THE STEREOSELECTIVE PHARMACOKINETICS OF THE ENANTIOMERS OF PERHEXILINE IN POOR AND EXTENSIVE METABOLISERS OF THE CYTOCHROME P450 2D6

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ABSTRACT

Perhexiline maleate was first introduced for the prophylaxis of exertional angina in the 1970s but reports of adverse reactions, including potentially fatal hepatotoxicity, increasingly restricted its application. By 1988 Australia and New Zealand were the only countries permitting its use, limited to the treatment of refractory angina pectoris conditional upon the therapeutic monitoring of patients, due to recognition of the concentration dependent nature of its efficacy and toxicity. An understanding of the extreme interindividual variability in the pharmacokinetics of perhexiline due to metabolism by the polymorphic Cytochrome P450 2D6 (CYP2D6) has prompted a recent resurgence of its use in Australasia and Europe.

Perhexiline is a chiral molecule and is administered as a racemic mixture. Prior to the publication of the papers that are the topic of this thesis the characterisation of the clinical pharmacology of the enantiomers of perhexiline had been limited to one pharmacokinetic study that suggested that the (+) enantiomer of perhexiline may display a smaller polymorphic effect in its metabolism than its optical antipode. The four publications that comprise this thesis describe a comprehensive investigation of the pharmacokinetics and metabolism of the enantiomers of perhexiline in extensive and poor metabolisers (EM and PM, respectively) of CYP2D6 in both an *in vitro* model and clinically. The aim was to determine if the CYP2D6 polymorphism affects the metabolism of (+)-perhexiline significantly less than (-)-perhexiline, such that the inherent variability observed in the pharmacokinetics of the racemic preparation used clinically might be overcome by administration of only (+)-perhexiline.

Although both the *in vitro* and *in vivo* studies determined that the involvement of CYP2D6 was proportionately greater in the total clearance of (-)- than (+)-perhexiline, the empirical data also demonstrated that the role of CYP2D6 in the metabolism of (+)-perhexiline was simply too pre-eminent for a chiral preparation of this enantiomer to significantly reduce the difference in clearance observed between EM and PM. An unexpected finding was that the enantioselectivity observed in the clinical pharmacokinetics of perhexiline in EM was, in fact, significantly greater in PM. Whilst the enantioselectivity in EM was attributable to metabolism by CYP2D6, the mechanism responsible for this in PM could not be determined, but was postulated to involve enantioselective biliary excretion. Because PM are effectively exposed to greater concentrations of (+)-perhexiline and lower concentrations of (-)-perhexiline, when the relative pharmacodynamic activities of the individual enantiomers have been established therapeutic drug monitoring may be improved by the development of specific enantiomer target concentration ranges in plasma. What is certain is that perhexiline will remain an essential option in the armamentarium for the treatment of refractory angina pectoris and therapeutic drug monitoring will remain obligatory due to the inter- and intra-subject pharmacokinetic variability attributable to the respective polymorphic and saturable metabolism of both enantiomers by CYP2D6.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by any other person, except where due reference has been made in the text.

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Publication 1

Enantioselective assay for the determination of perhexiline enantiomers in human plasma by liquid chromatography.

Davies BJ, Herbert MK, Culbert JA, Pyke SM, Coller JK, Somogyi AA, Milne RW, Sallustio BC.

J Chromatogr B Analyt Technol Biomed Life Sci. 2006 Feb 17;832(1):114-20.

Publication 2

Determination of the 4-monohydroxy metabolites of perhexiline in human plasma, urine and liver microsomes by liquid chromatography.

Davies BJ, Herbert MK, Coller JK, Somogyi AA, Milne RW, Sallustio BC.

J Chromatogr B Analyt Technol Biomed Life Sci. 2006 Nov 7;843(2):302-9.

Publication 3

CYP2B6, CYP2D6, and CYP3A4 catalyze the primary oxidative metabolism of perhexiline enantiomers by human liver microsomes.

Davies BJ, Coller JK, Somogyi AA, Milne RW, Sallustio BC.

Drug Metab Dispos. 2007 Jan;35(1):128-38.

Publication 4

Steady-state pharmacokinetics of the enantiomers of perhexiline in CYP2D6 poor and extensive metabolizers administered *Rac*-perhexiline.

Davies BJ, Herbert MK, Coller JK, Somogyi AA, Milne RW, Sallustio BC.

Br J Clin Pharmacol. 2008 Mar;65(3):347-54.

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INTRODUCTION

1. Clinical Use of Racemic Perhexiline Maleate

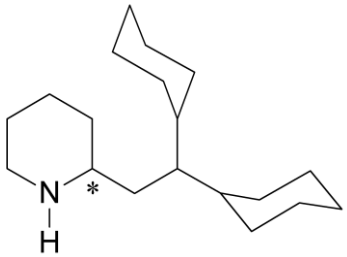

Figure 1

Chemical structure of perhexiline. The chiral carbon is indicated with an asterisk.

Perhexiline (2-(2,2-dicyclohexylethyl)piperidine) consists of a $CHCH_2$ backbone with one piperidine and two cyclohexyl moieties. It is a chiral molecule due to asymmetry of the second carbon of the piperidine ring (Fig. 1). Racemic perhexiline maleate was developed by Richardson-Merrell in the late 1960s (Ashrafian et al, 2007) and first introduced for routine clinical use as a monotherapy in the prophylaxis of exertional angina in the 1970s (Burns-Cox et al, 1971). The original dose recommendation was 400 mg per day, although reports of severe peripheral neuropathy, papilloedema and potentially fatal hepatotoxicity prompted the restriction of its use to specialists only and a reduction in the starting dose to 100 mg per day (Shah, 2006). Following continuing safety concerns, in April of 1985 the United Kingdom was the first country to withdraw perhexiline from its market (Bakke et al, 1995). The withdrawal was ultimately worldwide in 1988, except in Australia and New Zealand, where Pexsig® (Sigma Pharmaceuticals, Clayton, Victoria, Australia) remains licensed for the treatment of refractory angina conditional upon therapeutic monitoring of patients (Inglis and Stewart, 2006). Recognition of the concentration dependent nature of the efficacy and toxicity of perhexiline has prompted a resurgence of its use in Australasia (Gardiner and Begg, 2005) and Europe (Ashrafian et al, 2007), with prospective Cytochrome P450

2D6 (*CYP2D6*) genotyping (Barclay et al, 2003, Davies et al, 2006) and continued CYP2D6 phenotyping (Sallustio et al, 2002, Davies et al, 2006) being gradually adopted clinically for dosage individualisation. Although clinical use of perhexiline is increasing, little is known about the pharmacokinetics and pharmacodynamics of its individual enantiomers.

2. Coronary Artery Disease and Angina Pectoris

Since the mid nineteenth century the pattern of illnesses in the western world has changed from predominantly infectious to more culturally influenced diseases, such as coronary artery disease (DeJongste et al, 2004). Atherosclerotic plaque formation in a coronary artery gradually reduces the luminal diameter until it upsets the balance between myocardial oxygen consumption and coronary blood flow; myocardial ischaemia is the result (Kim et al, 2002). The chest discomfort characteristic of angina pectoris may begin when the narrowing is more advanced and is the initial clinical manifestation of ischaemic heart disease in approximately one half of patients (Marzilli et al, 2006).

2.1 Treatment of Coronary Artery Disease

Aspirin is recommended for all patients with coronary artery disease; in patients where aspirin is contraindicated, clopidogrel or ticlopidine may be employed as an alternative antiplatelet therapy (Kim et al, 2002). Platelets have been implicated in the disruption of atherosclerotic plaques, with subsequent thrombus formation possibly resulting in the development of myocardial infarction, unstable angina or both (Kim et al, 2002). Long term statin therapy to lower low density lipoprotein-cholesterol has been show to

decrease the recurrence of coronary events in patients with coronary artery disease (Evans et al, 2004).

2.2 Conventional Treatment of Angina Pectoris

Given the aetiology of angina pectoris, treatment strategies are focused on increasing coronary blood flow and decreasing myocardial oxygen demand. Conventional approaches include lifestyle modifications, haemodynamically acting pharmacological agents and surgical revascularisation techniques (Marzilli, 2004, Marzilli et al, 2006). The primary aim of lifestyle modification as a component of cardiac rehabilitation is the recovery of the capacity to perform the activities of daily living. This typically requires overweight reduction, elimination of tobacco use and initiation of an exercise program. Obesity increases myocardial oxygen demand and smoking lowers the angina threshold by reducing the oxygen carrying capacity of blood. The benefits of physical activity are many and include a reduction in cardiac mortality, a delay in the progression of atherosclerotic disease and improved myocardial perfusion (Mannheimer et al, 2002).

2.2.1 Haemodynamic Antianginal Agents

Beta-blockers are the first class of drug chosen for the treatment of angina pectoris (Kim et al, 2002) because they increase exercise tolerance and decrease heart rate and contractility during stress to reduce myocardial oxygen demand. The diastolic filling time available for coronary perfusion is also increased due to the slower heart rate (Kim et al, 2002). However, the use of beta-blockers may be limited by contraindications (chronic obstructive pulmonary disease, severe congestive heart failure, bradycardia and/or conduction disturbances) or adverse side effects (fatigue, depression, sleep disturbance and sexual dysfunction) (Jackson, 2001). Beta-blockers are frequently

combined with benzothiazepene or phenylalkylamine calcium channel blockers, titrated to the lowest heart rate and blood pressure level tolerated. They have negative inotropic and chronotropic effects that retard the increase in myocardial oxygen demand at submaximal loads to increase the anginal threshold (Yang et al, 2004). Dihydropyridine calcium channel blockers may also be prescribed. They produce negligible decreases in myocardial oxygen demand; rather, they increase myocardial oxygen supply by increasing coronary blood flow. Nitrates dilate vascular smooth muscles, particularly systemic veins and conductance arteries, to increase myocardial oxygen supply and reduce demand (Kim et al, 2002). Long acting nitrates are useful provided an interrupted dose schedule is applied to prevent nitrate tolerance (Yang et al, 2004). Short acting nitrates are available as sublingual formulations for both the immediate relief of exertional angina (Kim et al, 2002) and as a prophylactic before commencing strenuous activities (Mannheimer et al, 2002).

Although haemodynamically acting pharmacological agents are effective in controlling anginal symptoms, there is no conclusive evidence that they reduce morbidity and mortality. Furthermore, patients treated with combinations of these drugs often do not receive greater symptomatic or prognostic benefits, but are much more likely to experience adverse effects (Jackson, 2001).

2.2.2 Surgical Revascularisation

Patients with angina refractory to medical treatment are usually referred for surgical revascularisation to improve symptoms and prevent myocardial infarction and death. This includes coronary artery bypass grafting (CABG) or percutaneous coronary interventions (PCI) such as balloon angioplasty, stents and atherectomy techniques.

However, elderly patients frequently have comorbidities that exclude them from revascularisation procedures or have a history of previous revascularisation that excludes them as candidates for additional procedures (Conti, 2006).

Revascularisation is expected to improve anginal symptoms and prevent myocardial infarction and death, although a review of the data suggests that this may not be the case; only a minority of patients is free from angina and antianginal medications following revascularisation. Incomplete revascularisation, graft failure or disease progression in native coronary arteries may explain the persistence of angina after a revascularisation procedure, although the prevalence of angina after successful procedures suggests that mechanisms unrelated to epicardial coronary obstructions contribute to the pathogenesis of ischaemia (Gowda et al, 2005).

Although treatment guidelines advocate initial management of angina with intensive medical therapy and lifestyle intervention, the early use of PCI has become common. The Clinical Outcomes Utilising Revascularisation and Aggressive Drug Evaluation (COURAGE) study was a multi-centre, randomised trial designed to determine whether early intervention with this surgical procedure combined with optimised medical therapy was superior to medical therapy alone. Over 2000 patients were followed for a median of 4.6 years and in this time PCI demonstrated no incremental advantage with respect to the risk of myocardial infarction, other major cardiovascular events or death (Boden et al, 2007).

2.3 Refractory Angina Pectoris

Approximately 5 to 15% of patients with angina meet the criteria for refractory angina pectoris, characterised by coronary insufficiency in the presence of coronary artery disease which cannot be controlled by conventional medical treatment, PCI or CABG, and where reversible myocardial ischaemia has been clinically established as the cause of symptoms (Mannheimer et al, 2002). Although effective in the treatment of stable angina, unstable angina and acute coronary syndromes (Willoughby et al, 2002), perhexiline is generally reserved for the treatment of refractory angina due to its narrow therapeutic index and complex and variable pharmacokinetics (Ashrafian et al, 2007).

3. Antianginal Mechanism of Action of Perhexiline

Most antianginal agents produce haemodynamic changes, such as a reduction in systemic vascular resistance, coronary vasodilatation or negative inotropism, to address the imbalance between myocardial oxygen supply and demand. Drugs that exert primarily a metabolic action with little effect on coronary haemodynamics, such as perhexiline, trimetazidine, ranolazine and etomoxir, have considerable potential as adjunctive therapy for angina (Lee et al, 2004).

The development of perhexiline as a metabolic antianginal agent began in the early 1960s when the related compound hexadylamine demonstrated increased cardiac oxygen efficiency in isolated perfused heart preparations (Rowe et al, 1963). Subsequent study of perhexiline in a canine right heart perfusion model found that intravenous administration produced an increase in the ratio of the change in cardiac output to the change in oxygen consumption. Specifically, although perhexiline caused a reduction in left ventricular work, measured as the product of cardiac output and aortic

pressure, largely as a result of a fall in systemic blood pressure, it also produced a fall in myocardial oxygen consumption that was proportionately greater than the reduction in left ventricular work (Cho et al, 1970).

Similarly, in 21 patients with angiographically proven coronary artery disease, treatment with 200 mg of perhexiline twice a day for at least 10 days produced an increase in cardiac output despite a lower left ventricular filling pressure during atrial pacing. Consistent with the animal models that preceded it, perhexiline increased lactate extraction without increasing oxygen extraction (Pepine et al, 1974) and was evidence of increased cardiac efficiency due to enhanced myocardial carbohydrate utilisation.

Jeffrey et al (1995) measured direct substrate utilisation by isolated, working rat hearts perfused with substrates at their physiological concentrations and demonstrated that perhexiline, at a concentration of 0.6 mg/L, produced a significant decrease in fatty acid oxidation matched by a significant increase in lactate utilisation and a significant increase in cardiac output for an insignificant increase in oxygen consumption. The authors suggested that because endogenous substrate utilisation had fallen with exposure to perhexiline, this endogenous pool probably came from a fatty acid source, namely triglycerides, rather than a carbohydrate source, and that the change in substrate utilisation was probably attributable to inhibition of fatty acid metabolism rather than stimulation of lactate utilisation.

The molecular mechanism that accounted for the metabolic action of perhexiline was the discovery that it was a potent *in vitro* inhibitor of carnitine palmitoyltransferase-1 (CPT-1), a key regulator of long-chain fatty acid metabolism (Kennedy et al, 1996).

Fatty acids are more reduced molecules than carbohydrates so they generate more adenosine triphosphate (ATP) per weight of substrate, although the relatively higher reduced flavin adenine dinucleotide to reduced nicotinamide adenine dinucleotide ratio compared with the oxidation of glucose or lactate translates into a 10-15% lower yield of ATP via the electron transport chain for a given oxygen consumption (Liedtke, 1981).

Mammalian tissues have evolved to utilise fatty acids as their preferred fuel during stress, when the response to an acute challenge assumes an unlimited oxygen supply to support as much biomechanical work as possible (Wolff et al, 2002). Catecholamines increase circulating fatty acid concentrations by stimulation of hormone-sensitive lipase during stress (Khoo and Steinberg, 1975). Increasing levels of circulating fatty acids increase the rate of myocardial fatty acid influx (Vyska et al, 1991) and inhibit the myocardial uptake of carbohydrates (Boden et al, 1994) and the conversion of pyruvate to acetyl-CoA (Lopaschuk et al, 1993). However, the reduction of oxygen delivery to the heart is itself the primary stress during angina pectoris and by inhibiting CPT-1, perhexiline facilitates a shift in myocardial metabolism towards glucose utilisation, preserving ATP synthesis when oxygen delivery is limited.

Classical kinetic studies of mitochondrial fractions revealed that cardiac preparations were significantly more sensitive to inhibition of CPT-1 by perhexiline than hepatic preparations, although the increasing level of inhibition at higher perhexiline concentrations could not be described by simple Michaelis-Menten kinetics (Kennedy et al, 1996). The authors suggested that the curvilinear Dixon plots (Fig. 2) might have been due to positive cooperativity in binding of perhexiline to the inhibitory site of the

enzyme or to its binding to multiple inhibitory sites. Only the liver isoform of CPT-1 is expressed in the liver (Weis et al, 1994), so although the atypical kinetics of the inhibition of the cardiac mitochondrial fractions may have been due to the presence of both the muscle and liver isoforms of CPT-1, this possibility may be discounted because similar atypical kinetics were observed in the liver. Another possibility that was not considered is that the observed kinetics may have reflected different inhibition affinities of the (+) and (-) enantiomers for the isoforms of CPT-1. Thus it is also possible that the perhexiline enantiomers may exhibit differential selectivity for cardiac or hepatic tissue.

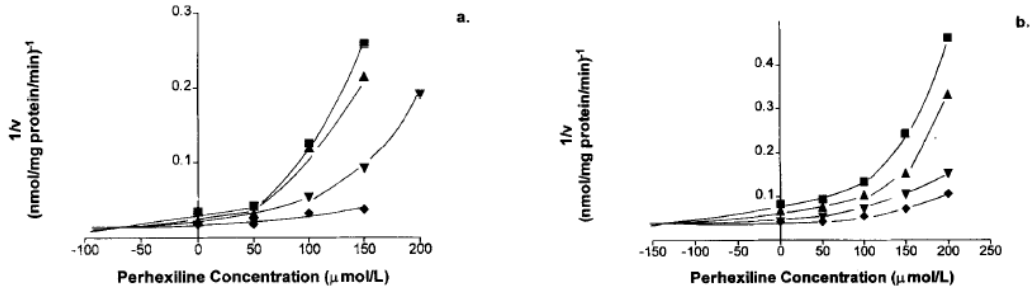

Figure 2

Dixon plot of inhibition of mitochondrial CPT-1 in (a) cardiac and (b) hepatic preparations by perhexiline at various palmitoyl-CoA concentrations. The enzyme preparations were preincubated with perhexiline or vehicle for 15 minutes then incubated with 400 μmol/L carnitine and palmitoyl-CoA at 25 (squares), 50 (triangles), 100 (inverted triangles), and 200 (diamonds) μmol/L. Example of one of three experiments. K_i values: (a) 86 (74-99), and (b) 146 (127-169) μmol/L, N = 3.

Reproduced from Kennedy et al, Biochemical Pharmacology, 1996.

More recent work from the laboratory of Kennedy et al (1996) by Unger et al (2005) suggested that some of the increased myocardial efficiency produced by perhexiline may be independent of the inhibition of CPT-1. In non-ischaemic working rat hearts

acute exposure to perhexiline had no effect; in contrast, 24 hours pretreatment with perhexiline significantly increased cardiac work and efficiency without significant effects on palmitate oxidation, although no explanation for this observation was suggested by the results obtained. Whether this effect also occurs in ischaemic working rat hearts remains to be investigated.

The microvesicular steatosis observed in rat hepatocytes cultured in a medium containing perhexiline is consistent with decreased mitochondrial oxidation of fatty acids by inhibition of the liver isoform of CPT-1. Activated long-chain fatty acids are instead esterified into triglycerides that subsequently accumulate as lipid droplets (Deschamps et al, 1994). Similarly, in two patients who died from cirrhosis after being prescribed perhexiline for at least two years, lysosomal phospholipidosis and pseudoalcoholic liver lesions were described (Pessayre et al, 1979). It has been postulated that cationic amphiphilic drugs like perhexiline bind to phospholipids to create a complex that is resistant to degradation by phospholipases (Reasor et al, 2006). The pathogenesis of phospholipidosis is due to the imbalance of phospholipid turnover allowing its intracellular accumulation and the subsequent appearance of membranous lamellar inclusions known as lamellar bodies (Hirode et al, 2008). Perhexiline associated hepatic injury is the most severe adverse reaction related to its use (Poupon et al, 1980).

4. Antianginal Efficacy of Perhexiline

Clear evidence of the clinical efficacy of perhexiline as an antianginal agent came from a double-blind, randomised, placebo-controlled, crossover multi-centre trial conducted in the United Kingdom and Ireland (Burns-Cox et al, 1971). Perhexiline produced a

highly significant reduction in anginal attacks and glyceryl trinitrate consumption during a period of four or eight weeks of active treatment in 55 patients withdrawn from other long-acting antianginal agents. The study was continued by Pilcher et al (1973), who followed 46 of the patients for approximately one year. Similar efficacy was maintained, although elevated serum aspartate aminotransferase, an hepatic enzyme and a marker of toxicity, was common on the 400mg daily dose and reversible upon dose reduction. The authors recommended a starting dose of 200mg daily since most patients found it as effective as the higher dose in reducing anginal frequency. Importantly, perhexiline proved to be efficacious in cases where anginal symptoms had proved resistant to therapy with beta-blockers.

White and Lowe (1983) investigated the antianginal efficacy of perhexiline in patients with angina refractory to maximal beta-blocker therapy, except that all medications on entry to the trial were continued unchanged. The double-blind, placebo-controlled crossover study found significant incremental reductions in anginal frequency with parallel reductions in glyceryl trinitrate consumption and increases in mean exercise duration, from an initial dose of 100mg of perhexiline per day up to the maximally tolerated dose or 400mg per day, whichever was lower. While incremental therapeutic effects were evident up to the maximum dosages, a significant benefit was evident after 100mg of perhexiline per day for two weeks. Five of the 20 patients became asymptomatic, although eight patients developed adverse effects requiring a reduction in perhexiline dose. No adverse effects were manifested on the lowest dose of perhexiline. Neurotoxicity was evident in one patient as paresthesiae and hepatotoxicity was evident in another as elevated serum transaminase. Both patients were receiving 400mg of perhexiline per day and liver function returned to normal within a week of

ceasing perhexiline. Importantly, this study demonstrated the efficacy of perhexiline for the treatment of angina in combination with beta-blockers without producing any additive adverse haemodynamic effects.

Because Singlas et al (1978) had demonstrated that elevated plasma perhexiline concentrations were found to be associated with severe toxicity, Pilcher et al (1985) performed a study to determine whether toxicity could be avoided in the long term by regular monitoring of plasma perhexiline concentrations. Forty-one patients continued to receive drug treatment established before the trial in conjunction with perhexiline therapy for up to 70 months. An improved therapeutic response was positively related to plasma perhexiline concentration and severe side effects did not occur at levels less than 1.50mg/L. Because the wide variation of plasma perhexiline concentrations obtained in patients receiving the same dose suggested that substantial interindividual differences in perhexiline metabolism existed, the authors proposed that patient dosage should be adjusted to achieve a therapeutic range of between 1.00 and 1.50mg/L.

Horowitz et al (1986) examined correlations between perhexiline dose, plasma drug concentrations, efficacy and development of toxicity in patients either unresponsive or unsuitable for other forms of treatment, to which perhexiline was added to previously prescribed anti-anginal medication. In patients treated chronically with 50-400mg/day, dosage increases were made to minimise anginal symptoms, whilst reductions in dosage were made if patients became asymptomatic. Of 19 patients, five became asymptomatic and a further nine experienced a reduction in frequency of anginal attacks, although nine patients developed evidence of hepatotoxicity or neurotoxicity, with concomitant plasma perhexiline concentrations of 0.72-2.68mg/L. By contrast, a second group of 22

similar patients were treated chronically with dosage adjusted to maintain plasma perhexiline concentrations below 0.60mg/L. Nine patients became asymptomatic and the remaining 13 all experienced a reduction in frequency of anginal attacks. None developed adverse effects, demonstrating that long-term toxicity could be markedly reduced without significantly compromising antianginal efficacy, as long as the lower limit of plasma perhexiline concentrations was maintained above 0.15mg/L.

Cole et al (1990) tested the plasma perhexiline concentration range of 0.15 to 0.60 mg/L against placebo in 17 patients with refractory angina who continued to receive maximal antianginal therapy during a randomised, double-blind, placebo-controlled crossover designed study conducted over six months. Patients given perhexiline achieved significantly greater increases in objective exercise testing criteria than patients given placebo. By blinded review of subjective measures, most patients noted an improvement while taking perhexiline, whereas no patient identified the placebo phase with improvement. Side effects attributable to perhexiline were minor and related to transient elevations of perhexiline concentrations above the target range.

It is now recognised that therapeutic drug monitoring of perhexiline concentrations in plasma is essential for guiding dosage due to the very large inter- and intra-individual variability in pharmacokinetics (Sallustio et al, 2002) and that maintaining concentrations within the range of 0.15 to 0.60 mg/L minimises the risk of clinically significant toxicity whilst still retaining clinical efficacy (Horowitz et al, 1986, Cole et al, 1990).

5. Pharmacokinetics of Perhexiline

The absolute bioavailability of perhexiline is unknown because it is only available as an oral formulation, although early studies by Richardson-Merrell (Wright et al, 1973) suggested that it was well absorbed from the gastrointestinal tract. Once in systemic circulation it is rapidly distributed to tissues and has a large volume of distribution (Leeson et al, 1969). Data collected by Hussein et al (2001) during routine therapeutic monitoring of perhexiline permitted the identification by pharmacokinetic modelling of two very different subpopulations within their sample population that exemplified the necessity of monitoring. The larger subpopulation, comprising 88% of subjects, had modal values for oral clearance (CL/*F*) and volume of distribution (V/*F*) of 21.8 L/h and 1470 L, respectively. In turn, the smaller subpopulation had modal values of 2.06 L/h and 260 L, respectively. The lower value of V/*F* for the second subpopulation suggested that this group had greater oral bioavailabilities as a consequence of reduced presystemic metabolism.

The major determinant of the oral clearance of perhexiline is hepatic metabolism, since the recovery of unchanged perhexiline in urine from patients maintained on therapeutic doses is generally less than 1% (Singlas et al, 1978). Hepatic oxidation of perhexiline hydroxylates the fourth carbon of one of the cyclo-hexyl moieties to form a single *cis*-OH-perhexiline metabolite (M1) or one of two *trans*-OH-perhexiline metabolites, *trans1*-OH-perhexiline (M3) or *trans2*-OH-perhexiline (Fig. 3) (Wright et al, 1973, Singlas et al, 1978, Beck et al, 2004). In urine the monohydroxy perhexiline metabolites account for 2-38% of a dose recovered at steady-state (Wright et al, 1973, Singlas et al, 1978). Secondary metabolism forms several 4,4'-dihydroxy metabolites that account for as little as 0.2% (Singlas et al, 1978) of a dose recovered in urine at steady-state to as

much as 37% (Wright et al, 1973). The concentration of dihydroxy metabolites in plasma is so low that to date they have only been detected in urine (Wright et al, 1973, Singlas et al, 1978).

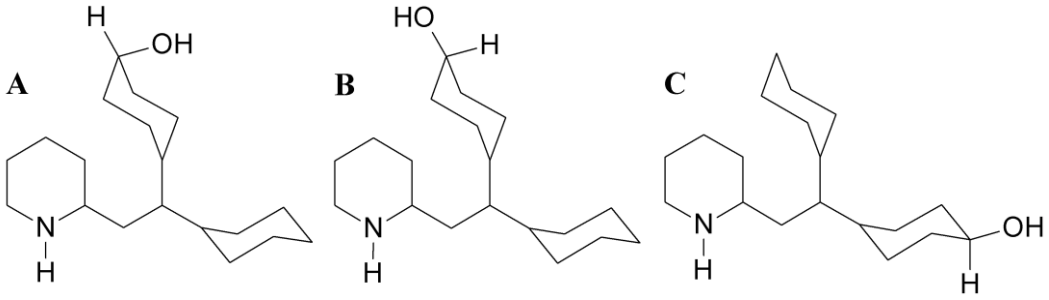

Figure 3

Chemical structures of the three isomers of 2-(2-cyclohexyl-2-(4-hydroxycyclohexyl)ethyl)-piperidine: *cis*-OH-perhexiline (A), *trans1*-OH-perhexiline (B) and *trans2*-OH-perhexiline (C).

5.1 Polymorphic Metabolism of Perhexiline

The primary 4-monohydroxy metabolites of perhexiline found in plasma are *cis*-OH-perhexiline and *trans1*-OH-perhexiline (Singlas et al, 1978, Amoah et al, 1984), except in poor metabolisers (PM) of CYP2D6, originally described by the debrisoquine 4-hydroxylation genetic polymorphism (Shah et al, 1982, Morgan et al, 1984). PM have a profoundly impaired capacity to form *cis*-OH-perhexiline (Cooper et al, 1984) due to the absence of CYP2D6 activity (Barclay et al, 2003, Sørensen et al, 2003) that is responsible for the bimodal distribution of oral clearance in patients maintained within the therapeutic range of perhexiline concentrations in plasma (Sallustio et al, 2002).

There are conflicting reports regarding the capacity of PM to form *trans1*-OH-perhexiline; either a reduced capacity in PM (Amoah et al, 1984, Cooper et al, 1986, Cooper et al, 1987) or no difference between PM and EM (Singlas et al, 1978, Cooper et al, 1984). No investigation of *trans2*-OH-perhexiline has been reported since its

initial characterisation (Wright et al, 1973), although clearance by formation of this metabolite and *trans1*-OH-perhexiline may account for the majority of perhexiline oral clearance in PM.

5.2 CYP2D6 Phenotype

CYP2D6 is a promiscuous drug metabolising enzyme, responsible for the metabolism of approximately 25% of commonly prescribed drugs, although interindividual genetic variability means its activity ranges from complete deficiency to excessive activity (Sistonen et al, 2007). Phenotyping of CYP2D6 activity is performed by determination of the urinary metabolic ratio of unchanged parent drug to CYP2D6-dependent metabolite and is a sensitive measure of the polymorphism due to its demonstrated relationship with the partial intrinsic clearance of the parent drug (Jackson et al, 1986). Using debrisoquine or sparteine as a probe drug for *in vivo* CYP2D6 phenotyping separates Caucasian populations into two groups, PM and extensive metabolisers (EM), clearly defined by an antimode (Zanger et al, 2004). A subgroup between the major EM mode and the PM mode of intermediate metabolisers (IM) is apparent in the frequency distributions of metabolic ratios and was mathematically defined by Bock et al (1994) and found to represent 10 to 15% of the Caucasian population. A fourth phenotypic group known as ultrarapid metabolisers (UM) is arbitrarily defined by metabolic ratios indicative of extremely high drug oxidation capacity. Although there is no distinct mode in the population distribution of metabolic ratios, there is some deviation from normality that distinguishes this clinically significant group (Zanger et al, 2004).

5.3 CYP2D6 Genotype

The human *CYP2D* locus was first sequenced by Kimura et al (1989). It is located on the long arm of chromosome 22 (q11.2-qter) and consists of the *CYP2D6* gene and 2 related genes, *CYP2D7* and *CYP2D8P*. Each contains 9 exons. *CYP2D7* has 97% homology with *CYP2D6* that extends into the upstream regulatory region. The insertion of a single thymine base into the first exon disrupts the reading frame such that any mRNA transcribed is incapable of translation into a functional P450. *CYP2D8P* has 92% homology with *CYP2D6* and is a pseudogene; it shares none of the upstream regulatory region and contains multiple deletions and insertions that produce a completely disrupted reading frame.

The incidence of over 50 gene duplication events within the *CYP2* family 400 million years ago coincided with the beginning of terrestrial animal and plant interaction, when plants evolved metabolites to make themselves unpalatable to animals, and animals, in turn, evolved new drug metabolising enzymes to detoxify them (Gonzalez and Nebert, 1990). Kimura et al (1989) speculated that because current human diets rely mostly on cultivation and selectively avoid toxic plants, without this evolutionary pressure many of the P450 genes, including *CYP2D6*, previously required for survival might be lost. The large number of insertions in the pseudogene suggested it had been inactive the longest, and gene conversions between *CYP2D6* and the pseudogene may hasten the introduction of inactivating mutations, gradually increasing the frequency of PM, although recent research suggests that the diversity observed at the *CYP2D6* locus reflects the same factors affecting variation at other autosomal markers (Sistonen et al, 2007).

PM possess two null *CYP2D6* alleles and hence no detectable CYP2D6 activity. They comprise approximately 5-10% of the Australian population (Peart et al, 1986, Sallustio et al, 2002). The most frequent null allele in Caucasians is *CYP2D6*4* and homozygous individuals account for approximately 80% of PM (Zanger et al, 2004). The IM phenotype does not simply reflect heterozygotes carrying one functional and one non-functional allele because Hardy-Weinberg equilibrium correctly predicts a heterozygote frequency of approximately 35 to 40% based on the observed PM frequency, yet the observed frequency of phenotypic IM is 10 to 15% (Bock et al, 1994). *CYP2D6*9*, **10* and **41* are associated with reduced activity and individuals carrying one of these alleles and a null allele account for the majority of IM. *CYP2D6*1* and **2* are the most common alleles and are both associated with normal activity. (Zanger et al, 2004). At the time of writing the nomenclature files for the *CYP2D6* alleles of the Human Cytochrome P450 Allele Nomenclature Committee may be viewed on the World Wide Web at: http://www.cypalleles.ki.se/cyp2d6.htm.

5.4 Clinical Significance of the CYP2D6 Polymorphism

Without any functional CYP2D6, PM are more likely to suffer excessive plasma perhexiline concentrations following a standard loading regimen (Davies et al, 2006) and require lower doses for long-term therapy (Sallustio et al, 2002, Barclay et al, 2003). However, the significant linear relationship between the metabolic ratio of the concentrations of *cis*-OH-perhexiline to perhexiline in plasma and oral clearance does not simply support the CYP2D6-dependent formation of *cis*-OH-perhexiline as the primary determinant of oral clearance; it permits use of this ratio as a tool to guide dosage individualisation. PM, defined as individuals with metabolic ratios less than 0.3 (Sallustio et al, 2002, Barclay et al, 2003), require maintenance doses of 10 to 25 mg

per day. The rest of the population, comprised of IM, EM and UM with corresponding metabolic ratios ranging from one to 30 and higher, require 100 to 500 mg of perhexiline per day to maintain its concentration in plasma within the therapeutic range (Sallustio et al, 2002).

Single dose pharmacokinetic studies performed by Horowitz et al (1981) with 150 and 300 mg oral doses of perhexiline in five patients revealed that the mean peak plasma concentration of perhexiline and the mean area under the plasma perhexiline concentration-time curves were 4.3 and 5.3 times greater, respectively, after the dose was doubled. Furthermore, the elimination half-life was concentration dependent and increased from 11.2±2.1 to 19.1±2.8 hours, indicating that the elimination pharmacokinetics of perhexiline were non-linear and metabolism was saturable in these patients, with resultant variation in the systemic availability of orally administered perhexiline. In three patients treated daily with perhexiline Wing et al (1982) reported that perhexiline oral clearance was concentration dependent within the range of plasma perhexiline concentrations achieved clinically. The apparent time taken to reach steady state increased from 3-4 weeks on a dose of 100 mg/day, to 4-6 weeks on 200 mg/day, and to 6-8 weeks on 250 or 300 mg/day.

CYP2D6-catalysed metabolism of perhexiline accounts for the non-linear pharmacokinetics observed in EM. Cooper et al (1985) noted the increased time required to attain steady state with increased dose and the different dose requirements of five EM patients to achieve given plasma perhexiline concentrations. All demonstrated changes in metabolism from first order to zero order kinetics, but the dose at which

saturation occurred varied considerably due to interindividual variation in the rate of *cis*-OH-perhexiline formation.

Saturability in perhexiline metabolism by EM was also reported by Sallustio et al (2002) and evidenced by the significant decrease in metabolic ratio from its initial measurement to steady-state. Notably, the ratio of PM remained effectively unchanged over the same interval, reflecting a lack of CYP2D6 metabolising capacity rather than saturated metabolism. Sørensen et al (2003) examined the *in vitro* enzyme kinetics of perhexiline 4-monohydroxylation using human liver microsomes and described a high affinity reaction catalysed by CYP2D6 and a low affinity reaction catalysed by an undetermined CYP isoform, consistent with the non-linear and linear pharmacokinetics observed *in vivo* in EM and PM patients, respectively.

6. Chirality

In 1848 Louis Pasteur made the discovery that tartaric acid existed in two forms with identical physico-chemical properties that differed only in their ability to rotate plane-polarised light whilst in solution. It was not until 1874 that van't Hoff and La Bell independently put forward the explanation that this optical activity was due to two different and non-superimposable tetrahedral arrangements of four different substituents attached to a central chiral carbon atom (Brocks and Jamali, 1995). Due to the different orientation of the substituents in a molecule containing one chiral carbon atom there are two possible stereoisomers known as enantiomers. Each may interact with their surrounding environment in stereospecific ways with subsequent stereoselective properties in biological systems (Burke and Kratochvil, 2002). Of a racemic compound, one enantiomer may influence desirable pharmacological properties, pharmacokinetic,

pharmacodynamic or both, while the other often exhibits a very different physiological role (Izake, 2007).

6.1 Pharmacokinetics of the Enantiomers of Perhexiline

At the time of writing the characterisation of the pharmacology of the enantiomers of perhexiline has been limited to two pharmacokinetic studies that are not the subject of this thesis (the findings of Inglis et al, 2007, of which the author of this thesis is a co-author, are included in the discussion) and an absence of any investigation of their respective pharmacodynamics altogether. In a 1986 report Gould et al administered single oral doses of 300 mg of each of the individual enantiomers of perhexiline to eight subjects. Although not stated, these subjects were EM due to their capacity to form CYP2D6-dependent monohydroxy metabolites. The oral clearance of the perhexiline enantiomer and the AUC of the corresponding *cis*-4-monohydroxy metabolite was 2.5- and 28-fold greater, respectively, following administration of (-)- than (+)-perhexiline. Whereas (+)-perhexiline was metabolised to the *cis*- and *trans1*-OH-(+)-perhexiline metabolites at similar rates, (-)-perhexiline displayed distinct stereoselective metabolism to its *cis*-4-monohydroxy metabolite. Thus, the authors suggested that the (+) enantiomer of perhexiline may display a smaller polymorphic effect in its metabolism than its optical antipode, although there are no reports in the literature describing the metabolism of perhexiline enantiomers by PM.

6.2 The 4-monohydroxy Metabolites of the Enantiomers of Perhexiline

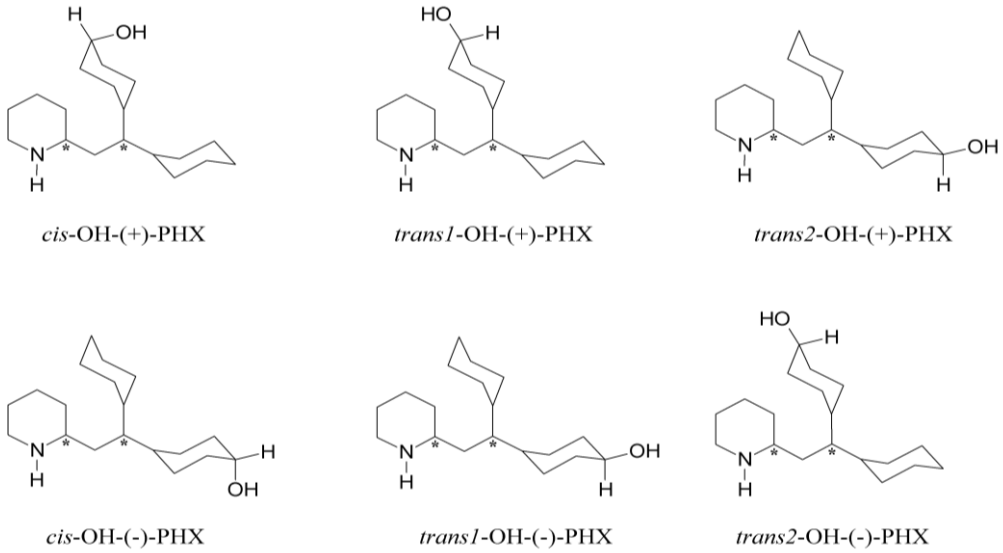

Figure 4

The chemical structures of the six possible 4-monohydroxy metabolites of the enantiomers of perhexiline (PHX). Chiral centres are indicated with an asterisk.

Because perhexiline is formulated as a racemic mixture of (+) and (-) enantiomers, hepatic 4-monohydroxylation introduces a second chiral centre into the molecule, making six conformations possible, composed of one pair each of *cis*-, *trans1*- and *trans2*-4-monohydroxy enantiomers (Fig. 4). To date, a chiral analytical method capable of resolving these metabolites has not been reported and the method for resolving their parents, the enantiomers of perhexiline, is one of the subjects of this thesis.

7. Aims and Hypotheses

Metabolism by CYP2D6 is clearly the primary contributor to the complex and highly variable clinical pharmacokinetics of racemic perhexiline and limits its therapeutic application. The following papers describe a comprehensive investigation of the pharmacokinetics of the enantiomers of perhexiline in EM and PM, in both an *in vitro* model and clinically, with the aim of determining if the CYP2D6 polymorphism affects

the metabolism of (+)-perhexiline significantly less than (-)-perhexiline. If this is the case it may be possible to overcome the inherent pharmacokinetic variability observed in the metabolism of the racemic perhexiline used clinically by administration of only the (+) enantiomer.

The overall aim was addressed by developing two HPLC assays utilising pre-column formation of fluorescent derivatives that facilitated both *in vivo* and *in vitro* pharmacokinetic studies: a chiral assay for perhexiline and an achiral assay for its 4-monohydroxy metabolites. Although not capable of resolving the six conformations of 4-monohydroxy perhexiline, the achiral assay characterised the metabolism of each of the pure enantiomers and their specific metabolites using human liver microsomal preparations from genotypic EM, IM and PM. These findings were then related to the stereoselective pharmacokinetics measured *in vivo* by the chiral assay in EM, IM and PM patients maintained at steady-state with racemic perhexiline.

The following specific hypotheses were tested:

1. CYP2D6-catalysed metabolism by EM liver microsomal preparations is enantioselective for (-)-perhexiline.

2. CYP2D6-catalysed metabolism by EM liver microsomal preparations accounts for a lower proportion of the total metabolism of (+)-perhexiline than (-)-perhexiline.

3. Metabolism of perhexiline by PM liver microsomal preparations is not enantioselective.

4. Systemic exposure in EM patients administered racemic perhexiline is greater for (+)-than (-)-perhexiline.

5. Systemic exposure in PM patients administered racemic perhexiline is similar for (+)- and (-)-perhexiline.

PUBLICATION 1

Enantioselective assay for the determination of perhexiline enantiomers in human plasma by liquid chromatography.

Davies BJ, Herbert MK, Culbert JA, Pyke SM, Coller JK, Somogyi AA, Milne RW, Sallustio BC.

J Chromatogr B Analyt Technol Biomed Life Sci. 2006 Feb 17;832(1):114-20.

Because no HPLC assay had been reported in the literature, a chiral assay capable of resolving the enantiomers of perhexiline in human plasma was developed to permit investigation of the *in vivo* pharmacokinetics of the individual enantiomers in patients maintained with the racemic preparation used clinically. Attempts to use enantioselective stationary phases were unsuccessful so chiral derivatising agents were trialled since pre-column derivitisation of perhexiline is obligatory to enhance its detection by conventional HPLC due to its poor UV absorbance and fluorescence. Reaction of racemic perhexiline with a solution of acetone containing 0.05% (*R*)-(-)-1-(1-napthyl)ethyl isocyanate at ambient temperature rapidly formed highly fluorogenic diastereomeric napthyl ethyl ureas that were resolved on a C18 stationary phase using a gradient of methanol and water, producing two peaks of equal areas. No racemisation of the individual enantiomers occurred when assayed separately.

A clinical study of one patient phenotyped as an EM demonstrated the application of the validated method and revealed that systemic exposure to (+)-perhexiline was indeed greater than to (-)-perhexiline in this EM, providing further impetus to study the effect in a larger sample size and to include PM (publication 4).

This publication also detailed the preparation of the hydrochloride salts of each of the enantiomers of perhexiline that were subsequently used in human liver microsomal incubations (publication 3). The (+)-perhexiline-(-)-1,1'-binapthyl-2,2'-diylhydrogenphosphate (BNPA) and (-)-perhexiline-(+)-BNPA salts, intermediaries in the preparation of the (+)- and (-)-perhexiline hydrochloride salts, were characterised by NMR spectroscopy and their enantiomeric relationship was established from the observation of their equal yet opposite optical rotation. The (+)- and (-)-perhexiline hydrochloride salts were characterised by NMR spectroscopy and mass spectrometry and their enantiomeric relationship again established by their respective optical rotations.

Davies, B.J., Herbert, M.K., Culbert, J.A., Pyke, S.M., Coller, J.K., Somogyi, A.A., Milne, R.W. and Sallustio, B.C. (2006) Enantioselective assay for the determination of perhexiline enantiomers in human plasma by liquid chromatography. *Journal of Chromatography v.832 (1), pp. 114-120, February 2006*

PUBLICATION 2

Determination of the 4-monohydroxy metabolites of perhexiline in human plasma, urine and liver microsomes by liquid chromatography.

Davies BJ, Herbert MK, Coller JK, Somogyi AA, Milne RW, Sallustio BC.

J Chromatogr B Analyt Technol Biomed Life Sci. 2006 Nov 7;843(2):302-9.

Because the six 4-monohydroxy metabolites of perhexiline could not be chromatographically resolved following reaction with a chiral derivatising agent, an achiral HPLC-fluorescent method was developed that adequately resolved the three pairs of 4-monohydroxy perhexiline enantiomers utilising pre-column derivatisation with dansyl chloride, a C18 stationary phase and a mobile phase composed of a gradient of methanol and water. Sufficient sensitivity was obtained to permit their quantification in the plasma and urine of EM and PM patients maintained with racemic perhexiline and in a human liver microsomal preparation from a genotypic EM incubated with racemic perhexiline.

This publication was the first to report the renal clearance of the 4-monohydroxy perhexiline enantiomeric pairs and the proportion recovered as glucuronidated conjugates. Importantly, this validated method demonstrated its potential utility for application to the *in vitro* model (publication 3), to characterise the kinetics of the 4-monohydroxylation reactions of the individual enantiomers of perhexiline in human liver microsomal incubations and identify the specific CYP isoform profile of these individual reactions.

Davies, B.J., Herbert, M.K., Coller, J.K., Somogyi, A.A., Milne, R.W. and Sallustio, B.C. (2006) Determination of the 4-monohydroxy metabolites of perhexiline in human plasma, urine and liver microsomes by liquid chromatography.
Journal of Chromatography v.843 (2), pp. 302-306, November 2006

PUBLICATION 3

CYP2B6, CYP2D6, and CYP3A4 catalyze the primary oxidative metabolism of perhexiline enantiomers by human liver microsomes.

Davies BJ, Coller JK, Somogyi AA, Milne RW, Sallustio BC.

Drug Metab Dispos. 2007 Jan;35(1):128-38.

Prior to this publication the *in vitro* enzyme kinetics of racemic perhexiline 4-monohydroxylation in human liver microsomes had been reported by Sørensen et al (2003). They simply described a high affinity reaction catalysed by CYP2D6 in EM and a low affinity reaction in PM, although the CYP isoforms responsible for the low affinity reaction were not identified.

By incubating *CYP2D6* genotyped human liver microsomal preparations from EM, IM and PM with each of the individual enantiomers of perhexiline and measuring the production of the resolved *cis*-, *trans1*- and *trans2*-OH-perhexiline metabolites specific to each, it was possible to characterise the kinetics of the individual reactions and calculate the total intrinsic clearance of each enantiomer by each genotype. In addition to P450 isoform-specific inhibitors, monoclonal antibodies directed against P450 isoforms and recombinantly expressed human P450 enzymes were used to completely define the P450 isoform profile of the 4-monohydroxylations. Calculation of the unbound fraction of each perhexiline enantiomer in the microsomal incubations prevented erroneous sigmoidal transformations of the reaction curves and permitted comparison with the unbound fraction in patient plasma, relating the affinities of the *in vitro* reactions to the respective linear and non-linear oral pharmacokinetics observed in patients (publication 4).

Davies, B.J., Coller, J.K., Somogy*i*, A.A., Milne, R.W. and Sallustio, B.C. (2007) CYP2B6, CYP2D6, and CYP3A4 Catalyze the Primary Oxidative Metabolism of Perhexiline Enantiomers by Human Liver Microsomes.
Drug Metabolism and Disposition v.35 (1), pp. 128-138, January 2007

PUBLICATION 4

Steady-state pharmacokinetics of the enantiomers of perhexiline in CYP2D6 poor and extensive metabolizers administered *rac*-perhexiline.

Davies BJ, Herbert MK, Coller JK, Somogyi AA, Milne RW, Sallustio BC.

Br J Clin Pharmacol. 2008 Mar;65(3):347-54.

Whilst the third publication suggested that enantioselective pharmacokinetics should only be observed in EM, the *in vivo* pharmacokinetic study described in the fourth publication reported that the significant enantioselective pharmacokinetics observed in EM also occurred in PM, with greater apparent oral clearances of (-)- than (+)-perhexiline. This was in agreement with Inglis et al (2007), although the apparent oral clearances of patients maintained at steady-state with *rac*-perhexiline were calculated from their respective AUC over an inter-dosing interval, rather than from the trough concentrations of each enantiomer in plasma. Furthermore, analysis of the steady-state plasma concentration-time profiles of the enantiomers of perhexiline was undertaken in order to attempt to elucidate the mechanisms underlying the observed enantioselectivity, particularly with respect to PM.

The concentration in urine of each enantiomer of perhexiline was determined for the five EM for whom a complete urine collection was obtained over their inter-dosing intervals by validating the method employed for plasma (publication 1) with urine. From this data the renal clearances of each enantiomer were calculated for the EM and subsequently assumed for the PM, for whom the inter-dosing interval was too large to make the complete recovery of urine practical.

Finally, this publication summarises the pharmacokinetics of the enantiomers of perhexiline and proposes a mechanism that explains the greater enantioselectivity observed in PM than EM and suggests how this may be further investigated.

Davies, B.J., Herbert, M.K., Coller, J.K., Somogy*i*, A.A., Milne, R.W. and Sallustio, B.C. (2007) Steady-state pharmacokinetics of the enantiomers of perhexiline in CYP2D6 poor and extensive metabolizers administered *Rac*-perhexiline. *British Journal of Clinical Pharmacology, v.65 (3), pp. 347-354, March 2008*

DISCUSSION

The main aim of the four publications contained within this thesis was to determine whether the CYP2D6 polymorphism affects the pharmacokinetics of (+)-perhexiline significantly less than (-)-perhexiline. The contribution of CYP2D6-mediated metabolism to the total clearance of each enantiomer of perhexiline was established from the difference between the observed pharmacokinetics of (+)- and (-)-perhexiline in EM in both an *in vitro* and an *in vivo* model and the pharmacokinetics of PM in the same models. Furthermore, it was achieved within the limitations of current chromatographic techniques and without simply administering the pure enantiomers to patients, for which ethical approval could not be obtained without first performing the preclinical experiments described by this thesis. If the apparent oral clearance of (+)-perhexiline in EM was significantly less than the apparent oral clearance of (-)-perhexiline in the same patients, yet not significantly different to the apparent oral clearance of (+)-perhexiline in PM patients, then it may be possible to overcome the inherent interindividual pharmacokinetic variability observed in the metabolism of the racemic perhexiline used clinically by administration of only the (+) enantiomer.

Whilst not directly addressing the primary aim of this thesis, the first publication served as the essential prerequisite for the following three studies. It described an enantioselective HPLC-fluorescent method that was validated across the range of concentrations expected to be observed in patients maintained within the therapeutic range of plasma (±)-perhexiline concentrations and demonstrated it had the necessary specificity for the complex plasma samples of patients receiving poly-drug therapy. A pilot study illustrating the different plasma (+)- and (-)-perhexiline concentration-time profiles over the course of a dosing interval in an elderly, hospitalised female patient

phenotyped as an EM and treated with racemic perhexiline for refractory angina pectoris served to confirm the enantioselective metabolism first described by Gould et al (1986), who had administered single doses of the individual perhexiline enantiomers to eight healthy volunteers. Importantly, this publication detailed the preparation and characterisation of the pure perhexiline enantiomers used in the *in vitro* study described in the third publication and in future pharmacodynamic studies not described in this thesis.

Similar to the first publication, the second publication served primarily to detail the quantitative methods employed for further studies, in particular the investigation of the *in vitro* kinetics of the metabolism of (+)- and (-)-perhexiline by human liver microsomal incubations described in the third publication. None the less, because the EM and PM patients had similar dose-corrected concentrations of racemic perhexiline in plasma, some general observations about CYP2D6 activity as it related to the formation of the 4-monohydroxy perhexiline metabolites were possible. The pairs of *cis*- and *trans1*-OH-perhexiline enantiomers were the primary metabolites detected in the plasma of the EM patients, in contrast to the PM patients where they were frequently below the lowest limit of quantification. This strongly suggested that the formation of at least one of the enantiomers of both *cis*-OH-perhexiline and *trans1*-OH-perhexiline was mediated by CYP2D6, in agreement with Amoah et al (1984) and Cooper et al (1986 and 1987). As previously discussed in the introduction of this thesis, the subjects of Gould et al (1986) were likely EM; when the results of the second publication are considered in conjunction with the stereoselective study of Gould et al (1986) it is likely that the CYP2D6-dependent *cis*-OH-perhexiline and *trans1*-OH-

perhexiline metabolites detected in the plasma of the EM patients treated with racemic perhexiline were principally derived from (-)- and (+)-perhexiline, respectively.

Although *trans2*-OH-(±)-perhexiline was the primary metabolite detected in the plasma of PM patients, it was only above the limit of quantification in the plasma of the EM patient with the lowest measured CYP2D6 activity. This was similar to the observations of Beck et al (2004), who detected both the *trans1* and *trans2* conformation metabolites in the plasma of the four PM patients of their study yet only one conformation in the plasma of the 26 EM patients using an achiral liquid chromatography-mass spectrometry assay. This suggested that the *trans2*-OH-perhexiline metabolites that occurred in plasma, whether they were derived from (+)- or (-)-perhexiline, were substrates of CYP2D6 and therefore must compete with one or both of the enantiomers of perhexiline for subsequent oxidation. It is certain that some of the 4-monohydroxy metabolites of perhexiline must undergo further oxidation, by CYP2D6 and perhaps other P450 isoforms, to account for the 11-37% of the 400 mg daily dose of racemic perhexiline recovered from the urine of five subjects at steady-state by Wright et al (1973) as dihydroxy perhexiline metabolites. Indeed, of the patients maintained on perhexiline and described by Singlas et al (1978), the 14 non-toxic patients received a mean daily dose of racemic perhexiline of 230 mg and excreted a mean of 9.5% of their dose in urine as the total of three 4,4'-dihydroxy perhexiline metabolites for which reference compounds were available, whilst in the 13 toxic patients, in whom PM were presumably over represented, they were receiving a mean daily dose of 300 mg of racemic perhexiline and recovering only a mean of 1.5% of it in the same manner. It is interesting to note that Wright et al (1973) reported that of the perhexiline metabolites recovered in urine and characterised by achiral gas chromatography-mass spectrometry

there were seven compounds with mass spectra consistent with dihydroxy metabolites; due to the possible conformations of these molecules up to 14 isomers may, in fact, be present. The consideration that any of these metabolites may have competed with the parent perhexiline enantiomers for oxidation further highlights the possible complexities of perhexiline pharmacokinetics.

The third publication comprehensively investigated the *in vitro* hepatic 4-monohydroxylations of each perhexiline enantiomer in order to test the first three hypotheses of this thesis. The kinetics in human liver microsomes obtained from genotypical EM, IM and PM were quantified and the exact P450 isoforms mediating these reactions were exhaustively described by utilising monoclonal antibodies and recombinantly expressed human P450 enzymes in addition to the prototypical P450 isoform-specific chemical inhibitors employed in the achiral analysis of Sørensen et al (2003). The most significant finding of this study was that it unequivocally demonstrated that both enantiomers of perhexiline were subject to significant polymorphic metabolism by CYP2D6. Furthermore, the mean intrinsic clearance attributable to CYP2D6 in the EM and IM microsomal incubations was 83 and 32% greater, respectively, for (-)- than (+)-perhexiline, confirming the enantioselectivity of CYP2D6-mediated metabolism for (-)-perhexiline proposed in the first hypothesis of this thesis and reported in the *in vivo* study of Gould et al (1986). Interestingly, CYP2D6 also exhibited distinct stereospecificity with respect to the conformation of the 4-monohydroxy metabolites it produced. Whereas formation of both *cis*-OH-perhexiline enantiomers was CYP2D6-dependent, only (+)-perhexiline was a substrate for 4-*trans1*-monohydroxylation by CYP2D6. Although CYP2B6- and CYP3A4-mediated metabolism contributed to *trans1*-OH-(+)-perhexiline production, the mean intrinsic

clearance for the formation of this metabolite by CYP2D6 in the EM and IM microsomal incubations was approximately 30- and 10-fold greater, respectively, than the total of the two corresponding minor pathways. The significant interindividual variability possible in the hepatic expression and hence activity of CYP2B6 and CYP3A4 (Urquhart et al, 2007) probably contributed to the disagreement that previously existed in the literature as to whether formation of *trans1*-OH-(±)-perhexiline was subject to polymorphic metabolism or not. While no antimode separated PM from EM in the distribution of the total recovery of *trans1*-OH-(±)-perhexiline in the urine of the 50 subjects of Cooper et al (1984), there was certainly a lower median recovery from the three PM than the EM, consistent with the current findings relating to the relative rates of formation of each of the *trans1*-OH-perhexiline enantiomers by CYP2D6, CYP2B6 and CYP3A4.

In common to all the *in vitro* CYP2D6-mediated reactions, with the exception of IM 36 who possessed an allele responsible for reduced enzymatic activity (described as *CYP2D6*2J* at the time of publication and subsequently renamed *CYP2D6*59* (Toscano et al, 2006) by the Human Cytochrome P450 (*CYP*) Allele Nomenclature Committee), was a very high affinity of the enzyme for both (+)- and (-)-perhexiline. The Michaelis-Menten constants calculated for the free concentration of each substrate in the microsomal incubations were within the range of the unbound concentrations of (+)- and (-)-perhexiline in the plasma of patients maintained within the therapeutic range of racemic perhexiline concentrations (Inglis et al, 2007). Whilst Sallustio et al (2002) demonstrated that CYP2D6 was responsible for the saturable metabolism of racemic perhexiline observed *in vivo*, the *in vitro* CYP2D6 kinetics were consistent with the non-linear pharmacokinetics observed for both perhexiline enantiomers in the EM

patients of Inglis et al (2007). Thus saturable as well as polymorphic metabolism by CYP2D6 complicates the pharmacokinetics of both (+)- and (-)-perhexiline.

Although the affinity of CYP2D6 for the enantiomers of perhexiline was similar among the EM and IM, with the noted exception due to the *CYP2D6*59* allele, for each perhexiline enantiomer there was variation in the V_{max} values for each reaction attributable to a gene-dose effect that has been well established for perhexiline metabolism by CYP2D6 *in vivo* (Barclay et al, 2003, Davies et al 2004 and 2006, Inglis et al, 2007). The affinity of the CYP2B6 and CYP3A4 mediated hepatic 4-monohydroxylation reactions was two orders of magnitude less than the reactions catalysed by CYP2D6 and suggested that these clearance pathways should not be subject to the saturability observed *in vivo* for CYP2D6 mediated metabolism. CYP2B6 and CYP3A4 are minor contributors to P450-mediated hepatic clearance except in PM. Their activity was not enantioselective and thus confirmed the third hypothesis of this thesis, although the absence of distinct enantioselectivity was unable to explain the significantly greater enantioselectivity observed *in vivo* in PM than EM patients in the study of Inglis et al (2007) and the fourth publication included in this thesis.

Of the total mean intrinsic clearance of (-)-perhexiline, CYP2D6 accounted for 99 and 89% in the EM and IM microsomal incubations, respectively. With regards to (+)-perhexiline, CYP2D6 similarly accounted for 97 and 76%, respectively, confirming the second hypothesis of this thesis, that CYP2D6 accounts for a lower proportion of the total hepatic P450-mediated oxidation of (+)- than (-)-perhexiline. Although the first two hypotheses are essential prerequisites to support the possibility that the inherent interindividual pharmacokinetic variability observed in the metabolism of racemic

perhexiline could be overcome by administration of only (+)-perhexiline, the empirical data does not support this notion; the pre-eminence of CYP2D6 in the metabolism of (+)-perhexiline is simply too great. The total intrinsic clearance of (+)-perhexiline by the microsomes prepared from the liver containing only one fully functional *CYP2D6* allele (IM 36) was still more than six-fold greater than the mean total intrinsic clearance of the PM microsomes. The mean difference increased to greater than 20-fold for the EM microsomes and would reasonably be expected to be greater yet for UM derived liver microsomes. This conclusion was supported by the *in vivo* findings of the fourth publication of this thesis, where the median total apparent oral clearances of (+)- and (-)-perhexiline were 94 and 89% lower, respectively, in the PM patients than the EM patients. This fraction represents the proportion of the total apparent oral clearance of each enantiomer of perhexiline metabolised by CYP2D6 in EM patients maintained within the therapeutic range of racemic perhexiline concentrations in plasma and is almost as large for (+)- as it is for (-)-perhexiline.

The fourth and final publication of this thesis examined the steady-state plasma perhexiline enantiomer concentration-time profiles over the course of an interdosing interval in six EM and two PM patients. Although the last two hypotheses had effectively been tested by the *in vivo* study of Inglis et al (2007), who determined that systemic exposure to (+)-perhexiline was greater than (-)-perhexiline in both EM and PM patients treated with racemic perhexiline, the finding related to PM patients was completely unexpected following the distinct absence of enantioselectivity displayed by this genotype in the *in vivo* study and warranted further investigation. Because Inglis et al (2007) had measured only the trough plasma perhexiline enantiomer concentrations, plasma sampling was undertaken over the entire interdosing interval in both EM and

PM patients in order to attempt to elucidate the processes responsible for the observed enantioselectivity, with particular respect to the PM patients. Furthermore, urine collections were completed over the entire interdosing interval of five of the EM patients, permitting calculation of the renal clearance of each perhexiline enantiomer once the assay described in the first publication had been revalidated for this matrix.

Despite the variability in the renal clearance of each of the enantiomers of perhexiline among the five EM patients for whom it was calculated, it accounted for less than 2% of the total apparent oral clearance of each enantiomer in each patient, consistent with a previous finding for racemic perhexiline administered to patients at steady-state (Singlas et al, 1978), and exhibited a distinct lack of enantioselectivity. The mean renal clearances were subsequently assumed for the PM patients, since the difference between the two genotypes was reasonably attributable only to the expression of a hepatic enzyme, and accounted for 9 and 4% of the median total apparent oral clearance of (+)- and (-)-perhexiline, respectively. Because Cooper et al (1987) recovered two orders of magnitude more racemic perhexiline in the first 24 hours after the administration of a single 300 mg dose from the bile than from the urine of two patients who had undergone cholecystectomy, it was considered that biliary excretion of perhexiline could be a significant contributor to the total apparent oral clearance of PM patients. The absolute clearance by this route would be the net difference between canalicular secretion into bile and subsequent reabsorption and efflux from and into the gastrointestinal tract. One of these processes may have been highly enantioselective, however there were no secondary peaks evident in the plasma (+)- and (-)-perhexiline concentration-time profiles of the two PM patients to support the notion of enterohepatic recirculation. This may have been obscured by the relatively large time

intervals in the sampling protocol for these patients due to their weekly interdosing intervals, although an examination of the plasma (±)-perhexiline concentration-time profile of the only PM patient in the study of Jones et al (2004), where plasma was sampled at 1, 2, 4 and 6 hours post-dose, did not provide evidence of this phenomenon either.

The ratio of the median apparent oral clearance of (-)- to (+)-perhexiline in the EM and PM patients of the fourth publication was 1.3 and 2.1, respectively, similar to the corresponding results of Inglis et al (2007) of 1.5 and 2.3. What was most remarkable about these findings was not just that both EM and PM exhibited enantioselective apparent oral clearance, but that the magnitude of the enantioselectivity in PM was in fact significantly greater than in EM, despite the absence of enantioselectivity attributable to CYP2D6-mediated metabolism. There must be a highly enantioselective clearance pathway, greater than renal clearance and unmasked by the absence of CYP2D6-mediated metabolism in PM patients, that is responsible for their more enantioselective apparent oral clearance than EM patients. However, given that the median age of the patients in the fourth publication is 80 years and they have advanced cardiovascular disease and comorbidities frequently requiring hospitalisation for treatment, investigation should be undertaken in animal and *in vitro* models or in healthy human volunteers.

Although both patient genotypes displayed enantioselective pharmacokinetics, the ratio of the apparent oral clearance of (-)- to (+)-perhexiline was significantly greater in PM patients than in EM patients. Dosage adjustment based upon the results of therapeutic drug monitoring targets a racemic concentration of perhexiline in plasma and thus

exposes PM patients to significantly different concentrations of each perhexiline enantiomer than EM; systemic concentrations of (+)-perhexiline are approximately 50% higher and (-)-perhexiline concentrations approximately 30% lower. As discussed in the introduction to this thesis, the atypical kinetics observed in the inhibition of both cardiac and liver derived CPT-1 by Kennedy et al (1996) may have been due to different inhibition affinities of each enantiomer of perhexiline for both the muscle and liver isoforms of CPT-1. Thus it is possible that (+)- and (-)-perhexiline may exhibit differential selectivity for target enzymes in cardiac and hepatic tissues. Once the relative pharmacodynamic activities of the individual enantiomers have been established, therapeutic drug monitoring may be improved by developing specific enantiomer target concentration ranges in plasma for the racemic preparation of perhexiline or by developing a target concentration for a chiral preparation. What is certain is that perhexiline will remain as an essential option in the armamentarium for the treatment of refractory angina pectoris and therapeutic drug monitoring will remain obligatory, whether the preparation used clinically contains one enantiomer or both, due to the considerable involvement of CYP2D6 in the metabolism of both enantiomers.

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