). The cause of this 3-4 fold higher protein binding of omeprazole and proguanil is not known. However, pH differences are known to result in changes in drug-protein binding between serum and plasma (Brørs et al. 1984; Ohshima et al. 1989). Alterations in the drug-protein binding caused by the difference in pH may be due to the conformational change in the protein, changes in ionisation (charge) or modification in the charge distribution of the protein binding site (Ohshima et al. 1989). Whether pH differences in the cancer patient samples are the cause of this unexpected change in free drug fraction is not known. The fraction unbound of the probe drug in the patients was lower than the predicted value observed from spiking normal healthy plasma. To confirm this result, the fraction unbound should be measured following the direct addition of the drug into individual cancer patient drug-free plasma. This was not feasible in this study, since no drug free-plasma (zero time point) was collected in the clinical studies to avoid additional blood withdrawal from the patients.

The lower fraction unbound observed in the patients could also be due to changes in the levels of the acute phase response proteins which can influence drug-protein binding. Acute phase response proteins previously described as drug-binding proteins include albumin, alpha$_1$-acid glycoprotein and lipoproteins. Omeprazole is an acidic drug (pK_a = 3.97) (BrándstRöm et al. 1985) and was previously reported to be primarily bound to albumin, with an additional role for alpha$_1$-acid glycoprotein (Regårdh et al. 1985). The extent of omeprazole binding in plasma was independent of drug concentration (Regårdh et al. 1985). The studies within this chapter have confirmed that albumin was the major binding protein for omeprazole and that alpha$_1$-acid glycoprotein could also bind to omeprazole. However alpha$_1$-acid glycoprotein had a lower relative contribution to omeprazole binding than low-density lipoprotein. In contrast to omeprazole, proguanil is a basic drug (pK_a = 10.4; (Findlay 1951), and basic drugs were assumed to bind preferentially to alpha$_1$-acid glycoprotein (Paxton 1983; Kremer et al. 1988). However, the studies in this chapter could not find any evidence for a major role of alpha$_1$-acid glycoprotein in proguanil binding. In contrast, the major proteins responsible for binding proguanil appeared to be high-density and low-density lipoproteins as well as albumin. It is important to note that proguanil, although considered to be a basic drug, does have two pK_a values, 2.3 and 10.4 (Abdi et al. 2003), and may be a ampholyte, which may explain its ability to bind to proteins other than alpha$_1$-acid glycoprotein.

C-reactive protein is an acute phase response protein that had been previously associated with compromised CYP3A4 metabolic status in cancer patients (Rivory et al. 2002). It was not known if C-reactive protein could bind drugs; however, C-reactive protein was demonstrated to not bind the probe drugs even at the high plasma concentrations observed during the acute phase inflammatory response in the cancer patients.

Although both omeprazole and proguanil were demonstrated to be bound by albumin, there was no associated decrease in unbound drug as the concentrations of albumin increased in the patients. It is important to note, however, that few patients in the current studies were clinically hypoalbuminemic. Many other proteins in the plasma, such as transferrin and globulins, are known to bind drugs (Piafsky 1980; Allardyce et al. 2002). The effect of these other drug-binding proteins on the fraction unbound of the probe drugs was not determined directly. In order to account for the combined effect of multiple drug-binding proteins, the relationship between fraction unbound and total plasma proteins was determined in the patients. If protein binding has an effect on the fraction unbound of omeprazole and proguanil, a negative correlation would be expected, i.e. as the concentration of total plasma proteins increase, the fraction unbound of drug should decrease. However, this relationship was not observed; moreover, the significant inverse association between total plasma protein concentrations and metabolite formation suggests that total plasma protein could be an additional biomarker of inflammation. The lack of association between CYP2C19 metabolic status and total plasma proteins in patients tested with omeprazole may be due to the relatively small sample size in this study. Since more than 200 proteins have been detected in the plasma (Anderson et al. 2002), it is not known which specific protein(s) are appropriate biomarkers. However, previous data (chapter 4) have suggested some influence of CRP, IL-6 and an inverse association with TNF-α and TGF-β on CYP2C19 activity.

Importantly, when hepatic clearance (CL_H) of probe drug was individually calculated for each patient based on the free fraction of drug measured in each patient, it was clear that there was no difference in the CL_H between concordant and discordant individuals for either probe drug.

Interestingly, patient #1009 (Table 6.8 and Table 6.9) had a relatively low hepatic clearance (CL_H = 0.21 mL/min) prior to tumour resection. Immediately after surgery, the hepatic clearance had increased to 0.64 mL/min, which was similar to the predicted

"healthy" hepatic clearance value for proguanil. This change in hepatic clearance (0.21 versus 0.64 mL/min) following resection of the tumour was reflected in a change in metabolic ratio (12.22 versus 0.50).

The following table (Table 6.10) summarises the evidence for whether or not the increased metabolic ratio (poor metaboliser status) of omeprazole and proguanil in the cancer patients could be due to changes in drug-protein binding (unbound drug).

Scenario	Covariate	Omeprazole	Proguanil
Protein-binding limits the availability of the probe drug for hepatic CYP2C19 metabolism	Total plasma protein concentration versus measured unbound drug fraction in patients	No decrease in fraction unbound as protein concentrations increase	No decrease in fraction unbound as protein concentrations increase
	Albumin concentration versus measured fraction unbound of drug in patients	No decrease in fraction unbound as albumin concentrations increase	No decrease in fraction unbound as albumin concentrations increase
	Individualised hepatic clearance (based on free drug fraction) relative to metabolic ratio	Concordant and discordant patients have same hepatic clearance but different metabolic ratios	Concordant and discordant patients have same hepatic clearance but different metabolic ratios
Summary		**No evidence**	**No evidence**
Changes in plasma proteins may be biomarkers of cancer-associated inflammation	Albumin concentration versus metabolite formation	No association	No association
	Total plasma protein concentration versus metabolite formation	Protein concentration is not inversely associated with metabolite formation	Protein concentration is significantly inversely associated with metabolite formation
Summary		**No evidence**	**Some evidence**

Table 6.10. ***A summary of the results of chapter 6.***

From both first principles and direct measurement of drug protein binding, it has been demonstrated that the most likely cause of an altered drug/metabolite ratio in the discordant patients is decreased CYP2C19 activity rather than changes in drug protein binding. In conclusion, the use of either probe drug (omeprazole or proguanil) to detect compromised CYP2C19 activity appears to be a valid approach.

CHAPTER 7

General discussion and conclusion

7.1 General discussion and conclusion

It has been long recognised that inter-individual differences exist in patients with respect to their ability to metabolise drugs (phenotype) and this may impact on therapeutic effectiveness and/or toxicity. There are a large number of drug metabolising enzymes existing that are known to have polymorphic expression (Nebert 1997; Bosch et al. 2006), with the majority of the work in this area focussed on the genetic basis for such variability (genotype). One such enzyme is CYP2C19. A number of cancer chemotherapeutic drugs, such as cyclophosphamide, bortezomib, thalidomide and tamoxifen, are metabolised at least in part by CYP2C19 (Ando et al. 2002b; Pekol et al. 2005; Timm et al. 2005; Uttamsingh et al. 2005; Schroth et al. 2007; Helsby et al. 2010b; Li et al. 2011; Lim et al. 2011). Hence, any disturbance in CYP2C19 activity in cancer patients may affect drug efficacy or toxicity, particularly in those drugs with a narrow therapeutic index such as bortezomib (Papandreou et al. 2004). Compromised CYP2C19 activity may affect the toxicity of drugs in a similar manner to that observed with docetaxel and vinorelbine in cancer patients with low CYP3A4 activity (Slaviero et al. 2003). Hence, it may be important to determine the CYP2C19 activity of an individual prior to chemotherapy in order to prevent over- or under-dosing. The aim of the studies described in this thesis was to investigate the reported discordance between CYP2C19 genotype and phenotype in a cancer population and to explore the potential mechanisms for such discordance.

The majority of the CYP2C19 poor metabolisers can be accounted for by the *CYP2C19*2* and *CYP2C19*3* genetic defects. However, there are rare mutants of the *CYP2C19* allele including *CYP2C19*4*, *CYP2C19*5*, *CYP2C19*6*, *CYP2C19*7* and *CYP2C19*8* which may also account for null CYP2C19 activity (Ferguson et al. 1998; Ibeanu et al. 1998a; Ibeanu et al. 1998b; Ibeanu et al. 1999). The incidences of these rare mutant alleles were not determined in the studies in this thesis. The first clinical study in this thesis (chapter 3) was conducted in a small cohort of patients with advanced incurable (terminal) cancer. This published study (Helsby et al. 2008) demonstrated that CYP2C19 genotype-phenotype discordance exists in 37% of the cancer patients. The discordance was further investigated in a separate clinical study conducted in patients with stage IV disease or no-evaluable disease (resected tumour with no reoccurrence) as described in chapter 4. In accordance with the first study, a genotype-phenotype discordance was observed in 27% and 14% of patients with stage IV or no-evaluable

disease respectively. Moreover, there was a general decrease in the incidence of the discordance with disease severity.

However, in the second study, each patient was phenotyped on three separate occasions and some patients changed metaboliser categories (section 4.2.8), i.e. from extensive metaboliser to a poor metaboliser and vice versa. This further demonstrates that the compromised CYP2C19 activity observed in cancer was not due to the patients' genotype (i.e. rare mutant alleles). These two independent studies build upon a previous report of an increased incidence of phenotypic poor metabolisers in cancer patients (Williams et al. 2000).

The initial clinical study described in this thesis was conducted in a group of patients with terminal cancer who did not respond to treatment and were no longer on active chemotherapy (chapter 3). However, compromised CYP2C19 activity was also observed in patients at earlier stages of therapy (i.e. first-line treatment) and earlier disease progression (chapter 4). This was an important finding because, if significantly compromised activity only occurred in terminal cancer patients, there would be limited relevance to cancer therapy.

The first study in this thesis included patients with all types of cancer, including breast, lung and lymphoma, whereas the second clinical study recruited patients with colorectal cancer. Again, it was important to note that the observation of CYP2C19 genotype-phenotype discordance occurred in patients with a wide range of solid tumour types, as this indicates potential relevance to the general oncology population rather than a specific tumour type.

In the previous reported study (Williams et al. 2000), several patients had increased circulating levels of liver enzymes such as AST, ALT or ALP and decreased levels of albumin, which is indicative of liver damage (Sheehan et al. 1979). In addition, 9 of the 16 patients had liver metastases. Liver diseases such as cirrhosis and hepatitis are known to decrease the hepatic clearance of the CYP2C19 probe substrate omeprazole (Andersson et al. 1993b; Rost et al. 1995; Ohnishi et al. 2005) and increase the hydroxylation index of this drug (Kimura et al. 1999). Moreover, it has been reported that there is decreased clearance of antipyrine (a global marker of CYP activity) in patients with liver metastases (Sotaniemi et al. 1977). Thus the effect of liver damage/disease or

liver metastases on the increased incidence of omeprazole poor metabolism in this previous report could not be discounted.

However, the two clinical studies described in this thesis excluded patients with 'abnormal' liver and kidney function, as determined by routine clinical measures. Abnormal liver function indicates pathological change due to necrosis and the lysis of hepatocytes which cause the release of liver enzymes into the circulation. The observed poor metabolism of CYP2C19 probe drugs was therefore unlikely to be due to liver damage. Moreover, although 17 out of 45 patients in the two studies had liver metastases, no relationship was observed between those patients with liver metastases and the incidence of CYP2C19 poor metaboliser status. In addition, one patient had a primary colorectal adenoma (37.1 mm) with no liver metastases (section 4.2.8). Prior to resection, this patient had a discordant poor metaboliser status. In contrast, eighty-four days after surgery, the phenotype was concordant with the genotype. This was highly suggestive of a causal relationship between extra-hepatic tumour burden and changes in hepatic CYP2C19 activity. Furthermore, the observation of a cancer-associated compromised metabolic activity of CYP2C19 is a similar phenomenon to that reported for CYP3A4 activity in cancer patients (Slaviero et al. 2003).

One of the mechanisms proposed for the decrease in CYP2C19 activity in cancer was the inflammatory status of the patients. The studies described in this thesis were the first to attempt to correlate inflammatory status and CYP2C19 activity in cancer in the clinical situation. However, no relationships between cytokine or C-reactive protein (CRP) levels and CYP2C19 activity were observed in the patients (section 3.2.4 and section 4.2.7). This lack of relationship may be due to the NZ cancer patients having relatively less inflammation, since the overall C-reactive protein concentrations were low compared with the greater range reported previously (Rivory et al. 2002). In that study, a significant inverse relationship between the CYP3A4 activity and the acute phase response protein, C-reactive protein, was reported in forty patients with various types of primary tumour (Rivory et al. 2002).

Although the work in this thesis was the first to study the relationship between inflammation and CYP2C19 activity in cancer patients, an earlier clinical study reported a significant correlation between CYP2C19 activity and cytokines in congestive heart failure (Frye et al. 2002). Increasing concentrations of TNF-α were associated with a poor metaboliser status (low metabolite recovery). Conversely, increased TNF-α

concentrations in the NZ cancer patients was associated with an improved metaboliser status (low metabolic ratio) as described in section 4.2.7. The reported concentrations of TNF-α in congestive heart failure were similar to the concentrations observed in the NZ cancer patients. However, the sample size in the congestive heart failure study was also small (n = 16), hence these conflicting findings may simply be due to stochastic error. Therefore, additional independent studies in larger cohorts are required to determine any association between inflammatory markers and CYP2C19 metaboliser status.

Increased TGF-β concentrations were associated with an extensive metaboliser status in the cancer patients described within this thesis (section 4.2.7). This was surprising, since a significant decrease in *CYP2C19* mRNA expression in hepatocytes following incubation with TGF-β has been reported (Aitken et al. 2007). However, the concentration of TGF-β used in that *in vitro* study was 10 ng/mL (Aitken et al. 2007), which is 10-fold higher than the maximum TGF-β concentration observed in the cancer patients. Interestingly, it has been reported that GATA-4, a transcription factor for *CYP2C19* (Mwinyi et al. 2010b) is increased by TGF-β (Haveri et al. 2009), hence this may be an alternative mechanism by which TGF-β could influence CYP2C19 expression and activity. Therefore, low levels of TGF-β would lead to low GATA-4 levels and decreased transcription of *CYP2C19.*

Interestingly, the observed CYP2C19 genotype-phenotype discordance does not seem to be cancer-specific. Resected colorectal patients with no current evidence of cancer (no-evaluable disease) also had compromised CYP2C19 activity, although the incidence of the genotype-phenotype discordance was lower in comparison with patients with stage IV or advanced incurable (terminal) disease (section 4.2.3). In these resected patients, there was a significant relationship between increasing body mass index or arm circumference measurements and the poor metaboliser status (section 4.2.6). Obesity was also known to affect drug metabolism by cytochrome P450 enzymes (Kotlyar et al. 1999). Moreover, inflammation, in particular increased TNF-α concentration, was also associated with obesity (Yudkin et al. 1999; Zahorska-Markiewicz et al. 2000; Hui et al. 2004; Olszanecka-Glinianowicz et al. 2004). Hence, inflammation due to any pathology may be a possible factor in the compromised CYP2C19 activity. Indeed this agrees with the data from the previous reported study in congestive heart failure (Frye et al. 2002).

As already discussed, the lack of association between CYP2C19 activity with cytokine and CRP concentrations may be due to the relatively small cohort sizes of the studies in this thesis and others in the literature. However, accuracy of the cytokine determinations may also be a factor. For example, the reported cytokine concentration in "healthy" individuals also varies widely in literature (Table 7.1). This may be due to the short circulating half-life and the presence of soluble receptors which may underestimate free circulating cytokine levels (Beutler et al. 1985; Castell et al. 1988; Klapproth et al. 1989; Fernandez-Botran 1991; Rose-John et al. 1994). Furthermore, there were fluctuations in the cytokine levels over time (Munoz et al. 1991; Togo et al. 2009). Hence, the cytokine concentrations determined in the patients' samples may not be an accurate measure of their inflammatory state.

Subjects	IL-1α	IL-1β	IL-6	TNF-α	IFN-γ	TGF-β	CRP	Reference
n = 15		0.98 ± 0.22	6.98 ± 1.96	7.31 ± 0.94			8.75 ± 0.84	(Dülger et al. 2004)[a]
n = 15		30.5 ± 4.7	10.3 ± 5.1	12.7 ± 4.9			0.31 ± 0.26	(Jablonska et al. 1997)[b]
n = 15		4.3 ± 0.2	4.4 ± 0.2	6.3 ± 1.3			0.41 ± 0.15	(Kuku et al. 2005)[b]
n = 20	28 ± 6	318 ± 40	11 ± 2	0			0.4 ± 0.2	(Macciò et al. 1998)[a]
n = 16	700 ± 700		0.5 ± 1.4	16.6 ± 5.4				(Mantovani et al. 2000)[b]
n = 378	13.1 ± 3.2	40.2 ± 8.8	22.8 ± 7	34.32 ± 11.46				(Yurkovetsky et al. 2007)[a]
n = 26			4.3 ± 3.1	20.4 ± 12.1	12.1 ± 3.6			(Zeh et al. 2005)[a]
n = 29		31.5 ± 13.2	20.0 ± 11.6	8.4 ± 2.6	2.1 ± 0.7			(Hathaway et al. 2005)[a]
n = 19			1.53 ± 0.30	3.36 ± 0.19		8.83 ± 1.18		(Adler et al. 1999)[a]
n = 50						22.21 ± 7.4		(Li et al. 2008)[b]
n = 40				0		18		(Fuhrmann-Benzakein et al. 2000)[c]
n = 6					1764 ± 316	70.8 ± 27.4		(Bellone et al. 1999)[b]

Table 7.1. ***The reported concentrations of various cytokines and C-reactive protein of healthy individuals.*** All units are pg/mL except for TGF-β (ng/mL) and CRP (mg/L). [a] reported as mean ± SE, [b] reported as mean ± SD, [c] reported as median.

Many of the reported cytokine concentrations in the literature were determined using commercially available ELISA assays that measure a single analyte per assay. In contrast, a multiplex assay was used in this thesis to determine numerous cytokines simultaneously. This system has the advantage of being cost- and time-effective as well as minimising sample volume requirement. Numerous studies have demonstrated good correlations between the multiplex assay and its corresponding ELISA measurements; however, the absolute concentration of each cytokine appears to vary between different assays (DuPont et al. 2005; Elshal et al. 2006; Richens et al. 2010). Hence, for consistency purposes, it is important that only one system is used throughout all cytokine determinations within a single study. The determination of CRP is an internationally standardised robust assay used by clinical laboratories and the normal concentration was reported to be < 10 mg/L in healthy individuals (Kyle 2008). However, there may also be a cyclical variation in the concentration of CRP, which is a protein with a relatively short half life (Coventry et al. 2009). It is apparent that the ability to measure the inflammatory markers accurately and appropriately is important and during the *chronic* inflammation associated with cancer, dynamic changes in cytokines may be missed during the sampling period. There is a large body of evidence demonstrating that *acute* inflammation can decrease CYP activity, for example in trauma and sepsis (Shedlofsky et al. 1994; El-Kadi et al. 1997; Morgan 1997; Carcillo et al. 2003; Renton 2004; Aitken et al. 2006; Morgan et al. 2008). Hence, it cannot be discounted that chronic inflammation may also result in down-regulation of CYP2C19 activity in cancer patients. A suitable increase in sample size in future studies may elucidate any possible associations with the inflammatory cytokines and CYP2C19 activity.

The mechanism(s) by which acute inflammation may influence hepatic drug metabolism have been postulated to involve the constitutively activated receptor (CAR). The promoter region of the *CYP2C19* gene contains a binding site for CAR (CAR response elements; CAR-RE), which can bind to CAR and pregnane X receptor (PXR) and this may up-regulate *CYP2C19* constitutively (Gerbal-Chaloin et al. 2001; Raucy et al. 2002; Chen et al. 2003; Urquhart et al. 2007). However, IL-6 has been shown to decrease CAR and PXR mRNA expression *in vitro* (Pascussi et al. 2000). Hence, any increase in IL-6 concentration during acute inflammation may down-regulate CAR and PXR and thus may decrease *CYP2C19* expression. The effects of chronic exposure of cytokines on CAR or CYP2C19 have not been studied. However, there is low or negligible *CYP2C19* expression in human primary hepatocyte or HepG2 cell cultures (Westerink et al. 2007;

Guo et al. 2011), hence it may be difficult to determine the effects of cytokines on CYP2C19 *in vitro*.

The expression of CYP is not solely due to transcriptional regulation as it is also influenced by post-translational events. Whilst transcription can be altered by gene mutations, epigenetic changes or alterations in transcription factors, translational changes can be influenced by factors which alter the stability or degradation of the gene product.

This thesis also attempted to determine whether the CYP2C19 enzyme could undergo post-translational changes that may influence the function of the protein. It has been reported previously that the inflammatory mediator nitric oxide can inhibit CYP activity (Wink et al. 1993) and also that proteasomal degradation of CYP proteins can occur (Aitken et al. 2008; Lee et al. 2008; Lee et al. 2009a). The studies reported in this thesis were the first to examine the effect of nitric oxide on CYP2C19. The preliminary results indicated that nitric oxide (released from nitric oxide donors) can transiently inhibit CYP2C19 activity *in vitro* (section 5.3.2.2). Longer exposure to nitric oxide resulted in significant loss of CYP2C19 activity, which was prevented by the proteasome inhibitor MG132 (section 5.3.3). This suggested that the observed decrease in CYP2C19 activity in the presence of nitric oxide could be the result of increased proteasomal degradation of the protein. However, further investigations are required to confirm this effect. Such studies should also directly measure the amount of nitric oxide released by the donors in the cell culture system as well as increasing the number of experimental replicates.

There was no correlation between the total plasma nitrate concentrations and CYP2C19 activity in patients in this study (section 5.3.4). However, there were difficulties in using circulating plasma nitrate concentrations as a marker of exposure of the cellular CYP protein to nitric oxide, since intracellular nitric oxide concentration may not be accurately reflected in the circulation (Bryan et al. 2005). In addition, plasma nitrates appear to be highly variable in many other study populations (Moshage et al. 1995; Bakan et al. 2002; Fiorenza et al. 2005; Novotny et al. 2007) and this biological variability may preclude associations in small cohorts. However, a post-translational decrease in CYP2C19 activity due to nitrosative damage during inflammation is a hypothesis that requires further investigation.

The measurement of CYP2C19 activity requires the determination of the plasma concentrations of a probe drug and metabolite, thus it is important that this drug to metabolite ratio is robust and not influenced by any changes in drug-plasma protein binding. A false negative phenotype test could appear as genotype-phenotype discordance. One of the consequences of an acute phase response due to inflammation was the altered synthesis of hepatic proteins such as albumin and $alpha_1$ acid glycoprotein (Moshage et al. 1987; Gabay et al. 1999). Both of these proteins are important in the plasma protein binding of drugs (Goldstein 1949; Meyer et al. 1968; Grandison et al. 2000). The studies undertaken demonstrated the robustness of the plasma drug/metabolite ratio of omeprazole and proguanil as a measure of metabolic (hepatic) clearance of drug by CYP2C19. For example, the individualised hepatic clearance of both probe drugs based on each patients' free drug fraction in the plasma was the same in both concordant and discordant patients (section 6.2.3). Hence changes in the concentration of proteins such as albumin and $alpha_1$ acid glycoprotein in the patients were unlikely to influence their free plasma drug concentrations to the extent that an apparent change in metabolic clearance would occur. Thus the drug to metabolite ratio for both omeprazole and proguanil is a robust measure of intrinsic (hepatic) clearance independent of any drug-protein binding changes. Interestingly, one outcome of this data analysis was the observation of a significant inverse correlation between total plasma protein concentration and metabolite formation. Plasma contains at least 289 proteins (Anderson et al. 2002). In addition to the complexity of the mixture, there is also a large dynamic range in protein concentrations, with high abundance proteins such as albumin and low abundance inflammatory mediators such as cytokines. Changes in plasma proteins associated with inflammation are likely to vary extensively over time, such changes in individual proteins may not be easy to detect in "snapshot" patient samples. However, a change in the balance of inflammatory biomarkers, including changes in albumin, may be associated with total plasma protein concentration. Future studies to investigate the potential of total plasma protein as a correlate of CYP2C19 activity are required.

Studies using various techniques to examine the human plasma proteome have been reported recently. Such techniques include ^{1}H NMR, protein microarrays, LC-MS/MS and Q-TOF (Saha et al. 2009; Thongboonkerd et al. 2009; Backshall et al. 2011). The proteomic result allows the identification of the plasma proteins that have been altered in diseases in order to understand its complex pathophysiology. It was observed that the plasma levels of twenty-seven proteins were altered during the early phase of sepsis in a

porcine model using differential proteomics analysis (Thongboonkerd et al. 2009). In addition, the intrinsic changes in plasma proteome specific to different subtypes of breast cancer have been reported (Nakshatri et al. 2009). More recently, metabolic profiling (metabonomics) of patients with inoperable colorectal cancer enabled the prediction of severity of toxicity to capecitabine (Backshall et al. 2011). Hence the use of pharmacometabonomics may further elucidate the role of various plasma proteins including inflammatory cytokines in the downregulation of CYP2C19 activity in cancer patients.

In conclusion, although a genotyping approach can identify patients who are true CYP2C19 poor metabolisers, a discordance between CYP2C19 genotype and phenotype has also been demonstrated in a cancer population. There is preliminary evidence presented in this thesis that inflammation may play a role in this compromised metabolic activity. This finding indicates that compromised CYP2C19 activity may not only occur in a cancer population but also in other diseases which are associated with a high degree of inflammation. Hence, the same drug at the same dose may have profound variability in clinical effectiveness or toxicity in different patients at a given time. Therefore, it is important that a phenotyping approach is also adopted in order to correctly identify CYP2C19 poor metabolisers prior to treatment with drugs metabolised by CYP2C19.

Future studies that have arisen from this thesis include:

- To focus on patients with tumour types that are treated with CYP2C19 substrates, such as breast cancer and multiple myeloma which are treated with the CYP2C19 substrates cyclophosphamide and bortezomib respectively. None of the chemotherapy regimens for treatment of colorectal cancer include known CYP2C19 substrates. However, it would be intriguing to determine whether other hepatic drug metabolising enzymes important in the metabolism of 5-fluorouracil are also dysregulated in some cancer patients.

- To investigate whether there is increased toxicity for drugs with a narrow therapeutic index in those individuals who are CYP2C19 poor metabolisers. In addition, studies to determine whether the poor metaboliser phenotype influences lack of efficacy for prodrugs such as cyclophosphamide. However, these studies will require large cohorts of patients to detect any associations due to confounding factors that may include somatic mutations associated with tumour resistance and the complex interplay of chemotherapy regimens.

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