THE STEREOSELECTIVE PHARMACODYNAMICS OF
THE ENANTIOMERS OF PERHEXILINE

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Abstract
In the past decade there has been growing interest and evidence that altered myocardial energy metabolism plays a major role in the onset and/or progression of ischaemic heart disease and heart failure. The anti-anginal agent perhexiline, marketed in Australia as 50:50 racemic mixture of (+)- and (-)-enantiomers, was one of the original metabolic agents introduced for clinical use in the 1970’s. Its mechanism of action is thought to involve manipulation of cellular energetics by inhibiting fatty acid metabolism through its interaction with the carnitine palmitoyltransferase system. This allows for a ‘switch’ in energy towards utilisation of carbohydrates, resulting in a greater net production of ATP per unit of oxygen used. However, perhexiline has a narrow therapeutic index. Hence clinical usage requires careful dosage individualisation using therapeutic drug monitoring in order to minimise adverse effects such as peripheral neuropathy and hepatotoxicity associated with elevated plasma perhexiline concentrations. There are currently no studies investigating the toxicity and efficacy of the individual enantiomers of perhexiline in vivo and my aim was to address this considerable gap in knowledge.

Study 1: This study investigated the effects of (+) and (-)-perhexiline on superoxide formation by NADPH oxidase in intact neutrophils from healthy subjects and patients with symptomatic stable angina pectoris.

(-)-Perhexiline showed a greater inhibition of superoxide formation than (+)-perhexiline (P<0.05, Wilcoxon matched paired test) in neutrophils from 11 healthy volunteers, with median IC50 values of 1.19 and 1.64µM, respectively. In 6 patients with angina, neutrophil superoxide formation was also significantly inhibited by both (+)- and (-)-perhexiline with IC50 values of 2.07µM (1.04-2.95µM) and 1.35µM (1.08-1.86µM) respectively; however there was no significant difference between the two enantiomers.
This study demonstrates that both enantiomers dose dependently inhibit superoxide formation by neutrophil NADPH oxidase and are both likely to contribute to the beneficial anti-oxidant properties of perhexiline.

Study 2: This study compared female Dark Agouti (DA) and Sprague Dawley (SD) rats, as models of CYP2D6 metabolism and hepatotoxicity after administration of perhexiline.

Median (range) perhexiline concentrations in liver and plasma of the SD strain (n=4) were 5.42 (0.92 – 8.22) µg/g and 0.09 (0.04 – 0.13) mg/L, respectively. The DA strain (n=4) showed higher plasma (0.50 (0.16 – 1.13) mg/L, P<0.05) and liver (24.47 (9.40 – 54.70) µg/g, P<0.05) perhexiline concentrations, and cis-OH-perhexiline concentrations in plasma and liver of DA rats were 2.5 and 3.7 fold higher compared to SD rats. In both strains the plasma concentration ratio of (+):(-) enantiomers was approximately 2:1. When compared to their controls, DA rats treated with perhexiline had significantly higher plasma LDH concentrations (2-fold, P<0.05), whilst there were no changes in biochemical liver function tests in the SD group.

Study 3: The aim of this study was to administer the enantiomers of perhexiline to the DA animal model. The hypothesis was that there is enantioselectivity in the toxic effects associated with perhexiline in a clinical setting.

Racemic perhexiline did not affect hepatic lipid or glycogen content. However, (-)-perhexiline induced a concentration-dependent increase in glycogen content, while (+)-perhexiline induced concentration-dependent increases in lipid and decreases in glycogen content. Racemic (p<0.001) and (+)-perhexiline (p<0.05) induced peripheral nerve dysfunction, while (-)-perhexiline induced no change. Thus, (+)-perhexiline, but not (-)-
perhexiline displayed the toxicity (hepatic steatosis and neuropathy) associated with clinical use. Mean (s.e.m.) plasma perhexiline concentrations in rats treated with racemate, (+)- or (-)-enantiomer were 0.84 (0.40), 0.67 (0.07) and 0.29 (0.04) mg/L, respectively, corresponding to the mid-upper clinical therapeutic range.

To my knowledge the studies performed throughout this thesis are the first to test the anti-inflammatory as well as toxic effects of the individual enantiomers of perhexiline. The results suggest that the administration of racemic perhexiline is indeed the administration of two drugs with very different pharmacokinetic as well as pharmacodynamics properties. This not only helps us breach the gap in the current knowledge on how racemic perhexiline may exert its toxic effects, but also introduces the realistic possibility that the use of an enantiomeric formulation of perhexiline may increase efficacy or decrease toxicity providing a safer alternative to racemic perhexiline use.
Declaration

Name: ........................................................ Date: ........................................

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to John Licari and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Signature: ........................................................ Date: ........................................
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throughout the years and without whom I don’t think I would have been able to finish this thesis. Late in the thesis preparation, we welcomed our son, Natale, whose birth has made me the happiest person in the world and inspired the completion of this thesis.
Lack of Clinically significant Stereoselectivity in the Inhibition of Human Neutrophil Superoxide Formation by (+)- and (-)-Perhexiline

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Study design

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Manuscript 2

Metabolism and Hepatotoxicity of the Myocardial Metabolic Agent Perhexiline in Sprague Dawley and Dark Agouti Rats

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Milne RW

Study design

Somogyi AA

Interpretation of data, critical revision of manuscript.

Sallustio BC

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Manuscript 3

The enantiomers of the myocardial metabolic agent perhexilone display divergent effects on hepatic energy metabolism and peripheral neural function in rats

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Milne RW

Study design, critical revision of manuscript

Somogyi AA

Interpretation of data, critical revision of manuscript.

Sallustio BC
Study conception and design, management and interpretation of the data, manuscript preparation, critical revision of the manuscript.
Abstracts and Presentations


This work has been subject to intellectual property and as such two provisional patent applications were lodged in September 2012. Australian patent No. 2012903850 and US patent No. 61697214: Use of enantiomers of perhexiline. As a result of the pending intellectual property over this thesis I was not able to submit any papers for publication at this time.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>AGPAT</td>
<td>Acylglycerol-phosphate acyltransferase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>CAT</td>
<td>Carnitine acyltransferase</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary Heart Disease</td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CPT</td>
<td>Carnitine palmitoyl-transferase</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450 enzyme</td>
</tr>
<tr>
<td>D</td>
<td>Dose</td>
</tr>
<tr>
<td>DA</td>
<td>Dark Agouti</td>
</tr>
<tr>
<td>DGAT</td>
<td>Diacylglycerol acyltransferase</td>
</tr>
<tr>
<td>EM</td>
<td>Extensive Metaboliser</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FACoA</td>
<td>Fatty acyl CoA</td>
</tr>
<tr>
<td>FACS</td>
<td>Fatty AcylCoA synthase</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FADH$_2$</td>
<td>Reduced form of FAD</td>
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<td>FFA</td>
<td>Free fatty acids</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>--------------------------------------------------</td>
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<tr>
<td>GLUT-1</td>
<td>Glucose transporter -1</td>
</tr>
<tr>
<td>GLUT-4</td>
<td>Glucose transporter 4</td>
</tr>
<tr>
<td>G-6-P</td>
<td>Glucose -6-phosphate</td>
</tr>
<tr>
<td>GPAT</td>
<td>Glycerol-phosphate acyltransferase</td>
</tr>
<tr>
<td>HepG2</td>
<td>Hepatoma cell line</td>
</tr>
<tr>
<td>HK</td>
<td>Hexokinase</td>
</tr>
<tr>
<td>HLM</td>
<td>Human liver microsomes</td>
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<td>HPG</td>
<td>Hydroxyphenylglyoxylate</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>IC</td>
<td>Inhibitory constant</td>
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<tr>
<td>IM</td>
<td>Intermediate metaboliser</td>
</tr>
<tr>
<td>IS</td>
<td>Internal standard</td>
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<tr>
<td>$K_m$</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>LCFA</td>
<td>Long chain fatty acids</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>n/a</td>
<td>Not available</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>reduced form of NAD</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NYHA</td>
<td>New York Heart Association</td>
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<tr>
<td>$O_2^-$</td>
<td>Super oxide</td>
</tr>
<tr>
<td>ONOO$^-$</td>
<td>Peroxynitrate</td>
</tr>
<tr>
<td>OH</td>
<td>Hydroxy</td>
</tr>
<tr>
<td>PAP</td>
<td>Phosphatidic acid phosphohydrolase</td>
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<tr>
<td>PDH</td>
<td>Pyruvate dehydrogenase</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>PHR</td>
<td>Peak height ratio</td>
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<tr>
<td>PM</td>
<td>Poor metaboliser</td>
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<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PX</td>
<td>Perhexiline</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SD</td>
<td>Sprague Dawley</td>
</tr>
<tr>
<td>TCA</td>
<td>Tri-carboxylic acid</td>
</tr>
<tr>
<td>TDM</td>
<td>Therapeutic drug monitoring</td>
</tr>
<tr>
<td>TQEH</td>
<td>The Queen Elizabeth Hospital</td>
</tr>
<tr>
<td>UM</td>
<td>Ultra-rapid metaboliser</td>
</tr>
</tbody>
</table>
List of Figures

Chapter 1

Figure 1. Chemical structure of perhexiline. The star refers to the chiral centre of the molecule.

Figure 2. The energy metabolics of the myocardial cell. Red arrows demonstrate the pathway FA undertake to eventually lead to the production of ATP. Blue arrows indicate the pathway taken by carbohydrates eventually leading to the production of ATP.

Figure 3. The fate of FA as they enter the myocyte. Free FA are converted to FACoA by FACS. At this point it can either undergo β-oxidation, eventually leading to the production of ATP via the ETC, or it can divert to the storage pathway leading to the eventual formation of triglycerides via enzymatic processes. FA-Fatty Acid, FACS-Fatty AcylCoA synthase, FACoA- Fatty AcylCoA, GPAT- Glycerol-phosphate acyltransferase, AGPAT- Acylglycerol-phosphate acyltransferase, PAP- phosphatidic acid phosphohydrolase, DGAT- Diacylglycerol acyltransferase, ETC- Electro Transport Chain, ATP- Adenosine Tri-Phosphate

Figure 4. Illustration on transport of FACoA to the mitochondrial membrane. The CPT system consisting of CPT-1, located on the outer mitochondrial membrane and CPT-2 located on inner mitochondrial membrane, aids in the passage of fatty acyl CoA into the mitochondria via the addition of a carnitine group.

Figure 5. Metabolism of FACoA. In the mitochondrial matrix FACoA, undergoes a series of enzymatic processes to produce acyl-CoA, the entry substrate of the citric acid cycle. During
this process FAD and NAD are oxidised to FADH$_2$ and NADH. The final step involves the addition of a CoA molecule to form acyl-CoA.

**Figure 6.** This is a diagrammatic representation of the cross talk between the two major pathways for energy metabolism. The products of β-oxidation (NADH and Acetyl-CoA) have a negative feedback on the activation of PDH, thus inhibiting the conversion of glucose to energy and favouring FA utilisation as the preferred energy source. Similarly PDH regulates its own activation in a negative manner as a result of excessive pyruvate conversion to Acetyl-CoA.

**Figure 7.** The major metabolic pathway for perhexiline and formation of OH- perhexiline metabolites.

**Figure 8.** CYP2D6 phenotype definition and distribution in a random European population (N=316). The numbered arrows indicate the range of the sparteine metabolic ratio associated with number of functional genes. Taken from Zanger et al.

**Figure 9.** The mono- and di- hydroxyl metabolites resulting from perhexiline metabolism.
Chapter 2

**Figure 10.** The preparation and resolution of the (+) and (-) enantiomers of perhexiline from racemic perhexiline maleate

**Figure 11.** Sample chromatogram from the method described. As shown on the chromatogram, the retention time for the internal standard was 8.5 min, the (+) enantiomer was 21.6 min, and the (-) enantiomer was 22.7 min.

**Figure 12.** Sample chromatogram for the method described. The retention times of the internal standard (fendiline) a) are 18.8 min, and the OH perhexiline metabolites cis- b) 10.8 min trans-1- c) 12.4 and trans-2- d) 13.6 min respectively.

**Figure 13.** Various phases following centrifugation of the cellular fraction of blood through the Lymphoprep® medium.
Chapter One: Introduction
1.1 Clinical History of Perhexiline

Perhexiline [(±)-2-(2, 2-dicyclohexylethyl) piperidine] (Figure 1) was developed by Richardson-Merrell Pharmaceuticals in the late 1960s [1] and was first introduced for clinical use in the treatment of exertional angina in the 1970s [2]. Perhexiline was recognised as one of the more effective anti-anginal treatments at the time [3], with additional desirable effects which facilitated its use including no negative inotropic effects on blood pressure or heart rate. Despite all the positive outcomes from taking the drug, there were severe adverse events reported in the late 1970s to mid 1980s, particularly hepatotoxicity and neurotoxicity, that eventually resulted in its withdrawal from the market [4]. However, it was still prescribed in other countries, especially Australia and New Zealand, for the treatment of refractory angina but only when therapy was accompanied by therapeutic drug monitoring, initially to identify the phenotype of the patient, but also to maintain plasma perhexiline concentrations within a narrow therapeutic range associated with efficacy and lack of significant toxicity [3].

A double-blinded cross-over trial, conducted in the 1970s showed that perhexiline reduced the effects associated with exercise-induced tachycardia in healthy volunteers [5] and was also responsible for reducing the occurrence of anginal symptoms in patients with a history of coronary heart disease. In so doing, the frequency of nitroglycerine tablet intake was noted to reduce. Clinically, perhexiline displays improved oxygen utilization, no increase in heart rate or blood pressure as well as improvement in myocardial oxygen consumption [5, 6].

In the late 1980s after the discovery that perhexiline toxicity was associated with elevated concentrations in plasma [7], a double blind placebo-controlled cross-over clinical study revealed the efficacy of perhexiline in patients who were already receiving maximal tolerated pharmacological therapy [8]. To participate in the study, all patients needed to have
diagnostic evidence of coronary artery disease or a history of myocardial infarction as well as being unsuitable for revascularisation procedures. All patients included were on maximal tolerated doses of combinations of β-blockers, nitrates, calcium antagonists or all three. The results showed, as in the earlier trials, perhexiline was associated with little change in hemodynamic effects, but did produce an increase in myocardial efficiency and a decrease in the frequency of angina attacks [5, 6, 8]. This trial confirmed that by titrating doses to keep plasma concentrations below 0.6mg/L, perhexiline not only shows efficacy in the absence of any adverse effects but could be used safely in combination with conventional anti-anginal agents without having to drastically alter the current dosing regimen, providing there is strict therapeutic drug monitoring.

The treatment of stable and refractory stable angina was and still is the primary indication for perhexiline; however, more recently it has been shown to be efficacious in the treatment of other forms of cardiovascular disease such as atherosclerosis [9], endothelial dysfunction [9, 10], unstable angina [11], aortic valve disease [12] and heart failure [13], the mechanisms of which will be presented further on in the chapter.
Figure 1. The chemical structure of perhexiline. The star refers to the chiral centre of the molecule.

1.2 Coronary heart disease

Within the past century, according to the Australian Institute of Health and Welfare (AIHW) cardiovascular disease has grown to become the most prevalent disease in the world today [14, 15]. Coronary heart disease (CHD) is the most prevalent form of cardiovascular disease. It is a culmination of a series of events that present as a result of diseased coronary vasculature, which is also commonly referred to as coronary artery disease (CAD). In most cases, CAD clinically manifests in the form of angina and/or a myocardial infarction (MI), which are primarily due to impedance in coronary blood flow to the myocardium as a result of the formation of atherosclerotic plaques within the vascular arterial wall itself.
Endothelial dysfunction has been increasingly regarded as the underlying cause of atherosclerosis, diabetes, hypertension and heart failure. It is characterised by an imbalance of nitric oxide (NO) availability to the endothelial wall, which results in endothelium-dependent vasodilation impairment. NO is produced by NO synthase (NOS) which is specific to the environment where it is located, for example NO produced in the endothelium is synthesised by endothelial NOS (eNOS) [16]. Free radicals, such as the reactive oxygen species (ROS) in particular the superoxide anion (O$_2^-$) have been shown to react with NO reducing its bioavailability and therefore reducing its vascular effects. It undergoes an extremely rapid reaction with nitric oxide to form peroxynitrite (ONOO$^-$), which is a potent oxidising agent in its protonated form, peroxynitrous acid.

Chemical reaction for the formation of ONOO$^-$

$$O_2^- + \cdot NO \rightarrow ONOO^-$$

1.2.1.1 NADPH Oxidase

NADPH oxidase is a multicomplex enzyme which when active is the major source of O$_2^-$ in the cardiovascular system. The complex itself is made up of a flavocytochrome b$_{558}$ unit comprising of two membrane bound subunits p22$^{phox}$ (the oxidase) and gp91$^{phox}$ (also known as Nox 2), responsible for the stability and the activity of the enzyme, and at least four cytosolic subunits p47$^{phox}$, p67$^{phox}$, p40$^{phox}$ and Rac 1 or 2 which when activated translocate the membrane and interact with the flavocytochrome b$_{558}$ unit [17, 18]. Upon activation by
various stimuli such as angiotensin II, inflammatory cytokines or hypoxia, the oxidase uses NADPH as an electron donor to generate $O_2^-$ from molecular oxygen [19].

There have been 4 isoforms of Nox identified (Nox 1-5), which are encoded by separate genes and have differential expression throughout the cardiovascular system. The predominant isoforms in cardiomyocytes [20], endothelial cells [21] and fibroblasts [22] are Nox 2 and 4, whereas in vascular smooth muscle cells Nox 1 and 4 are preferentially expressed [23].

1.2.2 Angina

Angina pectoris is a result of the classical situation of supply and demand. It occurs when the demand for oxygen exceeds the rate at which blood is able to deliver it to the myocardium. The supply is greatly affected in patients suffering from CAD as they are at greater risk from developing atherosclerosis, the major contributor of ischaemic heart diseases.

Heavy feeling, tightness, sharp stabbing pain or squeezing of the chest are characteristic symptoms associated with angina presentation. The pain experienced in angina is one of gradual increase that can last from a few minutes to thirty minutes. The pain experienced is not localized to the heart, but may be referred pain that can radiate to the shoulder, arms, neck, throat, jaw and teeth [24].

Patients suffering from angina are classified as having stable, unstable or variant forms according to the degree of impairment the disease has on physical activity.

The clinical presentation of stable angina is commonly associated with chest pain that is short in duration (typically less than 15 minutes). The onset of chest pain does not occur while at
rest, but becomes progressively worse upon exertion. Rest or nitroglycerine therapy usually relieves this pain [25].

In contrast to stable angina, unstable angina manifests itself when at rest, as unpredictable chest pain often of lengthy duration. Upon examination, patients suffering from unstable angina often present with transient ischemic, ST-segment and T-wave abnormalities upon ECG analysis. Patients suffering from unstable angina are at high risk of the disease progressing to MI. The mechanism underlying the development of unstable angina is a atherosclerotic plaque rupture accompanied by thrombus formation [25].

As the name suggest, the onset of variant angina is unpredictable. The pain associated with this angina syndrome can occur either at rest or during normal physical activity, but is not brought about by exercise. The frequency of variant angina attacks seems to also be time dependent, with ischemic episodes manifesting with mild exercise in the early morning, whilst other episodes occur at rest between midnight and early morning. In a patient suffering from variant angina, ECG analysis commonly reveals ST-segment elevation with reciprocal depression. The mechanism underlying variant angina is believed to be due to coronary artery spasm that is an effect of endothelial dysfunction [26, 27].

1.2.3 Incidence of CAD

CHD is the most common form of heart disease in Australia. The 2001 national health survey revealed that approximately 1.9% (approx. 355,600) of Australians suffered some form of CHD, and of these, 266,700 suffered from angina and the remainder reported having a MI [15]. CHD is the largest single cause of death in Australia, accounting for 26,063 deaths in 2002. However, data collected over a 12-year period between 1991 until 2002 have indicated
that death rates have declined by approximately 41% in males and 40% in females [15]. The survey data also revealed that hospitalisation as a result of CHD was greatest in Australians who were 60 years of age and over, and hospitalisation rates had increased by 11.7% between 1993-94 and 2001-02 [15].

These findings were further explained by the international Monitoring Trends and Determinants in Cardiovascular Disease (MONICA) project. This was a 10-year study that monitored cardiovascular disease in 37 populations over 21 countries (including Australia) and its aim was to establish factors affecting mortality and morbidity rates. The main findings were that a decrease in mortality was largely determined by interventions that affected and changed coronary event rates [28].

It has been suggested, based on trends in the early 2000s, that by 2020 CHD will be the single leading health problem of the world [15].

1.2.4 Ischemic Heart Disease

Coronary Artery Disease (CAD) is one of the major contributors of myocardial ischemia. Myocardial ischemia is defined as a reduction in coronary blood flow such that there is an imbalance between the body’s oxygen supply and demand [29]. As a result of this imbalance, myocardial function and in particular energy metabolism, is greatly reduced. In order to restore balance, myocardial function and energy metabolism have been the target of many therapeutic interventions.
Reduction of coronary blood flow will affect oxygen supply and demand and as such will be the determining factor for whether the cell will utilise carbohydrates or fatty acids (FA) as the primary source of energy (Figure 2).

**Figure 2.** The energy metabolics of the myocardial cell. Red arrows demonstrate the pathway FA undertakes to eventually lead to the production of ATP. Blue arrows indicate the pathway taken by carbohydrates eventually leading to the production of ATP. CPT= carnitine palmitoyl-transferase, PDH= pyruvate dehydrogenase, GLUT= glucose transporter, TCA= tricarboxylic acid cycle, ETC= electron transport chain, HK= hexokinase
1.3.1 Fatty Acid Metabolism

FA metabolism is a complex process involving many enzymes. It begins with the transport of FA to the heart as albumin-bound FA via the blood stream. Once in the myocyte, free FA and acyl-CoA synthetase together with ATP, catalyse the formation of long-chain fatty acyl-CoA (LCFA). The LCFA can undertake one of two pathways, they can be converted to triglycerides by lipoprotein lipase as an energy storage option, or they can undergo β-oxidation in the mitochondria leading to the production of ATP (see Figure 3) [30, 31].

LCFA is impermeable to the mitochondrial membrane. In order to facilitate its uptake, the mitochondria have evolved a carnitine palmitoyl-transferase system, involving the enzymes carnitine palmitoyl-transferase (CPT)-1 and -2 and carnitine acyltransferase (CAT) [32, 33]. CPT-1 esterifies acyl-CoA to carnitine to form long-chain acyl-carnitine in the outer membrane. It is transported from the outer mitochondrial membrane by carnitine acyltransferase (CAT), to the inner mitochondrial membrane where it is converted back to LCFA and carnitine by CPT-2.
Figure 3. The fate of the fatty acid as it enters the myocyte. Free FA is converted to FACoA by FACS. At this point it can either undergo β-oxidation, eventually leading to the production of ATP via the ETC, or it can divert to the storage pathway leading to the eventual formation of triglycerides via enzymatic processes. FA-Fatty Acid, FACS-Fatty AcylCoA synthase, FACoA- Fatty AcylCoA, GPAT- Glycerol-phosphate acyltransferase, AGPAT- Acylglycerol-phosphate acyltransferase, PAP- phosphatidic acid phosphohydrolase, DGAT- Diacylglycerol acyltransferase, ETC- Electro Transport Chain, ATP- Adenosine Tri-Phosphate
1.3.1.1 The Carnitine Palmitoyltransferase (CPT) System

This system is one that has evolved to facilitate the entry of free FA into the mitochondrial matrix, via a series of enzymes. The first enzyme encountered by free FA is CPT-1, the key and rate limiting enzyme involved in the transfer of fatty acyl-CoA into the mitochondria, located on the outer mitochondrial membrane [32, 34]. Here the fatty acyl groups undergo esterification with carnitine to form fatty acyl-carnitine, which is then able to cross the outer membrane where it reaches the inner mitochondrial membrane. Acylcarnitine-carnitine translocase facilitates the entry of fatty acyl-carnitine into the matrix where it is converted back to fatty acyl-CoA and carnitine by CPT-2 [31, 33, 35] (Figure 4).

CPT-1 is a single polypeptide, 88 kDa in size and has both inhibitor and catalytic domains. Studies have found that CPT-1 consists of two hydrophobic domains H1 and H2. The orientation of positively and negatively charged residues in the H1 domain, suggests that the NH$_2$ terminus faces the cytosol [36]. Expression of a truncated NH$_2$ terminus of CPT-1 involving yeast studies, showed a decrease in the activity of the CPT-1 regulator malonyl-CoA [37], suggesting a pivotal role of the cytosolic NH$_2$ terminus in the regulation of CPT-1 [38].

CPT-2 is a 71kDa protein containing a 25 amino acid NH$_2$ terminal. It is located inside the inner mitochondrial membrane and its function is to reform the fatty acyl-CoA and carnitine from fatty acyl-carnitine [38, 39] (Figure 4).

There are two isoforms of CPT-1 found in both human liver and muscle cells and therefore have been named L-CPT 1 and M-CPT 1 respectively. They have different affinities to carnitine with the L-CPT 1 having a $K_m$ of 30 µM and the M-CPT 1 a $K_m$ of 500 µM [40].
Malonyl-CoA is a physiological inhibitor of CPT-1 synthesised in the sarcolemma by acetyl-CoA carboxylase (ACC), which requires acetyl-CoA as a substrate [41]. Studies have shown that malonyl-CoA binding to CPT-1 is non-competitive but also has allosteric properties. Rat liver mitochondria treated with proteases, which specifically digest the region of the protein responsible for allosteric inhibition without damaging the catalytic centre, have shown a reduced response to malonyl-CoA while the catalytic site was still active [38, 42, 43]. In contrast, the administration of trypsin caused inactivation of the catalytic site but little or no reduction of malonyl-CoA binding.
**Figure 4.** Illustration of the Carnitine Palmitoyltransferase System consisting of CPT-1, located on the outer mitochondrial membrane and CPT-2 located on inner mitochondrial membrane, which aids in the passage of fatty acyl CoA into the mitochondria via the addition of a carnitine group.
1.3.1.2 Beta Oxidation

In the mitochondria, LCFA undergo the process of β-oxidation. The rate of fatty acid β-oxidation is dependent upon the availability of the exogenous FA and the energy requirement of the tissue [32, 44]. β-Oxidation involves the repeated cleavage of two carbon acetyl-CoA units, as a result of the enzymatic activity of acyl-CoA dehydrogenase, enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase, leading to the formation of NADH and FADH$_2$ [31, 44] (Figure 5).

The final step in this process is the entry of acetyl-CoA into the tricarboxylic acid cycle (TCA), where it is eventually oxidised to form NADH, FADH$_2$ and CO$_2$. NADH and FADH$_2$ are reoxidised by the mitochondrial electron transport chain, thereby generating ATP. The flux through the TCA is highly regulated by the concentrations of NAD$^+$/NADH and ADP/ATP as they have potential inhibitory effects on the key enzymes in this cycle [35].
Figure 5. Metabolism of FACoA. In the mitochondrial matrix FACoA, undergoes a series of enzymatic processes to produce acyl-CoA, the entry substrate of the citric acid cycle. During this process FAD and NAD are oxidised to FADH$_2$ and NADH. The final step involves the addition of a CoA molecule to form acyl-CoA.
1.3.2 Carbohydrate Metabolism

Carbohydrates are the cell’s alternate energy source. This source accounts for about 10-40% of the energy produced in the cell. Entry of glucose in the cell is tightly regulated by enzymes and is dependent upon factors such as the concentration of transporters and by the transmembrane glucose gradient [45]. The two main transporters involved in the uptake of glucose under normal physiological conditions are GLUT 1 and GLUT 4, which are located on the sarcolemmal membrane and the intracellular microsomal vesicles [46, 47]. These transporters facilitate the entry of glucose into the cell where it is phosphorylated to glucose-6-phosphate (G-6-P) by the enzyme hexokinase (HK). G-6-P can either undergo glycogenolysis and stored as glycogen to be called upon by the cell whenever it is needed, or it can undergo glycolysis and be broken down to form ATP. Glycogenolysis is regulated by the amount of G-6-P and indirectly by insulin, which causes the activation of the glycogen synthetase enzyme [31, 47].

The pathway contributing to the energy production in the metabolism of carbohydrates is that of glycolysis. G-6-P undergoes a series of enzyme-catalysed reactions. The key enzymes phosphofructokinase-1, phosphofructokinase-2, and glyceraldehyde 3-phosphate dehydrogenase all act to convert G-6-P to pyruvate while at the same time producing ATP molecules [48]. Lactate can also be used as a source of pyruvate in extreme conditions due to its conversion by lactate dehydrogenase (LDH).
1.3.2.1 ATP Derived From Pyruvate

Pyruvate is transported into the mitochondria where it is converted to acetyl-CoA via decarboxylation by pyruvate dehydrogenase (PDH) (Figure 6). This is the key to an irreversible reaction in the metabolism of carbohydrates. The activity of PDH is highly dependent on its phosphorylation state and amount of substrate available. PDH is in its inactive form when phosphorylated by a PDH-kinase, whose activity depends upon the concentration of pyruvate, ADP, acetyl-CoA/CoA and NAD+/NADH. In its active form, PDH is dephosphorylated by a PDH-phosphatase and its activity is dependent upon Ca$^{2+}$ and Mg$^{2+}$ concentrations. Acetyl-CoA then enters the TCA cycle, as previously described in fatty acid oxidative metabolism [49-51].

1.3.2.2 Cross Talk Between Pathways

FA and glucose metabolism are not entirely independent processes. They require a degree of inter-relation in order to achieve optimum myocardial performance. In a healthy human heart, FA utilisation is favoured and therefore is primarily metabolised as a source of energy. β-Oxidation of FA produces compounds that have an inhibitory effect on carbohydrate metabolism. The high mitochondrial concentrations of acetyl-CoA/CoA and NAD+/NADH produced by FA oxidation activate PDH-kinases which then act to phosphorylate and thereby inhibit PDH, thus putting a halt on the key irreversible reaction in carbohydrate metabolism (Figure 6) [51, 52].
Figure 6. This is a diagrammatic representation of the cross talk between the two major pathways for energy metabolism. The products of β-oxidation (NADH and Acetyl-CoA) have a negative feedback on the activation of PDH, thus inhibiting the conversion of glucose to energy and favouring FA utilisation as the preferred energy source. Similarly PDH regulates its own activation in a negative manner as a result of excessive pyruvate conversion to Acetyl-CoA.
1.3.3 Metabolic Dysfunction During Ischemia

During periods of ischemia, the primary effect on the myocardium is mitochondrial metabolic dysfunction. As the myocyte prefers to utilise FA as its primary energy source (see section 1.6.1 for more detail), impaired blood flow reduces not only the concentration of free FA available, but also reduces the supply of oxygen to the myocardium, thereby diminishing the cells’ ability to undergo aerobic respiration [31, 53, 54]. Under ischemic conditions, the free FA continue to undergo β-oxidation producing NADH and acetyl-CoA, activators of PDH kinase, which in turn phosphorylates PDH resulting in its inhibition. The lack of oxygen leads to a decrease in FA flux through the electron transport chain and TCA cycle, resulting in decreased ATP formation and an increase in cytosolic acetyl-CoA concentrations resulting in further inhibition of PDH. As the cell detects a decrease in ATP formation, it switches energy source to carbohydrates. The lack of blood flow stimulates an increase in the concentration of the glucose uptake transporters GLUT-1 and -4 on the cell surface in order to facilitate the transport of more glucose into the cell. Glucose is then converted to pyruvate, which moves into the mitochondrial matrix to be decarboxylated by PDH [31, 50, 55] (see section 1.6.3 for further detail). However, PDH is inactive as a result of the NADH and acetyl-CoA produced by β-oxidation, causing pyruvate concentrations in the cell to rise. Under normal conditions lactate consumption would serve as an alternate source of pyruvate, however under ischaemic conditions, the increase of pyruvate causes the cell to go from a state of lactate consumption, to a state of lactate production via the enzyme LDH. An increase in lactate concentrations causes a decrease in cellular pH [50, 56].
As ATP concentrations are depleted, lactate concentrations increase and cellular pH decreases (↑H\(^+\)), cardiac efficiency also begins to decrease. At low pH the cell is unable to maintain Ca\(^{2+}\) homeostasis and requires more ATP to do so, leading to a decrease in contractile work, which also requires ATP [29, 57]. Therefore a reduction in ATP and a decrease in pH will not only cause the impairment of the Ca\(^{2+}\) pump but also a reduction in the contractile function of myocardial segments.

1.3.3.1 Myocardial metabolic Agents

In the last two decades, there have been significant advances towards the knowledge and understanding on the workings of the myocardium. However, despite advances in haemodynamic pharmacological therapies and surgical interventions, there are still significant proportions of refractory or relapsing IHD and HF patients. As mentioned earlier in this chapter, these disease states are characterised by an imbalance in energy production in myocytes. The myocardium’s ability to metabolise fatty acids is substantially reduced forcing the switch to its alternate energy source, carbohydrates. However, in severe disease, flux through this pathway is also severely impaired, potentially resulting in myocyte cell death. It is not well understood why this switch in substrate utilisation from FFA to carbohydrates takes place and whether this is an adaptive mechanism for cardio-protection or whether it contributes to the progression of the disease.

Recently, targeting myocardial energy metabolism has gained significant momentum as a novel approach in treating HF and IHD, and several studies have shown that the use of myocardial metabolic agents to modify myocardial substrate utilisation improves left ventricular ejection fraction (LVEF) [11], while reducing FFA metabolism and increasing
glucose metabolism [58], resulting in a more mechanically efficient, oxygen-sparing myocardium, characteristics that are deficient in IHD and HF.

Current agents used clinically to treat ischaemic conditions have shown to be metabolic modulators:

**Trimetazidine**

Trimetazidine (1-[2,3,4 – trimethoxybenzyl] piperazine dihydrochloride) is believed to exert its metabolic modulation via inhibition of the enzyme long-chain 3-ketoacyl coenzyme-A thiolase (LC 3-KAT), one that is involved in the β-oxidation pathway [59]. The advantageous characteristics of trimetazidine therapy are that it displays no negative inotropic effect either at rest or upon exertion and there is no need for therapeutic drug monitoring [59].

**Etomoxir**

Etomoxir is a potent CPT-1 inhibitor that was initially introduced as an antidiabetic agent due to its hypoglycaemic effects [60]. In a double blind randomized clinical trial, the therapeutic effects of etomoxir were studied. Although etomoxir displayed a trend for increased exercise tolerance at various doses, the trial had to be prematurely terminated as there were reports of elevated liver transaminase levels indicating the potential for hepatotoxicity [61].

**Ranolazine**

Ranolazine (±-N-(2,6-dimethylphenyl)-4-[2-hydroxy-3-(2-methoxyphenoxy)-propyl]-1-piperazineacetamide), is currently used for the treatment of chronic stable angina. MARISA (Monotherapy Assessment of Ranolazine in Stable Angina) was a placebo-controlled
randomized study to assess the efficacy of ranolazine in stable angina. The results showed that ranolazine increased exercise duration, while increasing the time between angina episodes without having any negative haemodynamic effects [62]. Ranolazine’s mechanism of action is not entirely understood, however studies to date have shown that may work via the inhibition of 3-KAT [63], a mechanism that is similar to that of trimetazidine.

1.4 Pharmacodynamics of Perhexilne

1.4.1 Racemic Perhexilne

Perhexilne is arguably one of the first myocardial metabolic agents used clinically and unlike other myocardial metabolic agents is clinically available in Australia and New Zealand. It has been recently reintroduced in the United Kingdom and also gained orphan status in the United States of America making it an appropriate platform for the development of new myocardial metabolic agents.

1.4.1.1 Anti-Inflammatory Mechanism of Perhexilne

Perhexilne has been shown to inhibit super oxide (O₂⁻) production via NADPH oxidase [9]. O₂⁻ derived from NADPH oxidase in phagocytic and endothelial cells is the major source of superoxide contributing to endothelial dysfunction. The NADPH oxidase complex is made up of four major units: two membrane bound subunits p22phox and gp91phox and two cytosolic components p47phox and p67phox [64]. This preassembled enzyme cannot produce O₂⁻ · , as it first needs to be activated. This is accomplished by phosphorylation of the p47phox subunit, which then interacts with the membrane subunits. [64, 65]. In a study on isolated human neutrophils, perhexilne significantly inhibited superoxide formation via NADPH oxidase by
up to 50% at clinically relevant concentrations. Furthermore, the same group was able to demonstrate that perhexiline had no effect on the assembly of the enzyme and it was only the pre-assembled complex that was affected [9].

1.4.1.2 Perhexiline In Heart Failure

There is a growing body of evidence to suggest that heart failure is associated with altered myocardial energetics. Studies have confirmed that the ratio of the high-energy phosphate storage molecule, phosphocreatine (PCr) and adenosine triphosphate (ATP), are significantly diminished in patients with HF, including non-occlusive hypertrophic cardiomyopathy (HCM) [66].

In a double blind, placebo-controlled study in HF patients, perhexiline significantly improved myocardial function and increased the PCr:ATP ratio from 1.27± 0.02 to 1.73± 0.02, significantly improving myocardium energetics [67]. Perhexiline was also recently shown to improve myocardial energetics and function in patients with HCM, a subtype of HF, for which there is currently no cure [13]. Thus, perhexiline may be a clinically useful pharmacotherapy in the management of heart failure.

1.4.1.3 Perhexiline In Aortic Stenosis

Perhexiline has been shown to provide symptomatic relief in elderly patients suffering from aortic stenosis [12]. Although this study was uncontrolled, Unger et al showed that administration of perhexiline to elderly patients (mean age 80 years, refractory to treatment and/or unable to undergo aortic valve replacement, as well as having baseline New York Heart Association (NYHA) classification scores of 3 or 4) relieved the symptomatic
characteristics of aortic stenosis in 13 of 15 patients over the first 3 months of treatment [12]. This finding has some major clinical implications, as there is currently no alternative to aortic valve replacement surgery. Perhexiline could provide another option for those patients who do not want to undertake the risk of open-heart surgery, who are of advanced age and/or frailty where the risk of surgery outweighs potential benefits, as well as patients whose disease state does not make them suitable candidates for aortic valve replacement.

1.4.1.4 Anti-anginal Action of Perhexiline

Early on in its history, the mechanism of action associated with perhexiline use was elusive and it was first described as a calcium, sodium and potassium channel inhibitor [1, 68]. It was discovered more recently that perhexiline's primary mechanism of action was not the inhibition of these ion channels, but rather appeared to be associated with effects on cellular metabolism [69].

It is now considered that perhexiline’s main mechanism of action is the inhibition of β oxidation via the carnitine palmitoyltransferase -1 (CPT-1) enzyme [1]. A study [69] using isolated cardiac and hepatic mitochondria (from Sprague Dawley rats) incubated with perhexiline found IC₅₀ (inhibitory constant) values for perhexiline that were lower for cardiac (77 µmol/L) than hepatic (148 µmol/L) CPT-1. The only compound that had a lower IC₅₀ was the naturally occurring malonyl-CoA. Perhexiline inhibition was of a non-competitive nature with respect to malonyl-CoA and another CPT-1 inhibitor hydroxyphenylglyoxylate (HPG). When the protease, nagarase, was added to the incubation mixture both malonyl-CoA and HPG lost their affinity for CPT-1 (nagarase destroyed the binding site on CPT-1) whereas perhexiline was not significantly affected, suggesting that although perhexiline is a
competitive inhibitor of palmitoyl-CoA, it may bind to an alternate site to that of the endogenous inhibitor malonyl-CoA.

Although the exact mechanism by which perhexiline inhibits CPT-1 is not conclusively known, its IC$_{50}$ values show a wider picture as to how this drug selectively inhibits cardiac CPT-1, thereby reducing fatty acid metabolism and as a result redirecting myocyte cellular metabolism towards carbohydrate utilization [10, 70].

1.5 Toxicity of Perhexiline

1.5.1 Clinical Toxicity

The chronic administration of perhexiline where supra-therapeutic concentrations are maintained in plasma, leads to severe toxic effects, principally peripheral neuropathy and/or hepatotoxicity in the form of micro- and macro-vesicular steatosis.

Case reports throughout the 1970s and 1980s all associated administration of perhexiline maleate with peripheral neuropathy in a sub-group of patients treated. In all instances patients suffered from paraesthesia, muscular weakness and, in severe cases, muscular atrophy, most of which subsided after cessation of perhexiline [71, 72].

An early study conducted by Morgan et al investigated four patients treated chronically with perhexiline who had developed liver disease. Three out of the four patients developed micronodular cirrhosis with severe hepatitis and steatosis, two of which also showed peripheral neuropathy. The other patient developed moderate hepatitis with peripheral neuropathy [73]. Although the report states the dosage of perhexiline used in each case, there is no indication of plasma perhexiline concentrations or those of its primary hydroxy metabolites (see
metabolism below on pg. 51), making it difficult to ascertain where these patients lie in the therapeutic range.

Other side-effects associated with the use of perhexiline include weight loss and hypoglycaemia (in diabetics) [73]. The theory as to why only a small sub-population of perhexiline administered patients suffered from the severe adverse effects was speculative. In these early studies, the general opinion was that these adverse effects were associated with elevated plasma perhexiline concentrations due to either: hepatic disease (possibly drug-induced) leading to impairment in the metabolism of perhexiline, or some genetic variable that impeded perhexiline metabolism [73, 74]. It was later realised this resulted from impaired metabolism of perhexiline due to a genetic polymorphism of the CYP2D6 enzyme present in approximately 7% of the Caucasian population (discussed late in this Chapter).

1.5.2 In Vitro Toxicity Studies

The administration of perhexiline to primary rat hepatocytes demonstrated a significant effect on β-oxidation. Perhexiline at low concentrations (5 µmol/L) seems to have no significant effect on ATP production, β-oxidation or triglyceride formation after being incubated for a 24-hour period. However after a 72-hour incubation period, perhexiline significantly decreased β-oxidation and increased ATP and triglyceride formation with no significant effect on oxygen consumption or cell death when compared to control [75].

At higher concentrations (25 µmol/L), perhexiline showed a substantial decrease in ATP production, β-oxidation and triglyceride formation after a 24-hour incubation period. This was accompanied by a diminished cell count, which suggests that at these concentrations, perhexiline exhibits toxic effects [75].
Concentrations of perhexiline in the mitochondria were found to be 20-fold higher than those of the initial incubate concentration [75] and may provide some further explanation to its toxic effects. The increase in mitochondrial perhexiline concentration is reliant on the mitochondrial membrane potential. As it enters the intramembranous space of the mitochondria, the uncharged perhexiline molecule becomes partially protonated due to the acidic environment, allowing it to cross the mitochondrial membrane down the electrochemical potential. Once inside the mitochondria, the alkaline conditions disassociate the proton from perhexiline and thus trapping the perhexiline molecule inside. [75].

At high concentrations (200 µmol/L) perhexiline seems to decrease mitochondrial respiration by completely inhibiting the respiratory chain. This eventually results in cell death mainly via oxidative dephosphorylation, which is a result of proton re-entry into the cell without producing any ATP via ATP-synthase and uncoupling of the respiratory chain, which in turn prevents the cell pumping protons back into the intramembranous space [75].

1.5.3 In Vivo Perhexiline Toxicity Studies

A study involving two different rat strains, the Dark Agouti (DA) and the Sprague Dawley (SD), demonstrated significant neuronal toxicity especially in the DA strain. In this study, the DA strain was proposed to represent a model of human CYP2D6 poor metabolism, whereas the SD was a model of extensive metabolism (see section 1.5.2 for more details). Perhexiline induced neuronal lipidosis in histological sections in the DA rat after 4 weeks treatment with 80 mg/kg/day given orally. At this dosage, the dorsal root ganglion taken from these rats revealed a considerable number of inclusion bodies, which were lamellar in appearance. A reduction in neuronal motor latency was also noted in perhexiline-treated DA rats when compared to control (P<0.05) or SD (P<0.025) rats. Hepatic biochemical analyses
revealed no significant differences when compared to controls. Furthermore, this lipidosis became more severe with prolonged exposure to perhexiline, whereas the SD rats showed no such inclusion bodies [76].

1.6 Pharmacokinetics of Perhexiline

1.6.1 Studies with Racemic Perhexiline

Studies in the 1970s with $^{14}$C - perhexiline revealed that 6-12 hr. after oral administration in humans absorption was greater than 88% [77], although absolute bioavailability is unknown as perhexiline is only available as an oral formulation. Perhexiline has a volume of distribution of approximately 1470 L [78] and is highly tissue (mitochondrial) [79] and plasma protein bound (>90%) [80].

Perhexiline is almost exclusively hepatically cleared by metabolism via CYP2D6 (with minor contributions of CYP3A4 and CYP2B6) to its major metabolites cis-OH-(±)-perhexiline, $trans_1$-OH-(±)-perhexiline, $trans_2$-OH-(±)-perhexiline [81]. The conversion of perhexiline to its cis hydroxyl metabolite is the major pathway in its metabolism (Figure 7) [81]. PMs (see below) are not able to produce the cis metabolite and as a result have substantially increased plasma perhexiline concentrations. [79, 82].

Previous studies have reported that patients who developed peripheral neuropathy had a (mean ± s.d) metabolic ratio (the ratio of unchanged parent drug to metabolite produced) of 3.78 ± 0.43, significantly higher (p<0.01) than patients that hadn’t developed the neuropathy with a metabolic ratio of 1.07 ± 0.18. The pharmacokinetic profile showed a higher
concentration of unchanged parent drug compared to metabolite in those patients suffering from peripheral neuropathy when compared to those that didn’t [74, 83].

**Figure 7.** The major metabolic pathway for perhexiline and formation of OH- perhexiline metabolites.
1.6.2 Cytochrome P450s

Cytochrome P450s (CYP’s) are a group of enzymes, which are involved in the degradation, and elimination of exogenous compounds from the body. They play a key role in the metabolism of substances or chemicals derived from either the environment or dietary intake (including pharmaceuticals/xenobiotics), converting these to more excretable compounds as a body defence mechanism [84, 85].

CYP enzymes are classified by a number (indicating the family they belong to), a subfamily letter and another number to identify the individual enzyme within the subfamily, and in some cases an asterix followed by a number at the end, to identify any allelic variants that may be associated with that gene, e.g. CYP2D6*4 [85].

Humans have 57 cytochrome P450 genes but only the encoded proteins from the CYP1, CYP2 and CYP3 families seem to be of great importance in terms of drug metabolism. Each CYP enzyme displays a unique characteristic, whether it is by demonstrating a higher affinity of a drug to a specific protein region or greater selectivity towards one particular enantiomer when drugs are administered as a racemic mixture [85].

Cytochrome P450 metabolism mainly occurs in the liver, however the epithelium in the small intestine also contains cytochrome P450 enzymes and may play a role in the bioavailability of a drug whose primary mechanism of elimination is via cytochrome P450 metabolism. In particular, CYP3A enzymes are concentrated in the tip of villi in the upper small intestine (as well as the liver) and can metabolise a wide array of compounds. As perhexiline is primarily metabolised by CYP2D6, this CYP3A family has only a minor effect on its clearance.
CYP2D6 has been one of the most widely studied cytochrome P450 enzymes with respect to polymorphisms. Although its expression in the liver is relatively low (2-4%), it is responsible for the metabolism of about 25% of currently available drugs [86], which include: antidepressants, antipsychotics, antiarrhythmics, antiemetics, β-blockers and opioids [85, 86].

The CYP2D6 enzyme is a result of the expression of the CYP2D6 gene located on chromosome 22q13.1 [87]. The gene is made up of 9 exons with an open reading frame of 1491 base pairs, which code for 497 amino acids. Due to its highly polymorphic nature, there is a large inter-individual variability in the activity of CYP2D6 within a population [88]. There are over 70 reported CYP2D6 allele variants [86] which result in altered enzymatic function by having no enzymatic activity, reduced enzymatic activity or by having increased enzymatic activity. This has resulted in the classification of subpopulations into poor metabolisers (PM), intermediate metabolisers (IM), extensive metabolisers (EM) and ultra-rapid metabolisers (UM) respectively (Figure 8) [86, 89]. In a Caucasian population UMs, EMs, IMs, and PMs account for approximately 3-5%, 70-80%, 10-17% and 5-10%, respectively [86].

### 1.6.2.2 Alleles Producing No Functional CYP2D6 Protein

non-functional protein due to the presence of these allelic variants, giving rise to the PM phenotype. This has a major consequence in clinical settings especially when administered a pharmacological agent that is highly reliant on CYP2D6 for its metabolism. Furthermore, this impaired ability to metabolise certain drugs will inevitably lead to drug-induced toxicity as a result of its accumulation, especially for those agents which have a narrow therapeutic index such as the antiarrhythmic drugs flecainide (therapeutic range 200-1000 ng/mL) [91] and mexiletine (therapeutic range of 0.5-2.0 μg/mL) [92].

1.6.2.3 Alleles Producing Functioning Protein with Decreased Activity

In some cases there are allelic variants, which produce a functional protein; however such proteins have significantly impaired catalytic activity. Alleles *10, *14, *17, *18, *36, *41, *47, *49, *50, *51, *54, *55 and *57 are responsible for the residual function of the resulting protein [86, 93]. A decrease in function is a result of decreased substrate affinity to the enzyme as well as the production of an unstable protein.

The presence of these allelic variants give rise to IMs, however, the highly polymorphic nature of CYP2D6 results in an overlap in the population distribution with EMs [93].
Figure 8. CYP2D6 phenotype definition and distribution in a random European population (N=316). The numbered arrows indicate the range of the sparteine metabolic ratio associated with number of functional genes. Taken from Zanger et al [89].
1.6.2.4 Multiple CYP2D6 Copies

Ultra rapid metabolisers (UM) are those that are able to metabolise probe substrates for CYP2D6 at a rate which surpasses that of the wild type CYP2D6*1 allele. The ability of this particular sub population to metabolise CYP2D6 substrates at an elevated rate is mainly due to the CYP2D6 gene being subject to copy number variations. As an extreme example, it has been reported that in a Swiss family, the father and two of his children had up to 13 copies of a functional CYP2D6 allele [94].

1.6.2.5 CYP2D6 and Perhexiline Metabolism

Perhexiline is a substrate for CYP2D6 and as such is subject to polymorphic metabolism. PM’s are at risk of toxicity due to excess plasma and tissue perhexilene concentrations following doses associated with efficacy in EMs. In contrast, UM’s may be too efficient at eliminating the drug and therefore struggle to attain target concentrations in plasma and risk having reduced or no clinical benefit; although this sub-population has been little studied. Horowitz et al., demonstrated almost no side effects with optimum efficacy in patients where plasma perhexiline concentrations were kept between 0.15 - 0.60 mg/L [7]. In clinical practice in this laboratory, the individualised dosage schedule to achieve this target concentration can range from 50 mg/week in PMs up to 1,000 mg/day in UM’s (i.e., a 140-fold range).

In a pharmacokinetic study by Horowitz et al. [95], five patients were administered a single oral dose of either 150 or 300 mg of perhexilene. The results of the study revealed that after the dose was doubled, the mean peak plasma perhexilene concentration, as well as area under the concentration versus time curve (AUC), was 4.3 and 5.3 times greater, respectively. They also showed that the mean elimination half-life was concentration-dependent with a half-life...
of 11.2 ± 2.1 hours after the 150-mg dose and 19.1 ± 2.8 hours after the 300-mg dose respectively. As perhexiline has a high hepatic clearance almost exclusively via CYP2D6, these results suggest that perhexiline metabolism was saturable and its pharmacokinetics nonlinear in nature. The narrow therapeutic range and such widely varying dosage requirements, calls for strict therapeutic drug monitoring (TDM) upon clinical administration of perhexiline maleate to individualise dosages in order to optimise efficacy without toxicity.

1.6.3 Animal Model CYP2D6 Poor Metabolism

The rat is commonly used as an animal model for the identification of toxicity and drug disposition in humans. They can be genetically modified or specifically bred between species to develop a unique characteristic that mimics a human condition or disease state. One such breed is the Dark Agouti rat, which is an established model of ‘poor’ metabolism involving the CYP2D enzyme family [96]. Debrisoquine is a commonly used substrate for the human CYP2D6 enzyme and a deficiency in the metabolism of this drug may be due to a defective enzyme as a result of mutations in its synthesis or a decrease in its expression, leading to lower enzyme activity and therefore a phenotypic poor metaboliser. Al-Dabbagh et al [96] used inbred rat strains in order to determine an appropriate model of human polymorphic drug metabolism, using the metabolic ratio; that is the ratio of parent drug to metabolite (4-hydroxy debrisoquine) recovered in urine over a set period of time. In this study they observed the metabolism of 5 mg/kg of debrisoquine over 24 hr. in Wistar, Lewis, Fischer A/GUS, PVG, BN and Dark Agouti rats with three rats of each strain. Of all the strains, the Dark Agouti and the Lewis showed drug and metabolite recoveries which suggested that the metabolic ratio of the Dark Agouti rat was consistent with that of human poor CYP2D metaboliser (PM), whereas the Lewis strain showed a metabolic ratio which was very similar to a human extensive metaboliser [96].
Not only does the Dark Agouti strain seem to be more deficient in the metabolism of debrisoquine than any other, but the female Dark Agouti shows greater impairment than its male counterpart suggesting a sex-dependent difference [97]. When debrisoquine 4-hydroxylase activity of the Dark Agouti rat was compared with that of the Fischer and Lewis strains (models of extensive metabolisers), it was significantly reduced by 3.5- to 5-fold, which was consistent with previous studies [97]. Interestingly Kahn et al. also showed that all female rats from each strain had significantly impaired 4-hydroxylase activity when compared to the male in the same strain, with the Dark Agouti female exhibiting the lowest activity of all [97].

In a later study by Gonzalez et al. it was discovered that the Dark Agouti rat possessed two CYP2D proteins, which were termed 2db1 and 2db2 and are now known as CYP2D1 and CYP2D2. In that study they put forward the notion that it was only the CYP2D1 enzyme that possesses the catalytic activity to metabolise debrisoquine and that CYP2D2 had no activity towards debrisoquine [98]. Furthermore CYP2D2 concentrations were 30 times less abundant in male Dark Agouti rats when compared to Sprague Dawley rats and almost absent in female Dark Agouti rats.

In 1989 Matsunaga et al., discovered three more CYP2D proteins termed CYP2D3, 4 and 5. The study also suggested that the inability of the Dark Agouti rat to metabolise debrisoquine to its metabolites was a result of dramatically reduced CYP2D1 concentrations and that CYP2D2, CYP2D3, CYP2D4 and CYP2D5 did not exhibit any metabolic activity towards the drug [99]. The theory that CYP2D1 was the only CYP2D enzyme with the capacity to metabolise debrisoquine in the rat was resolved by Yamamoto et al (1998) and Schulz et al (1999). Using immunochemical measurements with anti-CYP2D peptides it was revealed that there was no significant difference in CYP2D1 protein expression between the proposed poor
metaboliser model, the Dark Agouti and the proposed extensive metaboliser, the Sprague Dawley rat. However the expression of CYP2D2 was decreased between the two species with the Dark Agouti strain showing significantly lower protein content. In order to eliminate the possibility that the Dark Agouti rats’ inability to metabolise debrisoquine was the result of a deficient CYP2D1 protein, recombinant CYP2D1 and CYP2D2 was expressed in yeast. The result clearly showed that CYP2D2 was 20-fold more effective at metabolising debrisoquine than CYP2D1. The introduction of quinine (inhibitor of the CYP2D mediated oxidation of debrisoquine) reduced CYP2D2 mediated oxidation of debrisoquine by 53-67%, whereas CYP2D1 activity was inhibited by 0-6%. They concluded that the metabolism of debrisoquine in the Dark Agouti female rat was primarily mediated by the CYP2D2 enzyme [100-102].

However, this DA/SD rat model does not hold up for all human CYP2D6 metabolised drugs. In humans, propranolol is metabolised by CYPs 2D6, 1A2, 2C19 [102] and UDP-glucuronyltransferase [103], whereas in rats the main metabolic pathway is via the CYP2D isoform. In a report by Komura et al. they studied the effects of metabolism on propranolol and metoprolol in DA and Wistar rats. Administration of metoprolol to DA and Wistar rats coincided with the pharmacokinetic profiles of human PM and EM respectively, whereas the $K_m$ of propranolol for its high affinity enzyme in human liver microsomes was 3-4 µM, 20-50 fold higher than in the corresponding values in rats [104]. The low $K_m$ value together with the involvement of only the CYP2D isoform, may explain why the DA rat is not always an appropriate model of CYP2D6 human metabolism [104, 105].
1.7 Chirality

In 1811 Aarago [106] and in 1812 Biot [106], showed the effects of crystals on the plane of polarized light. In 1848 Louis Pasteur was the first to recrystallise racemic sodium ammonium salt of tartaric acid and discovered that 2 different crystalline structures were produced [107]. Pasteur went on to demonstrate that of these 2 different crystalline structures, only one was fermented by bacteria. It was not until 1847, when van’t Hoff showed that there was a relationship between three-dimensional structures, optical activity and an asymmetrical carbon and thus the concept of chirality began.

Chiral compounds have an asymmetric atom and thus form isomers, which are compounds that share the same molecular formula but differ in their spatial and structural arrangements. Enantiomers are stereoisomers that have identical chemical features, however they differ in their spatial arrangement and thus have a handedness property; that is they may exist as a right or left form and are not superimposable [108]. Enantiomer is the nomenclature given to each chiral compound form and these enantiomers may exist as dextro (+) and or levo (-), which can have different properties with respect to protein, enzyme or transporter interactions. R (Rectus) and S (Sinister) nomenclature often seen associated with enantiomers, refers to the configurations of the atoms attached to the chiral centre. Based on the sequence rule systems devised by Cahn, Ingold and Prelog in 1956, the atoms attached to the chiral centre are ranked based on their atomic number. Highest priority is given to the atom with the highest atomic number. If the priority sequence is to the right the nomenclature given is R and if the priority sequence is to the left then it is designated as S. The terms (+) and (-) or d and l can also be used, however these do not relate to structure but rather refer to the effects on polarised light.
Many commonly prescribed medications are currently in a racemic formulation and one could argue that these compounds contain a 50% impurity as one enantiomer may be responsible for all the therapeutic effects and the other may attribute to the toxic effects. An example is ketamine, $S$-ketamine is an anaesthetic agent, while its enantiomeric counterpart ($R$-ketamine) causes hallucinations [109]. It is clear that enantiomers have variable effects in different biological environments whether it may be due to an increase in affinity for a certain receptor or a different pharmacokinetic profile. As a result of this variability in activity it may actually be safer or more efficacious to market single enantiomers as the therapeutic compounds rather than the racemate mixture [110].

1.7.1 Pharmacokinetics of Perhexiline Enantiomers

Perhexiline is a chiral compound, which has a chiral carbon forming four different bonds and it exists as (+)- and (-)- enantiomers (Figure 1). The enantiomers are metabolised to cis-OH-(+)-perhexiline, cis-OH-(−)-perhexiline, trans1-OH-(+)-perhexiline, trans1-OH-(−)-perhexiline, trans2-OH-(+)-perhexiline and trans2-OH-(−)-perhexiline (Figure 9) [79, 82]. Although several studies have demonstrated enantioselectivity in the pharmacokinetics of perhexiline, to date there are no published papers which have investigated the pharmacodynamic effects of either the (+)- or (-)- enantiomers of perhexiline or consider that enantiomers may differ in their pharmacodynamic and toxicological properties.

The first report on the pharmacokinetics of perhexiline enantiomers was in 1986 by Gould et al [111] who conducted a study investigating the pharmacokinetic properties of the racemic and enantiomeric formulations of perhexiline. Eight healthy volunteers were given an oral dose of 300mg of racemic, (+)- or (-)- perhexiline on separate occasions. Blood samples were taken at an interval of 2 hr. for the next 24 hr. and urine was collected daily. Their results
showed that the peak plasma perhexiline concentration for the (+)- enantiomer was 2.5-fold higher than that of the (-)- enantiomer, which showed no significant difference to 300 mg of racemic formulation. The (-)- enantiomer seemed to be cleared much more rapidly than the (+)- enantiomer by not only showing a lower plasma perhexiline concentration, but also a 28-fold increase in metabolite production when compared to the racemic mix, which was also significantly greater than the (+)- enantiomer. These observations were confirmed by Davies et al [79] and Inglis et al [80], who also showed that both enantiomers displayed non-linear kinetics.

Studies with human liver microsomes suggested that CYP2D6 is the main enzyme involved in the metabolism of both the (+)- or (-)- enantiomers to their respective hydroxyl metabolites in EMs [79]; although elimination seems to be enantioselective for the (-)- perhexiline rather than (+)- perhexiline [79]. However CYP3A4 and CYP2B6 appear to be the main enzymes involved in metabolising the enantiomers to their metabolites in PMs [79].
Figure 9. The chiral structures of the mono- and di-hydroxyl metabolites resulting from perhexiline metabolism, taken from Davies et al [82].
The evidence suggests that the enantiomers of perhexiline are metabolised at different rates by different enzymes. This reveals certain gaps in our knowledge about the pharmacodynamic properties of perhexilene in the human body, as the correlation between the different pharmacokinetic and pharmacodynamic effects of the individual perhexilene enantiomers is yet to be described. For example, it is not known whether it is the (+)- or (-)- enantiomer that may be responsible for the peripheral neuropathy and/or hepatic steatosis seen in clinical toxicity of perhexilene, or whether just one of the enantiomers is responsible for the inhibition of CPT-1, or potentially whether the therapeutic effects of one enantiomer may be masked by the adverse effects of the other?

Hence, one of the aims of this study was to investigate the pharmacodynamic effects of the (+)- and (-)- perhexilene enantiomers.

1.8 Aims and Hypotheses

Perhexilene’s clinical use is complicated by its complex pharmacokinetics, which without therapeutic drug monitoring to individualise therapy, has the potential to cause severe hepatic and/or neurotoxicity in some patients. This thesis describes the investigation of the pharmacodynamic properties of (+)- and (-)- perhexilene in vitro and in vivo using a rat model with the intent of determining if (+)- perhexilene or its optical antipode is responsible for the steatosis and peripheral neuropathy seen in clinical practice. If either enantiomer demonstrates a superior pharmacodynamic profile, whether it is improved toxicity or efficacy, then it may be possible to develop an enantiomerically based formulation that is less toxic and/or more efficacious than the racemic compound currently prescribed.
The overall aim of this thesis was addressed by developing a rat model that would mimic the pharmacokinetics and metabolism of perhexiline in humans. By administering clinically relevant doses of either (+)- or (-)- perhexiline, it should be possible to determine whether either one, or both are responsible for toxicity. In addition, enantioselectivity with respect to one of perhexiline’s potential beneficial effects, inhibition of NADPH oxidase, was also tested in vitro.

The following hypotheses were tested:

1. That the enantiomers of perhexiline demonstrate different potencies for the inhibition of neutrophil NADPH oxidase induced superoxide formation.

2. That the enantiomers of perhexiline demonstrate enantioselectivity in hepato- and neuronal toxicity in the DA agouti animal model.
Chapter Two: Methods
2.1 (+)- and (-)-Perhexiline Preparation

The Chemistry Department at The University of Adelaide prepared the perhexilene enantiomers for this study. The (+)- and (-)-perhexiline were prepared by using a previously reported method from our laboratory [112]. Racemic perhexiline base was obtained from racemic perhexiline maleate source. Briefly, the maleate salt was removed by dissolving it in a sodium hydroxide solution, which was then extracted with ether. These extracts were then washed with water and dried with magnesium sulphate. The solvent was discarded to obtain pure racemic perhexiline.

Individual perhexilene enantiomers were obtained by adding, (S)-1,1'-Binaphthalene-2,2'-diyl hydrogen phosphate (S-BNPA) in acetone which resulted in the precipitation of a diastereomeric salt of (-)-perhexiline + (S)-BNPA. The isolation of the (-)-perhexiline enantiomer from the BNPA salt was achieved by the addition of a chloroform and ammonia solution. Using (+)- enriched perhexiline solution, the procedure was repeated with (R)-BNPA (Figure 10).
Figure 10. Preparation and resolution of the (+) and (-) enantiomers of perhexiline from racemic perhexilene maleate [112]
2.2 Ethics

The liver toxicity study was approved by the Animal Ethics Committee of The University of Adelaide (approval number M79/07) and the SA Pathology/CHN Animal Ethics Committee (approval number 120/07). The neutrophil studies were approved by The Queen Elizabeth Hospital Human Research Ethics Committee (approval number 2007056).

2.3 Animals

Female Sprague Dawley and DA rats (weight 180-200 g) were purchased from The University of Adelaide animal house (Adelaide, South Australia, Australia). The rats were individually housed at The Queen Elizabeth Hospital animal house. For the first week, the rats were allowed to acclimatise to their environment and were fed standard rat chow. During this period, behaviour and feeding patterns were observed and noted. The food pellets were weighed every evening and morning in order to determine the food consumption of these rats (being nocturnal feeders). The larger Sprague Dawley rats consumed around 21 g of food (approx. 7 large pellets), whereas the smaller DA strain consumed less food by comparison (4 pellets, approximately 13 g of food). All rats had ad libitum access to food and water at all times. The animals were kept in controlled conditions, in facilities which provided a 12 hr. day/night cycle at a temperature of approximately 22 °C.

2.4 Perhexiline Dosage Determination and Administration

The dosage regime for the toxicity study was modified from a previous report by Meier et al [76]. Meier et al orally dosed female DA animals for a period of 8 weeks at 80 mg/kg of perhexiline maleate and observed a mean plasma perhexiline concentration of 0.6 mg/L, the
upper limit of the perhexiline therapeutic range in humans. At this dosage, the dorsal root ganglion taken from these rats revealed a considerable number of inclusion bodies, which were lamellar in appearance.

My dosing regimen was based on the toxicity observed from the study of Meier et al. At 80 mg/kg, Mallory bodies accumulated in dorsal root ganglia giving rise to peripheral neuropathy associated with clinical toxicity following chronic perhexiline dosing. However the lack of biochemical changes in the liver, as well as plasma perhexiline concentrations that were near or within the human therapeutic range, indicated that an increase in dosage could achieve hepatic toxicity in the present study. The dose selected in the pilot study was 200 mg/kg, hence 2.5-fold greater than Meier et al [75].

Administration of the drug proved to be challenging. Initial attempts were made to incorporate the drug directly into the food pellets in order to mask its taste. Perhexiline maleate was dissolved in ethanol and the solution was injected into food pellets that were then left to allow the ethanol to evaporate overnight, leaving just the perhexiline maleate. Administration of these ‘perhexiline pellets’ was ineffective, as the rats appeared to only eat parts of the pellet, probably avoiding those areas that contained the drug. After seeking veterinarian advice, a mixture of peanut paste and perhexiline maleate was spread on the food pellet. This proved to be an effective way to administer perhexiline to these rats. During active treatment, each animal was housed individually and the required daily dose of perhexiline maleate was coated onto 7 rat chow pellets for SD rats, or 4 pellets for DA rats, placed in the cages at 4-5 pm each day, and left until the next dose. Food and perhexiline consumption was monitored daily and extra drug-free pellets provided if necessary.
2.5 Tissue Preparation for HPLC

Liver and heart tissue was dissected from the sacrificed rats, snap frozen in liquid nitrogen and stored at -80°C. The tissue was partially thawed in order to remove a slice, which was then completely thawed in saline solution. The smaller piece was dried and then weighed. A volume of cold phosphate buffer (0.15 M, pH 6) twice that of the tissue weight was added and the piece of tissue was further diced into smaller sections. A tissue homogenizer was used to reduce the solid tissue pieces resulting in a homogenates of heart and liver. The aliquots were taken and stored at -80°C.

2.6 Measurement of (+)- and (-)-Perhexiline in Plasma and Tissue

Perhexilene was extracted and quantified using the previously published method of Davies et al. [112], which had been validated in human plasma and liver microsomes. Briefly, 50 µl of a 10 mg/L solution of prenylamine (internal standard) as well as 50 µl of 2 M NaOH and 4 ml of a 10% ethyl acetate in n-hexane were added to 500 µl of liver or heart homogenate. This homogenate preparation was shaken for 15 min at 100 oscillations per min and then centrifuged at 3000 rpm for 10 min at 10°C. It was then placed in a dry ice/ethanol bath whereby the aqueous layer was snap frozen and subsequently discarded and the organic phase collected. The organic layer was transferred to 5 ml glass tubes and placed in a vacuum centrifuge to dry at room temperature. Once dried, 200 µL of the derivatising agent, naphthyl ethyl isocyanate (NEIC) in acetone (0.05%), was added, vortex mixed and incubated at room temperature for 5 min. Following this incubation, the addition of 200 µL of a 0.5M NaOH solution was followed by that of 3 mL of 10% ethyl acetate in n-hexane. The 5 mL tube was capped and vortex mixed for 5 min, then centrifuged at 2500 rpm for a further 3 min at 10°C. The aqueous phase of the mixture was snap frozen in a dry ice/ethanol bath and the organic
layer transferred to another 5 mL tube and the aqueous layer discarded. The tube was placed in a vacuum centrifuge at room temperature until it was completely dry. The resulting residue was reconstituted with 150 µL of the 80% methanol and 20% water mobile phase.

The HPLC conditions were as described in Davies et al. [112]. Briefly, analyses were conducted on an Agilent 1100 series HPLC apparatus (Agilent Technologies, Forest Hill, Victoria, Australia) operated by Chemstation for LC 3D software. The hardware consisted of a model G1322A degasser, a model G1311A pump operating at 1 mL/min, a model G1313A autosampler and a model G1321A fluorescence detector with excitation and emission wavelengths of 218 and 334 nm, respectively. Resolution of the diastereomers was achieved using a Merck Purospher RP-18E column (5 µm, 125 mm × 4 mm) at ambient temperature. The mobile phase was composed of 80% methanol and 20% water for the first 12 min, at which time the methanol was increased to 86% in a linear gradient over 1 min and maintained at that concentration for the remaining 16 min of each sample run time. An example of the chromatogram obtained under these conditions is shown in Figure 11.

The same method and conditions were used to determine concentrations of the perhexiline enantiomers in plasma.
2.6.1 Validation of HPLC Perhexiline Assay in Liver Homogenate and Plasma

Calibration and quality control (QC) samples were prepared using control liver and heart homogenates spiked with (±)-perhexiline maleate dissolved in 10% methanol with 0.1M HCL. As the method was a minor modification of a previously published assay established in our laboratory [112], an abbreviated revalidation was carried out by determining intra- and inter-assay accuracy and precision which were required to be within 15% except at the lower limit of quantitation (LLOQ) where 20% was acceptable. Calibration curves were only accepted if the $r^2$ was greater than 0.98.

The intra assay validation in liver homogenate for (+)-perhexiline demonstrated a coefficient of variation (CV) and a bias of 5 and 7% for the highest calibrator (2 mg/L, n=6) and 17 and 7% for the lowest calibrator (0.05 mg/L, n=6), respectively. (-)-Perhexiline had a CV and bias of 6% at the highest calibrator (2 mg/L, n=6), whereas the lower calibrator (0.05 mg/L, n=6) had a CV and bias of 18 and 6%, respectively. The inter assay validation for (+)-perhexiline had a CV of 10 and 8% and a bias of -5 and 15% for the highest and lowest calibrators respectively, whereas (-)-perhexiline had a CV of 13 and 16% and a bias of -7.0 and <0.1% for the highest and lowest calibrators respectively.

Two QC samples were run with each assay, comprising liver homogenate spiked to a concentration of 0.075 and 0.75 mg/L of (±)-perhexiline in QC1 and QC2, respectively. The inter assay CV and bias for QC1 was 14 and 13% for (+)-perhexiline, and 9 and 13% for (-)-perhexiline. For QC2, the CV and bias was 8 and 14% for (+)-perhexiline, 8 and 13% for (-)-perhexiline respectively.
As there was not enough heart homogenate to perform a validation assay in this tissue, we used calibration curves and QC’s that were made up in liver homogenate.

The plasma concentrations of perhexiline and its enantiomers were measured by a published method that was established and validated in our laboratory [112]. As the method was a minor modification of a previously published assay established in our laboratory, an abbreviated revalidation was carried out by determining intra- and inter-assay accuracy and precision. The intra assay validation in plasma for (+)-perhexiline demonstrated a coefficient of variation (CV) and a bias of 3 and 5% for the highest calibrator (2 mg/L, n=6) and 10 and 10% for the lowest calibrator (0.05 mg/L, n=6), respectively. (-)-Perhexiline had a CV and bias of 3 and 5% at the highest calibrator (2 mg/L, n=6), whereas the lower calibrator (0.05 mg/L, n=6) had a CV and bias of 10 and 10%, respectively. The inter assay validation for (+)-perhexiline (n=3) had a CV of 1 and 9% and a bias of -1 and -10% of for the highest and lowest calibrators respectively, whereas (-)-perhexiline (n=3) had a CV of 1.0 and 13% and a bias of -1 and -13% for the highest and lowest calibrators respectively.
Figure 11. A liver homogenate chromatogram from the method described. A) Represents a chromatogram from a blank sample without internal standard. B) Represents a chromatogram from a rat liver dosed with 200 mg/kg of racemic perhexiline. The chromatography conditions allows for separation of (+)- and (-)-perhexiline and the prenylamine internal standard, the retention time for the internal standard was 10.7 min, the (+) perhexiline enantiomer was 25.1 min, and the (-) perhexiline enantiomer was 27.1 min.
2.7 Measurement of OH-perhexiline in Plasma and Liver

Measurement of cis-, trans1- and trans2-OH-perhexiline was also based on a previously published method by Davies et al [82], which had been validated for plasma and liver microsomes. The assay was therefore revalidated to measure OH-perhexiline in heart, liver and plasma homogenate. Briefly, 100 µL of 10 mg/L of the internal standard (fendiline) was added to the liver homogenate. This was followed by the addition of 50 µL of 2M NaOH and 4 mL of a 30% dichloromethane in n-hexane. This homogenate preparation was shaken for 15 min at 100 oscillations per minute and then centrifuged at 3000 rpm for 10 min at 10°C. It was then snap frozen in a dry-ice/ethanol bath whereby the aqueous layer was discarded and the organic phase was decanted into 5 ml glass tube. It was then placed in a vacuum centrifuge to dry at room temperature. Once dried, 200 µL of the derivatising agent, dansyl chloride in acetone (0.015M), was added, vortex mixed and incubated at 40°C for 1 hr. Following this incubation, 200 µL of a 0.3M NaHCO₃ solution was added, followed by 3 mL of n-hexane. The 5 mL tube was caped and vortex mixed for 5 min, then centrifuged at 2500 rpm for a further 3 min at 10°C resulting in an organic and aqueous layer. The mixture was snap frozen in a dry-ice/ethanol bath and the organic layer was decanted into a 5 mL tube and the aqueous layer discarded. The tube was placed in a vacuum centrifuge at room temperature until it was completely dry. The resulting residue was reconstituted with 150 µL of the 80% methanol and 20% water mobile phase.

The HPLC instrument and analytical column were the same as for the enantiomer assay mentioned previously in section 2.6 with minor modifications. This assay was performed at a temperature of 30 °C and the mobile phase was 80% methanol and 20% distilled water for the first 14 min, at which time the methanol was increased to 100% for the remaining 8 min of each sample run time [82]. An example of the chromatogram obtained under these conditions
is shown in Figure 12.

A)

B)
**Figure 12.** Chromatogram of liver homogenate for the method described. A) Represents a blank sample without internal standard. B) Represents a chromatogram from a rat liver following administration of 200 mg/kg of (+)-, (-)- or racemic perhexiline. The retention times for the OH-perhexiline (Px) metabolites were: cis- 10.8 min, *trans-1*- 12.4 and *trans-2-* 13.6 min, with the internal standard (fendiline) at 18.8 min, respectively.
2.7.1 Validation of HPLC OH-Perhexiline Assay in Liver Homogenate

Pure cis- and trans2-OH-perhexiline were obtained from Sigma Pharmaceuticals (South Croydon, Victoria, Australia). Calibration and QC samples were prepared by spiking control liver homogenates with each metabolite prepared in 10% methanol with 0.1M HCL. The intra-assay validation for cis-, trans-1- and trans-2- OH-perhexiline had CVs of 2.39, 3.23 and 3.20 and biases of -3.33, -5.33 and -4.67% for the highest calibrator (5 mg/L), respectively. At the lowest calibrator cis-, trans-1- and trans-2- OH-perhexiline had CV’s 11.91, 12.45 and 10.65% and biases of -8, -12 and -16% respectively. The inter-assay validation for cis-, trans-1- and trans-2- OH-perhexiline had CVs of 0.25, 0.17 and 0.18% and biases of -1.80, -1.96 and -1.88% for the highest calibrator (5 mg/L), respectively. At the lowest calibrator (0.05 mg/L) the CVs were 6.25, 8.2 and 5.39% and biases were 5.2, 5.2 and -3.2% for cis-, trans-1- and trans-2- OH-perhexiline, respectively. Inter- and intra-assay validations were performed with 6 replicates.

Two QC samples were run with each assay. Control liver homogenate was spiked with cis-, trans-1- and trans-2- OH-perhexiline to final concentrations of 0.075 or 0.75 mg/L for QC1 and QC2, respectively. The inter-assay CV and bias at the lowest QC was 17 and 7% for cis-, 13 and 4% for trans-1- and 19 and 2% for trans-2- OH-perhexiline respectively. At the highest QC the CV and bias was 13 and 4% for cis-, 10 and 1% for trans-1- and 13 and -2% for trans-2- OH-perhexiline respectively.

Plasma OH-perhexiline concentrations were measured by a published method that’s established and validated in our laboratory [82]. As the method was a minor modification of a previously published assay established in our laboratory, an abbreviated revalidation was carried out by determining intra- and inter-assay accuracy and precision. The intra-assay
validation (n=6) for cis-, trans-1- and trans-2- OH-perhexiline had CVs of 7, 6 and 6% and biases of 1.6, 0.4 and 0.4% for the highest calibrator (5 mg/L), respectively. At the lowest calibrator cis-, trans-1- and trans-2- OH-perhexiline had CVs of 17, 19 and 19% and biases of 4, 16 and 8% respectively. The inter-assay validation (n=3) for cis-, trans-1- and trans-2-OH-perhexiline had CVs of 8, 5 and 6% and biases of -2, -5 and -5% for the highest calibrator (5 mg/L), respectively. At the lowest calibrator (0.05 mg/L) the CVs were 12, 13 and 13% and biases were -7, -13 and -13% for cis-, trans-1- and trans-2- OH-perhexiline, respectively.
2.8 Neutrophil Preparation

Neutrophils were prepared using a previously established method in our laboratory [113]. Briefly, 20 mL of blood was collected in 4 mL of 4.5% EDTA. The blood was centrifuged at 150 x g for 10 min, resulting in the separation of plasma from blood cells. The plasma was discarded and a volume of Hanks Balanced Salt Solution (HBSS) equal to that of the discarded plasma was added. The blood was then underlayed with Lymphoprep® (Axis-Shield, Oslo, Norway) and spun at 500 x g for 30 min.

**Figure 13.** This figure shows the various phases following centrifugation of the cellular fraction of blood through the Lymphoprep® medium.

The Lymphoprep separates the constitutive blood cells into different layers. As shown in Figure 13, at the top is the media layer, followed by the lymphocytes and monocytes layer, followed by the Lymphoprep and the red blood cell layer at the base. At the top of the red blood cell layer and just below the Lyphoprep, there is a much smaller layer of neutrophils. Layers above the neutrophil and red blood cell layers were aspirated and discarded. The red blood cells were lysed with lysis buffer, spun down at 500 x g for 10 min and lysed blood cells discarded, leaving only a neutrophil pellet. This was washed with 15 mL of HBSS
resuspended and counted using trypan blue exclusion. The cells were then plated at 1.6 x 10^6 and incubated with (+)-, (-)-, or racemic perhexiline for 1 hr. at 37°C (as described in chapter 3). The cells were dark adapted and incubated for a further 15 min following the addition of 10 µM of the chemiluminescent agent lucigenin. A picolite luminometer (Packard Instruments) was used to measure superoxide formation following the stimulation by 4 µM fMLP (as described in Chapter 3). Superoxide formation was measured over a 5 min period with the area under the chemiluminescence versus time curve (AUC) determined by the linear trapezoidal method after subtracting blank values.

Concentration response curves were fitted by comparing sigmoidal dose response models with a slope =1 or variable slope (hill equation). The sigmoidal dose response curve with a variable slope was determined to be the more appropriate model by demonstrating more stringent 95% confidence intervals as well as a greater coefficient of determination (R^2).
Chapter Three: Lack of Clinically significant Stereoselectivity in the Inhibition of Human Neutrophil Superoxide Formation by (+)- and (-)-Perhexiline
There is a growing body of evidence suggesting that inflammation may play a crucial role in the progression of cardiac disorders such as atherosclerosis, endothelial dysfunction and heart failure. With an ageing population it is becoming increasingly difficult to treat such patients without the use of invasive procedures that carry a higher morbidity and mortality rate thus impacting quality of life. Therefore there is substantial emphasis for the development of pharmacological agents to treat such patients.

The anti-anginal agent perhexiline has been reported to demonstrate anti-inflammatory properties at a clinically relevant concentration. Due to its chiral properties perhexiline is marketed as a racemate containing a 50:50 mixture of (+)- and (-)- perhexiline. However the use of this drug has been limited due to its hepatic and neuronal toxicity and as such it is only available in Australia and New Zealand under strict therapeutic drug monitoring.

There are currently no studies in the reported literature that investigate the toxicity of the individual perhexiline enantiomers (Chapter 5) or their anti-inflammatory effects.

In this study we investigated the effects of (+)- and (-)-perhexiline on neutrophils, (a major component in the inflammatory process responsible for the production of O$_2^-$, which is critical in the eventual damage of cellular membranes and DNA) from healthy volunteers and patients with stable angina. This study demonstrates that both enantiomers dose-dependently inhibit O$_2^-$ formation by neutrophil NADPH oxidase and that this inhibition is enantioselective favouring (-)-perhexiline, providing further impetus to study the effects of the individual perhexiline enantiomers in other disorders where inflammation may progress or even contribute to the disease.
Lack of Clinically Significant Stereoselectivity in the Inhibition of Human Neutrophil Superoxide Formation by (+)- and (-)-Perhexiline

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Introduction

Atherosclerosis, diabetes, hypertension and heart failure are all disorders that have been linked to endothelial dysfunction as a result of reactive oxygen species (ROS) [1]. NADPH oxidase is one of the major sources of superoxide production in cardiovascular disease [1, 2]. Phagocytes such as neutrophils use the reactive superoxide anion as a defence against invading pathogens via an inflammatory response. In addition, superoxide is also a crucial component in the regulation of key physiological processes and enzymes such as apoptosis and cellular signalling [3, 4]. The various isoforms of NADPH oxidase comprise differing subunits that make up the enzyme. Phagocytic NADPH oxidase is made up of two membrane bound subunits, p22\textsuperscript{phox} and NOX-2, and three cytosolic subunits, p40\textsuperscript{phox}, p47\textsuperscript{phox} and p67\textsuperscript{phox}. This preassembled enzyme requires activation, which is accomplished by phosphorylation of the p47\textsuperscript{phox} subunit allowing interactions with the membrane subunits [5, 6].

Perhexiline, 2-(2,2-dicyclohexylethyl)piperidine, is an efficacious antianginal drug approved for clinical use in Australia and New Zealand in patients who are refractory or contraindicated to conventional drugs and/or surgical therapy. Its antianginal actions are believed to be a result of the inhibition of carnitine palmitoyltransferase-1 (CPT-1), the rate limiting enzyme in fatty acid oxidation [7]. This inhibition facilitates a shift in myocardial metabolism from lipid to carbohydrate utilization, resulting in an oxygen sparing effect. This effect is highly advantageous especially in periods of myocardial ischemia, since carbohydrate metabolism utilises less oxygen to produce the same amount of adenosine tri-phosphate (ATP) when compared to the oxidation of fatty acids [8-11]. However recent studies have shown that the therapeutic effects of perhexiline extend beyond management of angina and the drug is also beneficial in patients with acute coronary syndromes [12], inoperable aortic valvular stenosis [13] and heart failure [14]. It has been shown that perhexiline increases platelet responsiveness to nitric oxide and also decreases superoxide release by neutrophils [15].
latter is of interest as there is increasing evidence that nitric oxide availability is considerably reduced as a result of its interaction with superoxide, leading to the formation of bioactive compounds such as peroxynitrite which has been linked to hypercholesterolemia, hypertension, endothelial dysfunction and heart failure [2, 16]. In a study by Kennedy et al., perhexiline was found to inhibit superoxide formation by NADPH oxidase present in human neutrophils and human endothelial cells [17]. Considering that these two cell types are the major source of superoxide in the cardiovascular system, this effect of perhexiline may contribute to its beneficial effects in treating cardiovascular disease [17]. However, despite its significant clinical efficacy, at high concentrations perhexiline can cause severe neurotoxicity [18, 19], and hepatotoxicity [20] and its clinical use is guided by therapeutic drug monitoring.

Perhexiline is a chiral compound and is formulated and marketed as a racemic (50:50) mixture of (+)- and (-)-enantiomers. Most studies involving perhexiline have been carried out using the racemic formulation so that the pharmacological actions of the individual enantiomers are relatively unknown. Enantiomers of the same drug can have differing effects in the same biological environment. A classic example of this is the anticoagulant drug warfarin, which was introduced for clinical use over 40 years ago. Warfarin exists as R- and S-warfarin enantiomers with the pharmacological activity residing predominately with the S-enantiomer [21]. In this study, we aimed to investigate the contribution of the individual enantiomers to the pharmacological effects of perhexiline on superoxide formation by human neutrophils from both healthy subjects and patients with stable angina pectoris.
Materials and Methods

Chemicals

Bis-N-methylacridinium nitrate (lucigenin), fMLP (N-formyl Met Leu Phe) and perhexilene maleate were purchased from Sigma Chemical Company (Castle Hill, NSW, Australia). (+)- and (-)-perhexiline (HCL salts) were synthesised and characterised as previously described [22].

Methods

The study was approved by the Ethics of Human Research Committee at the Queen Elizabeth Hospital. Eleven healthy subjects and 6 patients were recruited and gave written informed consent to participate. Inclusion criteria for healthy subjects required them to be free from illness and not taking any medication, including anti-inflammatory agents that might affect neutrophil function. Patients were included if they were aged 18 years or over, were scheduled for an angiogram or angioplasty at the Queen Elizabeth Hospital’s cardiac catheterisation laboratory, and had symptomatic stable angina. They were excluded from the study if they were receiving calcineurin inhibitors, corticosteroids, cytotoxic immunosuppressants, immunosuppressant antibodies, sirolimus derivatives, azathioprine, mycophenolate and any long acting nitrates or perhexilene.

Neutrophil Extraction

Blood (20 ml) was collected in a 50 mL tube containing 4% EDTA at a pH of 7.35 and neutrophils extracted as per Kennedy et al [17]. Briefly, the blood was centrifuged at 150 x g for 10 minutes and the subsequent plasma was discarded and replaced with an equal volume of Hanks Balanced Salt Solution (HBSS) pH 7.4. Using a Ficoll–Hypaque gradient (Axis-
Shield, Oslo, Norway), blood components were separated into three layers after centrifugation at 550 x g for 30 minutes. The upper and middle layers consisted of media, monocytes and lymphocytes and were discarded. The lower layer, consisting of red blood cells and neutrophils was resuspended, to the exact volume taken up by the upper and middle layers, with lysis buffer (ammonium chloride 155 mM, EDTA 100 µM, NaHCO₃ 10 mM), in order to lyse the red blood cells. The cells were then centrifuged at 500 x g for 10 minutes and the supernatant discarded, leaving the neutrophil pellet. Neutrophils were resuspended with lysis buffer re-centrifuged and then washed and resuspended in HBSS.

**Chemiluminescence assay of superoxide**

Extracted neutrophils were resuspended at 1.6x10⁶ cells in a final volume of 300 µL of HBSS. They were incubated for 45 min at 37°C in the presence of (+)-, or (-)-perhexiline at a final concentration of 0.2, 0.5, 1, 2 and 10 µM or vehicle, and then dark adapted for a further 15 min. Superoxide release was stimulated by the addition of fMLP (4 µM final concentration) and the response was measured in arbitrary chemiluminescent units by a luminometer (Berthold Technologies, AutoLumat Plus LB953, Germany) over a 5 min period with the area under the chemiluminescence versus time curve (AUC) determined by the linear trapezoidal method after subtracting blank values.

**Cell Viability**

Neutrophil viability was measured by trypan blue exclusion and was not affected by the incubation with (+)- or (-)-perhexiline, with up to 99% viability achieved (data not shown) when compared to controls.
Data analysis

For each subject, superoxide AUC in samples incubated with vehicle was used as the value for maximal superoxide formation and AUCs measured in the presence of test drugs were expressed as a percentage of the maximal AUC. Concentration-response curves were constructed describing percentage superoxide inhibition versus the log of the drug concentration and the Hill equation used to derive individual IC50 values. Data were not normally distributed and are presented as a median (range) IC50 values and paired comparisons analysed non-parametrically by the Wilcoxon matched-pairs signed rank test.
**Results**

Neutrophils were extracted from 11 healthy subjects (6 males, 5 females ranging in age from 25 – 50 years). Both (+)- and (-)-perhexilene inhibited superoxide formation in a concentration-dependent manner after stimulation by fMLP (Fig. 1). The median (range) IC50 of (+) perhexilene was 1.64 µM (0.54-3.58µM), which was significantly higher (P<0.05) when compared to that of (-)-perhexilene, 1.19 µM (0.55-2.01µM) (table 1).

Six patients diagnosed with angina participated in this study; their clinical status and medications are shown in Table 2. Both enantiomers of perhexilene also inhibited superoxide formation by neutrophils isolated from patients (Fig. 2). The IC50 values for (+)- and (-)-perhexilene were not significantly different, with median values of 2.07 µM (1.04-2.95 µM) and 1.35 µM (1.08-1.86 µM) respectively, and were similar to those observed with neutrophils from healthy subjects (Table 1).

Median baseline superoxide AUC’s in healthy volunteers, 1.92 x 10^8 (0.56-4.12), were not significantly different (P=1.00) to those in patient volunteers, 1.65 x 10^8 (0.64-4.58).
Discussion

Recent studies have suggested that the release of superoxide from activated neutrophils may play a major role in the progression of acute coronary syndromes and in ischemic reperfusion injury. Buffon et al. showed that in patients with unstable angina, neutrophils were activated as they crossed the coronary vascular bed [23]. Activated neutrophils release superoxide which not only damages the vascular endothelium contributing to its dysfunction, but also mediates the recruitment of more neutrophils via the increased expression of adhesion molecules [23]. Availability of nitric oxide, an important physiological vasodilatory factor in the myocardial endothelium, is reduced by superoxide. Its reaction not only decreases nitric oxide synthase expression but it also binds to free nitric oxide to produce peroxynitrite, a powerful oxidising agent capable of inflicting damage to the myocardial cells’ DNA and proteins [6, 16, 24].

The main finding of this study is that both (+)-perhexiline and (-)-perhexiline showed significant inhibition of superoxide formation by neutrophils isolated from both healthy subjects and patients diagnosed with myocardial ischemia. In both subject groups, the IC50 values for inhibition of superoxide formation were similar to those previously reported with the racemic formulation [17] and were within the range of total plasma perhexiline concentrations attained clinically. However, perhexiline is highly protein bound in plasma [25] and unbound concentrations are unlikely to exceed 0.1 µM. Therefore the clinical relevance of this effect may be small unless there is significant concentrations of perhexiline within the neutrophil membrane following longer term exposures. Perhexiline is highly concentrated within tissues [29] and it is possible that with chronic dosing, at steady state the intracellular concentrations achieved clinically may be comparable to those attained following the short-term (1hr) incubations utilised in this study.
In healthy subjects, (-)-perhexiline was a slightly more potent inhibitor of neutrophil superoxide formation, with a median IC50 approximately 30% lower than that of (+)-perhexiline (eudismic ratio = 1.37). However, this small difference is unlikely to be of clinical significance, since, due to enantioselectivity in their metabolism [26], systemic exposure to (+)-perhexiline is on average 1.5 to 2.5-fold greater than that of (-)-perhexiline. The higher concentration of circulating (+)-enantiomer may therefore compensate for its slightly higher IC50 values for superoxide inhibition. Thus, both enantiomers are likely to contribute to any clinical anti-inflammatory effects of perhexiline via inhibition of superoxide formation.

The lack of clinically significant enantioselectivity in the inhibition of neutrophil superoxide formation by (+)- and (-)-perhexiline is also supported by the observations in the patient group, where both enantiomers inhibited superoxide formation in a concentration-dependent manner with IC50 values comparable to those observed for healthy subjects. However, in the patient group, we were unable to detect the small difference in potency probably due to the smaller sample size. All of the patients participating in this study had hypertension and/or angina. Two patients had other metabolic disorders such as diabetes and hypercholesterolemia. All were treated with statins, 2 patients were treated with angiotensin converting enzyme inhibitors and 2 were given medication to manage their diabetes (Table 1). Despite the array of drugs these patients received, (+)- and (-)-perhexiline showed a similar pattern in the inhibition of superoxide formation when compared to healthy volunteers.

Racemic perhexiline has previously been shown to directly inhibit neutrophil NADPH oxidase activity [17]. Using phorbol myristate acetate (PMA) (a chemical which promotes the assembly of the NADPH oxidase subunits), Kennedy et al. demonstrated that racemic-perhexiline does not affect the assembly of the NADPH oxidase subunits in neutrophils [17], and that it does not react directly with superoxide [17]. In this study, the slight
enantioselectivity with respect to inhibition of superoxide formation in healthy subjects further supports direct inhibition of NADPH oxidase activity, since although enantiomers share the same physical and chemical properties, their structural differences can affect their orientation and interactions with proteins.

The inhibitory activity of perhexiline with respect to NADPH oxidase may extend to other cells and tissues that express the enzyme. Vascular endothelial cells have been shown to express the membrane bound NOX-2 and its subunits p40phox, p47phox, and p67phox, which are the same units that make up the NADPH oxidase present in neutrophils [27, 28], suggesting that NADPH oxidase may play an important role in controlling the tone of vascular smooth muscle and therefore consolidating its contribution to cardiovascular diseases. The current study has shown that both enantiomers of perhexiline inhibit the formation of superoxide by neutrophils isolated from both healthy volunteers and patients with angina. Furthermore, superoxide inhibition by (+)- or (-)-perhexiline did not appear to be affected by the co-administration of drugs used to manage angina or other common underlying diseases.
Table 1 Individual and median (range) IC50s and Hill slope coefficients for neutrophil NADPH superoxide inhibition from A) 11 healthy volunteers and B) 6 patients (* indicates P<0.05 versus (+)-perhexiline).

A)

<table>
<thead>
<tr>
<th>ID</th>
<th>(-)-Perhexiline</th>
<th>(+)-Perhexiline</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 (µM)</td>
<td>Hill Coefficient</td>
<td>IC50 (µM)</td>
</tr>
<tr>
<td>1</td>
<td>0.88</td>
<td>1.24</td>
</tr>
<tr>
<td>2</td>
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<td>0.92</td>
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<tr>
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<tr>
<td>6</td>
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<td>1.29</td>
</tr>
<tr>
<td>7</td>
<td>0.55</td>
<td>0.77</td>
</tr>
<tr>
<td>8</td>
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<td>2.29</td>
</tr>
<tr>
<td>9</td>
<td>0.59</td>
<td>2.42</td>
</tr>
<tr>
<td>10</td>
<td>1.92</td>
<td>1.45</td>
</tr>
<tr>
<td>11</td>
<td>0.59</td>
<td>1.21</td>
</tr>
</tbody>
</table>

Median
Range | 1.19* | 1.29 | 1.64 | 1.53 |
<p>| Median | (0.55 – 2.02) | (0.77 – 2.42) | (0.54 – 3.58) | (0.62 – 3.65) |</p>
<table>
<thead>
<tr>
<th>ID</th>
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<th>(+)-Perhexiline</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>IC50 (µM)</td>
<td>Hill Coefficient</td>
</tr>
<tr>
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<td>1.86</td>
<td>4.77</td>
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<td>2</td>
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<td>6</td>
<td>1.45</td>
<td>1.46</td>
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<tr>
<td>Median</td>
<td>1.34</td>
<td>1.65</td>
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<tr>
<td>Range</td>
<td>(1.08 – 1.86)</td>
<td>(0.96 – 4.77)</td>
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**Table 2** Patient’s clinical status and medications. Y.o= years old

<table>
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<tr>
<th>Patient</th>
<th>Age (years)/Sex</th>
<th>Medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Female 73</td>
<td>Simvastatin, irbesartan, esomeprazole</td>
</tr>
<tr>
<td>2</td>
<td>Male 59</td>
<td>Aspirin, atorvastatin, thyroxine, irbesartan, metformin, ketoprofen and diltiazem</td>
</tr>
<tr>
<td>3</td>
<td>Male 47</td>
<td>Perindopril, clopidogrel, aspirin, diltiazem and atorvastatin</td>
</tr>
<tr>
<td>4</td>
<td>Female 60</td>
<td>Aspirin, atenolol</td>
</tr>
<tr>
<td>5</td>
<td>Male 63</td>
<td>Aspirin, rosuvastatin, metoprolol</td>
</tr>
<tr>
<td>6</td>
<td>Male 86</td>
<td>Warfarin, esomeprazole, frusemide, clopidogrel, atorvastatin, candesartan, salbutamol, levodopa, meloxicam, tramadol and amitriptyline</td>
</tr>
</tbody>
</table>
Figure 1 Concentration-response curve for inhibition of superoxide formation in neutrophils from a) healthy subjects and b) patients after incubation with (+)● or (-)▲ perhexiline and stimulation by 4 µM fMLP. Data show median and range for 11 subjects. The median IC_{50} and Hill slope as in Table 2 were used to construct the line of best fit.
References


Chapter Four: Metabolism and Hepatotoxicity of the Myocardial Metabolic Agent Perhexiline in Sprague Dawley and Dark Agouti Rats

Text in manuscript. This manuscript is prepared for submission to and in accordance with the guideline of the journal Drug Metabolism and Disposition.
As we have previously shown enantioselectivity for (-)-perhexiline in its anti-oxidant effects, we now needed to test if (+)- or (-)- perhexiline demonstrated enantioselectivity towards toxicity in an \textit{in vivo} scenario. In order to do this an animal model that mimicked human perhexiline metabolism and toxicity needed to be established.

The two candidates I decided for this study were the DA and the SD rat strains. The DA strain has been extensively used as a poor metaboliser model for substrates for CYP2D6, whilst the SD strain has been used as a model of extensive metabolism for substrates for CYP2D6. Although a previous report involving dosing the DA rat with racemic perhexiline has been published, there was no investigation of the metabolites of perhexiline or the validity as a model of poor metabolism of perhexiline.

This publication was the first to report the metaboliser status of both the DA and SD strain with regards to perhexiline metabolism. Although not a model of CYP2D6 poor metabolism, the female DA rats attained plasma perhexiline concentrations in the mid-high end of the clinical therapeutic range, with enantioselective disposition similar to humans and with a biochemical marker of perhexiline-induced toxicity. Making the DA rat the preferable model to conduct future toxicity and efficacy studies on (+)- and (-)- perhexiline
Metabolism and Hepatotoxicity of the Myocardial Metabolic Agent Perhexiline in Sprague Dawley and Dark Agouti Rats

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2. Running Title Page

Metabolism and Hepatotoxicity of Perhexiline

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Text pages:  20
Tables:    1
Figures:   2
References:  36
Abstract:  279 words
Introduction:  847 words
Discussion:  964 words
Abbreviations:
ATP, adenosine triphosphate; CPT, carnitine palmitoyl transferase; CYP, cytochrome P450; DA, Dark Agouti; EM, extensive metaboliser; HPLC, high performance liquid chromatography; IM, intermediate metaboliser; PM, poor metaboliser; SD, Sprague Dawley; UM, ultra rapid metaboliser.
Introduction

The anti-anginal agent, perhexilene is thought to improve myocardial function via inhibition of the carnitine palmitoyltransferase (CPT) shuttle (CPT-1 and -2), causing a switch in myocardial energy metabolism from the β-oxidation of fatty acids to the utilization of carbohydrates (Kennedy et al., 2000), which require approximately 10-15 % less oxygen in order to generate an equivalent amount of ATP (Kodde et al., 2007). More recently it has become clear that perhexilene also exerts beneficial effects in acute coronary syndrome, heart failure, hypertrophic cardiomyopathy and inoperable aortic stenosis, which may involve additional mechanisms of action, including anti-oxidant effects (Horowitz et al., 2010; Lee et al., 2005; Unger et al., 1997; Willoughby et al., 2002). Despite these beneficial effects, perhexilene’s clinical use is limited, and requires therapeutic drug monitoring to individualise dose, due to its potential to cause severe, concentration-dependent (Horowitz et al., 1986), peripheral neuropathy (Singlas et al., 1978) and hepatotoxicity (Morgan et al., 1984). The latter initially presenting as raised plasma liver enzymes including aspartate transaminase and alkaline phosphatase and then developing into steatosis.

Perhexilene is a chiral molecule formulated as a racemic mixture of equal amounts of (+)- and (-)-enantiomers. In humans, it is almost entirely metabolised by CYP2D6 to its primary mono-hydroxy metabolites cis-OH-perhexilene, trans-1-OH-perhexilene and trans-2-OH-perhexilene (Davies et al., 2007; Wright et al., 1973). We, and others, have previously demonstrated significant enantioselectivity in its clinical pharmacokinetics, although both enantiomers were substrates for CYP2D6 (Davies et al., 2006a; Gould et al., 1986; Inglis et al., 2007). CYP2D6 is highly polymorphic (Ingelman-Sundberg et al., 2007; Sachse et al., 1997; Zanger et al., 2004; Zhou et al., 2009) and, as a result, there is an approximately 10- to 20-fold inter-individual variability in the apparent oral clearance of perhexilene (Sallustio et
metabolisers ranging from 100 mg/week, 100 - 200 mg/day and up to 500 mg/day, respectively (Sallustio et al., 2002). The plasma concentration ratio of cis-OH-perhexiline:perhexiline has been used to phenotype patients with respect to CYP2D6 metaboliser status, with a ratio < 0.3 indicating a CYP2D6 PM (Sallustio et al., 2002). Significantly PM subjects do not form cis-OH-perhexiline and the trace amounts of metabolites formed derive from CYP3A4 and CYP2B6 catalysed formation of the trans-OH metabolites (Davies et al., 2007).

Altered myocardial energy metabolism is now recognised as a major feature of many forms of heart disease and is being investigated as a novel therapeutic target (Fragasso et al., 2008; Horowitz et al., 2010). Perhexiline may be a clinically useful prototypic drug to further understand the mechanisms of action of myocardial metabolic agents. However, the pharmacodynamics of its individual enantiomers have not previously been investigated. An animal model with similar metabolism of perhexiline to humans is therefore needed to investigate their mechanisms of action and relationships with pharmacokinetics. The female Dark Agouti (DA) rat has been used extensively as a model of the human CYP2D6 PM phenotype, whilst Sprague Dawley (SD) and Wistar rats have been traditionally used as models of extensive metabolism (Schulz-Utermoehl et al., 1999). The aim of this study was to compare female SD and female DA rats as in vivo models of perhexiline metabolism and hepatotoxicity.
Materials and Methods

Materials
Perhexiline maleate, dansyl chloride, \((R)-(−)-1-(1\text{-napthyl})\text{ethyl isocyanate (NEIC)}\) and the internal standard prenylamine lactate were purchased from Sigma Chemical Company (Castle Hill, NSW, Australia), HPLC grade solvents (acetone, ethyl acetate, methanol and \(n\)-hexane) and sodium hydroxide were purchased from Merck (Kilsyth, Victoria, Australia). All other reagents were of analytical grade.

Animals- Dosing and Sampling
Animal ethics approval was granted from both the University of Adelaide and the Institute of Medical and Veterinary Science animal ethics committees. Female SD and DA rats were purchased from the University of Adelaide animal services. Animals were housed in a temperature-controlled room with free access to water. For the first week, all animals were fed standard rat chow and monitored for their daily consumption of food. The control group (n=4) was administered pellets coated with vehicle (peanut paste, used to mask the bitter taste of the perhexilne compound). During active treatment, 200 mg/kg of perhexiline maleate was mixed with peanut paste and coated onto 7 rat chow pellets for SD rats (n=4), or 4 pellets for DA rats (n=4), placed in the cages at 4-5 pm each day, and left until the next dose. Food and perhexilne consumption was monitored daily and extra drug-free pellets provided if necessary.

At the 8 week time point, the animals were euthanized under isoflurane (2-3%), blood was taken via a cardiac puncture and livers were collected, snap frozen in liquid nitrogen and stored at -80°C.
Biochemistry Assessments

Each blood sample was placed in a lithium heparin tube and plasma was obtained after centrifugation at 3000 rpm at 10°C. Liver function was assessed by measuring plasma concentrations of albumin, total bilirubin, alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and gamma glutamyl transpeptidase (GGT). The analyses were performed by the Clinical Chemistry Unit (Institute of Medical and Veterinary Science), on a Beckman Coulter instrument (Gladesville, NSW, Australia).

Preparation of Liver Homogenates

Livers were thawed at room temperature in saline, then dried and weighed. Ice cold 0.15 M phosphate buffer, pH 6.0 was added (2 ml per g liver), the liver was cut to smaller pieces, transferred to a 50 mL glass tube and homogenised on ice (IKA® T10 basic Ultra-Turrax). The homogenate was then poured into a glass tissue grinder on ice and ground with 5 passes into a fine homogenate that was then assayed for perhexiline and metabolites.

Quantitation of perhexiline and its cis- and trans-OH-metabolites.

Concentrations of (+)- and (-)-perhexiline in plasma and liver homogenate were determined using the method of Davies et al (Davies et al., 2006c). The concentrations of cis-, trans-1- and trans-2-OH-(±)-perhexiline in plasma and liver homogenate were determined separately by the method of Davies et al (Davies et al., 2006b). Although this assay resolves the cis-, trans-1- and trans-2-OH metabolites of perhexiline, each is the sum of the metabolites formed by both (+)- and (-)-perhexiline. Thus, plasma and liver perhexiline concentrations will also be presented as total (±)-perhexiline concentrations, and the ratio of (+)/(-) enantiomers will be shown separately. The assay for (+)-, (-)- perhexiline and OH-perhexiline was validated
with plasma and liver homogenate and demonstrated inter- and intra-assay coefficient of variation (CV) and bias, within ±15% for the highest limit of quantitation (HLOQ), 2 mg/L and within ±20% for the lowest limit of quantitation (LLOQ), 0.02mg/L. Samples above HLOQ were diluted accordingly in the same matrix, to fit within the calibration range.

**Electron Microscopy**

Liver tissue was dissected into cubes of approximately 0.5 mm$^3$, and fixed for one hour in fixative (4% formaldehyde and 1.5% glutaraldehyde in sodium cacodylate buffer, pH 7.2). The fixed tissue was post-fixed in 2% osmium tetroxide in sodium cacodylate buffer, *en bloc* stained with 2% uranyl acetate and dehydrated through 70%, 90% and 100% ethanol. The tissue was then processed through 1,2-epoxypropane, a 50/50 mixture of 1,2-epoxypropane and Procure 812 resin (Electron Microscopy Sciences, Fort Washington, USA) and two changes of 100% resin. Tissue and resin were transferred to Beem capsules and placed in an oven overnight at 90 °C.

Survey sections of tissue blocks were cut with glass knives and stained with Toluidine Blue. Thin sections were cut at approximately 100 nm thickness on a Porter-Blum ultra microtome (Sorvall, Newtown, USA) using a diamond knife (Micro Star Technologies, Huntsville, USA). Thin sections were stained with Reynolds’ lead citrate and examined in a Hitachi H-600 transmission electron microscope (Tokyo, Japan 1983). A minimum of 2-3 sections were examined for each tissue. Hepatic lipid vesicles and cytosolic glycogen were identified as described in previous papers, and content was measured as a percentage of the field of view.
Statistical analysis

Between-group comparison of plasma and tissue concentrations of perhexiline, OH-perhexiline, biomarkers for liver injury or glycogen and lipid contents was carried out using a Mann-Whitney U test. Statistical analysis was performed with GraphPad Prism version 4.02 for Windows (San Diego, California USA), significance level adopted was P<0.05, all data are presented as median (range).
Results

Plasma Perhexiline and OH-Perhexiline Concentrations

Plasma perhexiline concentrations were significantly different in DA rats compared to SD rats (Table 1). DA rats treated with racemic perhexiline had 5.6-fold higher median plasma concentrations compared to the SD strain (p<0.05). In both SD and DA rats treated with racemate, plasma (+)-perhexiline was always present at a higher concentration than (-)-perhexiline, with median ratios of (+)/(-) of 2.1 and 1.8, respectively (Table 1). Plasma cis-OH-perhexiline concentrations were 2.6-fold greater for the DA compared to the SD strain (p<0.05). Plasma concentrations of trans-1-OH- and trans-2-OH-perhexiline were below the limit of quantification in both the SD and DA rats, and in both strains were lower than the concentrations of cis-OH-perhexiline. The median ratio of plasma cis-OH-perhexiline:perhexiline concentrations in DA (0.89, 0.50-1.56) was not significantly different to that in SD (1.55, 0.44-5.50) (P=0.30).

Liver Homogenate Perhexiline and OH-Perhexiline Concentrations

Median tissue homogenate perhexiline concentrations were also significantly different between the two rat strains (Table 1). The DA rat showed perhexiline concentrations 4.5-fold higher than those of the SD strain (p<0.05). Similar to plasma, the ratio of (+)/(-) perhexiline concentrations in liver was also approximately 2:1 in both strains. Cis-OH-perhexiline was the major metabolite in both SD and DA livers (Table 1).

Relative accumulation of total (±)-perhexiline in liver versus plasma was 60- and 48-fold in the SD and DA rats, respectively. In the DA rat livers, median cis-, trans-1- and trans-2-metabolite concentrations were 28-, 31- and 33-fold higher than in plasma. In the SD strain
cis-OH-perhexiline concentrations were 23-fold higher in liver compared to plasma, however it was not possible to determine the relative tissue accumulation of the trans-OH metabolites as their plasma concentrations were below the limit of quantification. The median ratio of hepatic cis-OH-perhexiline:perhexiline concentrations in DA (0.66, 0.31-0.91) was not significantly different to that in SD (1.19, 0.13-5.26) (P=0.34).

**Biochemistry**

At 8 weeks, liver function tests on DA rats showed significantly elevated LDH (P<0.05) in the perhexiline-treated group (250 (221-352) U/L) compared to the control group (146 (105-159) U/L), but no significant differences in any of the other tests. The SD rats showed no significant differences in liver function tests.

**Histology**

Representative electron micrographs of livers from SD and DA rats are shown in figure 1. Cytoplasmic glycogen pools were clearly evident in the control SD rats (Figure 1A), and control DA livers, although not as intense or as large as those from SD control livers (Figure 1C). All other cellular structures and organelles appeared morphologically similar with 1 or 2 lipid droplets present in the field of view. Electron micrographs from perhexiline treated SD and DA rats are shown in Figures 2A and B, respectively. In each strain, glycogen and lipid content were assessed by measuring their area as a percentage of the field view. Figure 2, shows significantly lower (p<0.05) glycogen content in SD rats treated with perhexiline compared to SD controls.
Discussion

The DA rat has been extensively used in studies as a model of human CYP2D6 PM (Barham et al., 1994; Komura et al., 2005; Zysset et al., 1988). CYP2D6 is a major enzyme involved in the metabolism of many drugs and its highly polymorphic nature makes it difficult to predict the pharmacokinetics and pharmacodynamics of compounds that are extensively metabolised by CYP2D6, often resulting in adverse effects or lack of efficacy (Ingelman-Sundberg, 2005). The anti-anginal drug perhexiline is one such compound, with high plasma perhexiline concentrations increasing the risk of severe neurotoxicity and hepatotoxicity (Singlas et al., 1978), particularly in CYP2D6 PM.

In this study both the DA and SD strains had many similarities to humans with respect to perhexiline metabolism. For example, the ratio of (+)- to (-)-perhexiline in plasma and liver was approximately 2:1, consistent with previous clinical studies (Gould et al., 1986; Inglis et al., 2007). Furthermore, similar to studies in humans (Amoah et al., 1986; Davies et al., 2006b), cis-OH perhexiline was also the major metabolite formed in both the SD and DA strains. The 5-fold higher plasma perhexiline concentrations in DA versus SD rats suggest that the DA rat has a reduced capacity to clear perhexiline. However, unlike CYP2D6 PMs that do not produce any cis-OH-perhexiline (Davies et al., 2007), the cis-metabolite was still the major metabolite formed in the DA, with a plasma metabolic ratio of 0.89 similar to the SD strain (1.55) but higher than the ratio of 0.3 used to identify PM in humans. Thus, the DA rat is not as suitable a CYP2D6 PM model for perhexiline, consistent with previous observations that this strain’s suitability as a model for CYP2D6 PM is substrate specific (Komura et al., 2005). Despite the DA rat not being a true PM model we were able to attain clinically relevant plasma concentrations of perhexiline, whereas in the SD rat they were all below the lower limit of the clinical therapeutic range (0.15 mg/L).
In the rat, CYP2D1 and CYP2D2 enzymes perform the same function as human CYP2D6 (Hiroi et al., 2002; Matsunaga et al., 1989), catalysing the 4-hydroxylation of debrisoquine (Ohishi et al., 1993; Schulz-Utermoehl et al., 1999). Yamato et al., have shown by transfecting yeast with recombinant plasmids which encode for CYP2D1 and 2D2, that although both these enzymes are involved in the hydroxylation of debrisoquine (Yamamoto et al., 1998), CYP2D2 has a higher affinity for debrisoquine and therefore predominately catalyses its 4-hydroxylation. CYP2D1 has a lower affinity and may contribute to 4-hydroxylation at high substrate concentrations (Hiroi et al., 2002). Furthermore, immunoblotting studies revealed that hepatic CYP2D2 levels in the female DA rat were 30- to 40-fold lower than those reported in female SD and Wistar rats (Schulz-Utermoehl et al., 1999). Our observation of significant but lower metabolism of perhexiline in the DA rat suggests that its hepatic concentrations were sufficiently high to enable CYP2D1-catalysed metabolism of the OH-metabolites, accounting for the formation of cis-OH-perhexiline in the female DA rats. Indeed, there was significant hepatic accumulation of perhexiline in both strains, with similar liver:plasma concentration ratios in DA and SD rats of approximately 50:1 (Table 1). Alternatively, in the rat cis-OH-perhexiline may also be formed by CYPs other than the 2D family.

Elevated plasma perhexiline concentrations have been linked to adverse effects such as peripheral neuropathy and hepatotoxicity (Horowitz et al., 1986; Morgan et al., 1984; Singlas et al., 1978), with elevated biochemical markers of hepatotoxicity such as alkaline phosphatase and aspartate transaminase (Morgan et al., 1984). In the DA rats, LDH was the only biochemical marker to be significantly elevated (2-fold compared to control, p<0.05), whereas the SD strain showed no significant changes in any of the tests. Although LDH is a general marker of tissue damage and not a specific marker of liver dysfunction it indicates that the DA rat may be more sensitive to perhexiline toxicity, most likely due to the higher
systemic and liver exposure to perhexiline. The lack of clear biochemical evidence of hepatotoxicity probably reflects the fact that only one DA rat had plasma perhexiline concentrations above the clinical therapeutic range. However, Meir et al., also reported that there were no changes in biochemical liver function tests in the DA rat after chronic perhexiline administration (Meier et al., 1986). Alternatively the rat may be less sensitive to perhexiline-induced hepatotoxicity.

Upon histological analysis, livers from SD rats treated with perhexiline had lower glycogen contents (P<0.05) compared to vehicle treated animals (Figures 1 and 2), consistent with the inhibition of CPT-1 by perhexiline, leading to increased carbohydrate utilisation and depletion of glycogen stores. However, a similar effect was not observed in the DA rats. Liver biopsies from patients with perhexiline-induced hepatotoxicity typically show steatosis and phospholipidosis (Kopelman et al., 1977; McDonald, 1977). In this study, there was no statistically significant lipid accumulation in either rat strain.

In summary, our results suggest that the pharmacokinetic properties of the female DA rat make it a model of intermediate / extensive CYP2D6 metabolism of perhexiline. By utilising the female DA rat we were able to achieve perhexiline concentrations within the recommended human therapeutic range as well as demonstrate similar enantioselective pharmacokinetics of perhexiline as observed in humans, whereas the concentrations achieved with the SD rat were sub-therapeutic. In addition, the higher perhexiline concentrations in DA rats were also associated with biochemical toxicity suggesting that for future investigations of the toxicity or efficacy of perhexiline in vivo, the female DA rat may be the more suitable model.
Table 1: Plasma and liver concentrations of parent perhexiline and OH-metabolites in DA and SD rats dosed orally at 200 mg/kg/day for 2 months. (*P < 0.05, #P=0.057 compared to DA)

<table>
<thead>
<tr>
<th></th>
<th>DA strain</th>
<th>SD strain</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(±)-perhexiline (mg/L)</td>
<td>0.50 (0.16-1.13)</td>
<td>0.09 (0.04-0.13)*</td>
</tr>
<tr>
<td>Ratio (+):(-) perhexiline</td>
<td>1.83 (1.67-2.32)</td>
<td>2.13 (2.0-3.0)</td>
</tr>
<tr>
<td>Cis-OH-perhexiline (mg/L)</td>
<td>0.44 (0.25-0.57)</td>
<td>0.17 (0.04-0.23)*</td>
</tr>
<tr>
<td>Trans-1-OH-perhexiline (mg/L)</td>
<td>&lt;LLOQ</td>
<td>&lt;LLOQ</td>
</tr>
<tr>
<td>Trans-2-OH-perhexiline (mg/L)</td>
<td>&lt;LLOQ</td>
<td>&lt;LLOQ</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(±)-perhexiline (mg/Kg)</td>
<td>24.47 (9.4-54.70)</td>
<td>5.42 (0.92-8.22)*</td>
</tr>
<tr>
<td>Ratio (+):(-) perhexiline</td>
<td>2.25 (1.84-2.48)</td>
<td>1.87 (1.24-2.14)</td>
</tr>
<tr>
<td>Cis-OH-perhexiline (mg/Kg)</td>
<td>12.66 (7.50-14.48)</td>
<td>3.44 (0.71-8.50)#</td>
</tr>
<tr>
<td>Trans-1-OH-perhexiline (mg/Kg)</td>
<td>1.22 (0.58-1.36)</td>
<td>0.46 (0.29-1.00)</td>
</tr>
<tr>
<td>Trans-2-OH-perhexiline (mg/Kg)</td>
<td>1.14 (0.50-1.80)</td>
<td>0.15 (0.04-0.36)</td>
</tr>
<tr>
<td><strong>Liver:Plasma Concentrations</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(±)-perhexiline</td>
<td>53.6 (37.2-60.0)</td>
<td>55.6 (10.2-89.3)</td>
</tr>
<tr>
<td>Cis-OH-perhexiline</td>
<td>27.7 (23.7-35.7)</td>
<td>23.3 (3.1-212.5)</td>
</tr>
<tr>
<td>Trans-1-OH-perhexiline</td>
<td>31.0 (28.1-45.3)</td>
<td>N.D.</td>
</tr>
<tr>
<td>Trans-2-OH-perhexiline</td>
<td>33.3 (21.2-60.0)a</td>
<td>N.D.</td>
</tr>
</tbody>
</table>
Figure 1: Electron micrographs of liver tissue taken from A) the SD control group dosed with vehicle for a period of 8 weeks (n=4), B) the SD group treated with perhexiline maleate (200mg/kg/day), C) the DA control group treated with vehicle for 8 weeks (n=4), and D) the DA group treated with perhexiline maleate (200mg/kg/day) for a period of 8 weeks (n=4). Arrows represent glycogen stores within the hepatocytes. Magnification 30,000x.
**Figure 2:** Mean (s.e.m) of hepatic glycogen and lipid contents as a percentage of the field of view in both the DA (A) and SD rat (B) strains, following chronic dosing of 200 mg/kg of racemic perhexilene maleate (Rac) or vehicle control (Cont) over 8 weeks. * = P<0.05 versus control group (Mann Whitney) N=4 for each group.
Acknowledgments

John K Brealey, from the Electron Microscope Unit of The Queen Elizabeth Hospital, conducted the electron microscopy work in this project. John Pierides from the department of Surgical Pathology and Cytopathology carried out histological analysis for this project.
References


Chapter Five: The enantiomers of the myocardial metabolic agent perhexiline display divergent effects on hepatic energy metabolism and peripheral neural function in Rats

Text in manuscript. This manuscript is prepared for submission to and in accordance with the guideline of the journal Drug Metabolism and Disposition
As we have now established our animal model of perhexiline metabolism and toxicity, we now needed to examine if there is enantioselectivity in perhexiline induced toxicity. At a biochemical level, the clinical toxicity of perhexiline manifests as an increase in alkaline phosphatase and alanine transaminase suggesting a degree of liver injury. Histologically, perhexiline has been shown to increase hepatic lipid content in the form of macro- and microscopic steatosis, with appearance of lipid filled vesicles within the cytosolic compartment of the hepatocyte. Peripheral neuropathy is the other major clinical toxicity associated with perhexiline with patients experiencing loss of sensation in fingers as well as muscular weakness.

This publication was the first to report the toxic effects of (+)- or (-)-perhexiline in an animal model.

Perhexiline’s enantiomers exert substantially different effects on hepatic lipid and glycogen accumulation, and on neural function. Our data suggest that the toxicity of racemic perhexiline resides predominately in the (+)-enantiomer, consistent with inhibition of carnitine palmitoyltransferase-1, whilst (-)-perhexiline may offer greater potential long-term safety. Based on the finding of this study future development, with (-)-perhexiline as a prototype may lead to the development of a safer formulation of perhexiline one which would not require therapeutic drug monitoring.
The enantiomers of the myocardial metabolic agent perhexiline display divergent effects on hepatic energy metabolism and peripheral neural function in rats

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A list of non-standard abbreviations used in the paper

ATP, adenosine triphosphate; CPT, carnitine palmitoyl transferase; CYP, cytochrome P450; DA, Dark Agouti; HPLC, high performance liquid chromatography.

Key Words: Perhexiline, hepatotoxicity, neurotoxicity, enantioselectivity
Introduction

The anti-anginal agent perhexiline is approved in Australia for the treatment of angina pectoris refractory to other therapies (Horowitz et al., 1979), however it also has been shown to have clinically beneficial effects, including significant improvements in myocardial energetics, in heart failure (Lee et al., 2005) and hypertrophic cardiomyopathy (Abozguia et al., 2010), and it may also be useful in inoperable aortic stenosis (Unger et al., 1997). Perhexiline inhibits carnitine palmitoyltransferase 1 (CPT-1) (Kennedy et al., 1996), which forms part of the carnitine shuttle that translocates free fatty acids from the cytoplasm to the mitochondrion in order to undergo β-oxidation. CPT-1 is the rate-limiting enzyme for this pathway catalyzing the conversion of fatty acyl CoA to fatty acyl carnitine. Inhibition of CPT-1 causes a “switching” to carbohydrate utilization, the so-called Randle phenomenon (Hue et al., 2009): – it has been postulated that this switch is associated with an improvement of approximately 13% in the efficiency of myocardial oxygen utilization.

Although modulation of myocardial energy metabolism is an emerging therapeutic target for the treatment of cardiovascular disease (Lee et al., 2004) the clinical use of perhexiline has been limited to otherwise refractory cases because of the potential risk of hepato- and neurotoxicity during long-term administration (Morgan et al., 1984; Singlas et al., 1978). This has been attributed, in part, to lipid storage disorders in hepatocytes and Schwann cells, including steatosis and lysosomal phospholipidosis, which have also been described with other CPT-1 inhibitors (Guigui et al., 1988). Although this problem generally remains subclinical provided plasma perhexiline concentrations are kept within a defined therapeutic range (Horowitz et al., 1986), it remains a major impediment to the routine use of the drug.

Perhexiline is a chiral compound formulated as a racemate, with prior investigations of its enantiomers restricted to pharmacokinetic studies (Gould et al., 1986). Using an animal
model of potential perhexiline-induced hepato and neuro-toxicity, the aim of this study was to
test the hypothesis that the two enantiomers differ in their toxicological potential during long-
term therapy. Studies were performed in female Dark Agouti (DA) rats, which we have
previously shown are similar to humans with respect to perhexiline metabolism and
disposition, and the previously demonstrated facility for attaining clinically relevant plasma
perhexiline concentrations in this model (Licari et.al. manuscript in preparation).
Methods

Chemicals

(±)-Perhexiline maleate (racemate) was kindly provided by Sigma Pharmaceuticals (Rowville, Victoria, Australia). Pure (+)- and (-)-perhexiline were prepared as either the maleate or hydrochloride salts using a previously published method (Davies et al., 2006b). The derivatising reagent (R)-(−)-1-(1-napthyl) ethyl isocyanate (NEIC) and the internal standard prenylamine lactate were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Methanol, acetone, ethyl acetate, n-hexane and sodium hydroxide were purchased from Thermo Fisher (Scoresby, Australia); all solvents were of HPLC grade. All other reagents were of analytical grade. DA rats were purchased from the University of Adelaide animal facilities.

In vivo effects of (+)- and (-)-perhexiline in DA rats

Animals

The Adelaide University and the Institute of Medical and Veterinary Services animal ethics committees granted approval for this study. Pilot experiments were conducted with racemic perhexiline in order to develop dosing schedules associated with clinically relevant plasma concentrations and the development of steatosis (Licari et.al., manuscript in preparation). Thereafter, DA rats (n=4 in each group) were administered vehicle (peanut paste- control group), or 200 mg/kg daily of (±)-, (+)- or (-)- perhexiline maleate for a period of 8 weeks. After determining food intake (4 pellets for DA rats), perhexiline maleate was administered mixed with peanut paste and coated onto standard rat chow and left until consumption (overnight). On week 4 of dosing, blood was collected from the tail vein in order to assess liver function as well as to determine plasma (+)-, (-)- and OH-perhexiline concentrations. On
week 8 of dosing, animals were anesthetised and blood was collected via a cardiac puncture in order to assess liver function and determine the concentrations of perhexiline enantiomers and the OH-metabolites, as well as lactate, glucose and triglyceride concentrations. Animals were then euthanized and hepatic, cardiac and neuronal tissues were harvested in order to determine perhexiline enantiomer and metabolite tissue concentrations and morphological changes. Tissues were cut in half and either immediately snap frozen in liquid nitrogen or placed into fixative solution for electron microscopy analysis.

_Hepatic and Neuronal Morphology_

Hepatic and neuronal tissue was dissected into cubes of approximately 0.5 mm in each dimension and was fixed for one hour in electron microscopy fixative (4% formaldehyde and 1.5% glutaraldehyde in sodium cacodylate buffer, pH 7.2). The fixed tissue was post-fixed in 2% osmium tetroxide in sodium cacodylate buffer, en bloc stained with 2% uranyl acetate and dehydrated through 70%, 90% and 100% ethanol. The tissue was then processed through 1,2-epoxypropane, a 50/50 mixture of 1,2-epoxypropane and Procure 812 resin (Electron Microscopy Sciences, Fort Washington, USA) and two changes of 100% resin. Tissue and resin were transferred to Beem capsules and placed in an oven overnight at 90°C.

Survey sections of these hepatic and neuronal tissue blocks were cut with glass knives and stained with Toluidine Blue. Thin sections were cut at approximately 100 nm thickness on a Porter-Blum ultramicrotome (Sorvall, Newtown, USA) using a diamond knife (Micro Star Technologies, Huntsville, USA). Thin sections were stained with Reynolds’ lead citrate and examined in a Hitachi H-600 transmission electron microscope (Tokyo, Japan 1983). Glycogen and lipids were identified (as described by Singh et al., 1997) and total content was
measured as a percentage of the field of view. Two to three sections were examined for each tissue and the average value used.

Neuronal Function

At week 4 and 8, neuronal function was measured using von Frey filament testing of treated and non-treated groups (Chaplan et al., 1994). The thickness of each filament corresponds to a specific amount of pressure (in grams). Each animal was placed in a specially designed plastic container, with the bottom replaced by mesh flooring. The animal was left for 10-15 minutes to settle into its new environment. In ascending order, von Frey filaments were applied from beneath the mesh flooring to the plantar surface of the paw until the filament buckled and was held there for a total of 10 seconds. A positive response was noted if the paw was withdrawn within the 10-second period. If a filament induced 6 positive responses, the grams corresponding to that filament were recorded as the paw withdrawal threshold.

Measurement of perhexiline and OH-perhexiline in plasma, liver and heart

Heart and liver homogenate was prepared on ice in 0.15 M phosphate buffer at pH 7.4, using 2 ml of buffer per gram of tissue. The homogenate was then stored at -80°C until analysis. The concentration of (+)- and (-)- perhexiline in plasma and tissues (heart and liver homogenate) was measured according to the method of Davies et al (Davies et al., 2006b). The concentrations of cis-, trans1- and trans2-OH-perhexiline in plasma and liver homogenate were measured according to the method of Davies et al (Davies et al., 2006b)
Statistical Analysis

Between-group comparison of plasma and tissue concentration of (+)- and (-)-perhexiline were performed using Residual Maximum Likelihood (REML), treating treatments as fixed and rats as random to take into account the paired nature of (+)- and (-)-perhexiline concentrations following the administration of (±)-perhexiline, and the unpaired nature of between group comparisons. Analyses were carried out using GenStat 13th edition (VSN International Ltd, UK). Comparisons of cis-, trans1- and trans2-OH-metabolite formation following administration of (+)- and (-)-perhexiline was performed using a two-way ANOVA. Between-group comparisons of neuronal function, plasma liver function tests, and tissue glycogen and lipid contents were carried out using a one-way ANOVA, with a Bonferroni’s multiple comparisons post test. The relationship between (+)- and (-)-perhexiline concentrations, and hepatic lipid or glycogen contents were also assessed using multiple linear regressions. Data are expressed as mean ± S.E.M. All statistical analyses were performed with GraphPad Prism version 4.02 for Windows, GraphPad software (San Diego California USA), with a significance level of P<0.05 adopted.
Results

Plasma Biochemistry

Plasma liver function tests were carried out using samples collected at both the 4 and 8 week time points (Table 1). There were no significant (P>0.12) changes in biochemistry at 4 weeks. At 8 weeks, only plasma lactate concentrations were significantly higher in animals receiving (-)-perhexiline when compared to controls (P<0.05), (+)-perhexiline (P<0.01) and (±)-perhexiline (P<0.01).

Hepatic Histology

Representative electron micrographs for each treatment group are shown in Figure 1. Large numbers of lipid-laden vacuoles were associated with (+)-perhexiline treatment (Figure 1B) and large glycogen “pools” with (-)-perhexiline treatment (Figure 1C), whilst (±)-perhexiline treatment (Figure 1D) resembled controls (Figure 1A).

When measured as a percentage of the area of the field of view, hepatic lipid and glycogen content did not vary between control rats and those treated with racemic perhexiline. However, in (+)-perhexiline-treated rats only, there was 6-, 4- and 8-fold higher hepatic lipid content (p<0.05), compared to control, (-)-perhexiline and (±)-perhexiline, respectively (Bonferroni post hoc test) (Figure 2A). Conversely, rats treated with (−)-perhexiline produced 2- and 14-fold higher hepatic glycogen content compared to controls (p < 0.01) and (+)-perhexiline (p < 0.001, figure 2b), respectively.

In order to test the hypothesis that the enantiomers exert contrasting effects on lipid and glycogen accumulation, correlations between hepatic enantiomer concentrations and effects
were sought (including baseline data from controls and racemate-treated animals). As shown in Figure 3A, there was a direct correlation ($r = 0.79$, $p = 0.004$) between hepatic concentration of (+)-enantiomer and lipid content, while a direct correlation was also seen ($r = 0.78$, $p = 0.003$) between (-)-enantiomer concentration and glycogen content (Figure 3B). Furthermore, (+)-enantiomer concentrations were inversely correlated ($r = -0.66$, $p = 0.03$) with glycogen content (Figure 3C).

Stepwise multiple regression confirmed diverging effects of both (+)- and (-)-perhexiline concentrations on hepatic glycogen contents ($p = 0.02$ for (+)-perhexiline, $p = 0.0004$ for (-)-perhexiline, adjusted $R^2 = 0.76$), but only a significant effect of (+)-perhexiline on hepatic lipid contents ($p = 0.0004$, adjusted $R^2 = 0.62$).

**Neural function and histology**

Compared to controls, (-)-perhexiline treatment for 8 weeks (figure 2c) had no effect on peripheral neural function. In contrast, rats treated with racemic perhexiline or (+)-perhexiline revealed attenuation of response to the von Frey test compared to controls ($p < 0.001$ and $p < 0.01$, respectively) and to (-)-perhexiline ($p < 0.001$ and $p < 0.05$, respectively), consistent with the induction of peripheral nerve dysfunction. Nevertheless, electron microscopic examination of dorsal root ganglia revealed only occasional Mallory bodies, in approximately 1% of nerve cells from rats treated with (+)-perhexiline, with no Mallory bodies identified in other treatment groups.
**Perhexiline Concentrations**

DA rats administered pure enantiomers attained plasma (+)-perhexiline concentrations of 0.67 (± 0.07, range 0.52-0.80) mg/L, whereas the concentrations in animals administered (-)-perhexiline were 0.29 (± 0.04, range 0.22-0.39) mg/L. In rats treated with racemate, (+)-perhexiline plasma concentrations averaged 0.84 ± 0.40 mg/L. The ratio of plasma (+)- to (-)-perhexiline concentrations was approximately 2:1 irrespective of whether drug was administered as racemate or individual enantiomers.

In order to make comparisons between animals treated with racemate versus those treated with individual enantiomers, the plasma and tissue concentrations of (+)- or (-)-perhexiline were corrected for the 2-fold difference in enantiomer dose, and are shown in Figure 4A adjusted to the equivalent of a 100 mg/kg/day enantiomeric dose. When administered as individual enantiomers, the concentrations of (+)-perhexiline were higher (p <0.05) than those of (-)-perhexiline in liver and heart, but not plasma (Figure 4A). There was extensive (>20-fold) tissue accumulation of both enantiomers, with higher tissue:plasma concentration ratios for (+)- compared to (-)-perhexiline (p <0.05) in both heart and liver (Figure 4B). Administration of (±)-perhexiline revealed significantly higher (p<0.05) concentrations of (+)- compared to (-)-perhexiline in liver and plasma, but not heart (Figure 4A), with extensive accumulation of both (+)- and (-)-perhexiline in tissues, although the ratio of tissue:plasma concentrations was significantly higher (P<0.05) for (+)- compared to (-)-perhexiline only in the heart. In addition, although the tissue:plasma concentration of (-)-perhexiline in heart and liver following (±)-perhexiline was similar to that following the pure enantiomer, (+)-perhexiline accumulation in heart and liver was significantly lower (p <0.05) following the racemate compared to the pure enantiomer.
OH-Perhexilene Concentrations

In the DA rat perhexilene formed cis- and trans-mono-hydroxy metabolites (Figure 5). When administered as the individual enantiomers, the cis-OH metabolite was the major metabolite detected for both (+)- and (-)-perhexilene. Plasma cis-OH(-)-perhexilene concentrations were significantly higher (p<0.0001) than cis-OH(+)perhexilene concentrations, whereas the concentrations of trans-1- and trans-2-OH metabolites showed no significant difference between enantiomers (Figure 5A). In liver there was no significant difference in metabolite concentrations between (+)- and (-)-perhexilene (Figure 5B). The mean (± s.e.m.) total metabolite:parent ratio for (-)-perhexilene was significantly higher than that for (+)-perhexilene, in both plasma (2.34 ± 0.45 vs. 0.39 ± 0.07, p<0.05) and liver (1.11 ± 0.30 vs. 0.18 ± 0.03, p<0.05). When administered as the racemate, total cis-OH(±)-perhexilene was similarly the major metabolite detected in plasma and liver. Unfortunately, our cis/trans-OH-perhexilene assay did not resolve the metabolites of (+)- and (-)-perhexilene and therefore it was not possible to compare the metabolism of the enantiomers following administration of the racemate. However, it appears that following administration of racemic perhexilene, the majority of cis-, trans-1- and trans-2-OH-metabolites derive from (-)-, (+)- and (-)-perhexilene, respectively.
Discussion

Since its introduction in the 1970s, perhexiline has undergone meticulous scrutiny to determine its mechanism(s) of toxicity and efficacy. Horowitz et al., postulated that perhexiline was a potent CPT inhibitor and exerted its therapeutic effects via the inhibition of CPT1 (Kennedy et al., 1996). However, more recent studies have shown that perhexiline produced increased function in perfused rat hearts in the absence of any change in palmitate oxidation, suggesting that the inhibition of CPT alone may not account for improved myocardial function (Unger et al., 2005) and that other mechanisms, including its nitric oxide sensitizing and anti-inflammatory properties may also be important (Kennedy et al., 2006; Unger et al., 1997; Willoughby et al., 2002). Part of the difficulty in clearly determining the mechanisms of action of perhexiline is the lack of investigation of the pharmacodynamic properties of the individual enantiomers. This is the first study to investigate the effects of the individual enantiomers on in vivo neuronal and hepatic function, the main targets of perhexiline toxicity. We clearly show that, at clinically relevant concentrations, the two enantiomers have significantly different effects on both hepatic and neuronal function.

(+)-Perhexiline, clearly produced concentration-dependent hepatic lipid accumulation (figures 2 and 3), consistent with the proposed mechanism of action of perhexiline, inhibition of CPT-1, and previous clinical reports of hepatic steatosis (Lewis et al., 1979). In addition, (+)-perhexiline also resulted in concentration-dependent decreases in hepatic glycogen contents (Figures 2 and 3), also consistent with the inhibitory effects of this enantiomer on CPT-1 triggering a metabolic switch of energy sources from free fatty acid to carbohydrate utilization (Kennedy et al., 1996) resulting in decreased glycogen formation. Kennedy et al. have shown that racemic perhexiline inhibits CPT-1 with an IC50 of 77 and 148 µmol/L in isolated cardiac and hepatic mitochondria, respectively (Kennedy et al., 1996). We demonstrate that, following administration of the individual enantiomers, mean (s.e.m)
hepatic concentrations of (+)- and (-)-perhexilines were 104.9 (± 18.39) and 17.05 (± 1.4) µg/g tissue, respectively, or, assuming a tissue density of 1 g/ml, 375 µM and 61.6 µM, respectively. Thus, the hepatic concentrations of (+)-perhexiline were 2.5-fold higher than the reported IC50, again consistent with inhibition of hepatic CPT-1.

Surprisingly, histology from the animals treated with the (-)-perhexiline revealed a completely different metabolic effect, with (-)-perhexiline causing a concentration-dependent accumulation of glycogen (figures 2 and 3), but no effect on hepatic lipid contents (Figure 2A). Thus, it can be hypothesised that, unlike (+)-perhexiline, (-)-perhexiline does not inhibit hepatic CPT1, but may directly affect the pathways of carbohydrate utilization, a metabolic effect unique to (-)-perhexiline. However, as the hepatic concentrations of (-)-perhexiline were 2.5-fold lower than the reported IC50 for CPT-1 inhibition, we cannot rule out the possibility that at higher concentrations (-)-perhexiline may also inhibit hepatic CPT-1.

The mechanism by which (-)-perhexiline causes glycogen accumulation is unclear. Insulin stimulates glycogen synthesis and perhexilines may have some insulin sensitizing effects (Willoughby et al., 2002), by a mechanism which is yet to be clearly defined. An insulin like effect would be beneficial and similarly lead to enhanced myocardial glucose utilization and function (Fath-Ordoubadi et al., 1997). The observation in isolated rat hearts that perhexilines increased cardiac efficiency without any change in fatty acid oxidation, suggests a mechanism of action that may not be CPT-1 dependent, but may include an effect of (-)-perhexiline on carbohydrate/glycogen utilisation. Studies in primary hepatocyte cultures have also shown glycogen accumulation following exposure to perhexiline (Lageron et al., 1981), and there have been clinical reports linking the use of perhexilines to hepatic glycogen accumulation. In one report, liver biopsies from two patients receiving perhexiline maleate revealed characteristics similar to those seen in type-1 glycogen storage disease (Lageron et al., 1977).
Nonetheless, all of the metabolic changes in our study occurred without any significant changes in traditional liver function test, except for an increase in plasma lactate in animals treated with (-)-perhexiline.

Interestingly, (+)-perhexiline was also associated with neurotoxicity, since only animals treated with (+)- or (±)-perhexiline showed increased paw withdrawal thresholds in the Von Frey testing (Figure 5). This may suggest that inhibition of CPT-1 in neurons contributes to perhexiline-induced peripheral neuropathy. Mier et al., also reported that racemic perhexiline caused decreased peripheral neuronal function in DA rats, as well as inducing the accumulation of Mallory bodies within dorsal root ganglia (Meier et al., 1986). Electron microscopy of the dorsal root ganglion in our study revealed that only animals treated with (+)-perhexiline had similar inclusions and lamellar bodies, however these were only present in fewer than 1% of cells.

Formation of drug-induced Mallory bodies is a common characteristic seen in hepatic and neuronal tissues after administration of cationic amphiphilic drugs, which can diffuse through the lysosomal lipid membrane and become protonated in the intra-lysosomal acidic environment (Kornhuber et al., 2010). Protonated perhexiline accumulates in lysosomes forming complexes with phospholipids, preventing their degradation by lysosomal enzymes, eventually resulting in phospholipidosis and Mallory body formation (Deschamps et al., 1994). The development of hepatic steatosis has also been reported for other substances that inhibit mitochondrial oxidation of fatty acids such as ethanol (Grunnet et al., 1986), amiodarone (Fromenty et al., 1990) and ibuprofen (Freneaux et al., 1990). Deschamps et al., reported that, like other cationic amphiphilic drugs, perhexiline can also diffuse across the outer mitochondrial membrane along the mitochondrial membrane potential, becoming protonated in the intramembranous space where it accumulates and uncouples oxidative
phosphorylation causing a marked decrease in ATP levels as well as cell viability (Deschamps et al., 1994). However, this direct mitochondrial toxicity occurs at higher concentrations than those causing inhibition of β-oxidation (Deschamps et al., 1994). Although both lysosomal and mitochondrial accumulation are unlikely to be enantioselective as they depend on the amphiphilic nature of perhexiline, which should be the same for both enantiomers, the higher concentrations of (+)-enantiomer may theoretically contribute to a higher risk of lysosomal and mitochondrial dysfunction compared to (-)-perhexiline at the same dose.

All of the observed metabolic changes in the DA rats were attained with plasma perhexiline concentrations within the mid-high end of the clinical therapeutic range. We, and others, have previously demonstrated that (-)-perhexiline is cleared more rapidly than (+)-perhexiline in humans (Davies et al., 2007; Gould et al., 1986; Inglis et al., 2007), due to greater metabolism of the (-)-enantiomer by CYP2D6 (Davies et al., 2007). Similar to humans, we report significant enantioselectivity in the pharmacokinetics of perhexiline in DA rats. When administered as the racemate, plasma concentrations of (+)-perhexiline were significantly higher than those of (-)-perhexiline, consistent with greater clearance of the (-)-enantiomer, when administered as the pure enantiomers, although not statistically significant, plasma concentrations of (+)-perhexiline were again greater than those of (-)-perhexiline. However, the lack of statistical significance may have been due to the small sample size, since the total metabolite to parent ratio in both plasma and liver was statistically significantly greater for (-)-compared to (+)-perhexiline, again consistent with greater metabolism of the (-)-enantiomer.

There was extensive tissue distribution of both perhexiline enantiomers and the metabolites, with tissue:plasma concentration ratios >20, suggesting the involvement of uptake transporters and/or significant tissue binding. Other studies have also reported significantly
higher concentrations of perhexiline in tissues compared to plasma (Davies et al., 2006a; Meier et al., 1986). However, we demonstrate significantly different tissue distribution for (+)- and (-)-perhexiline when administrations as the pure enantiomer but not when administered as the racemate. In addition, administration of (+)-perhexiline as the pure enantiomer resulted in tissue:plasma concentration ratios 2- to 3-fold higher than administration of the racemic formulation. This suggests enantioselectivity in the net-uptake of perhexiline and possible inhibition of the net-uptake of (+)-perhexiline by (-)-perhexiline or the metabolites of (-)-perhexiline. Alternatively, (+)- perhexiline may have a higher affinity for tissue binding than (-)- perhexiline, and may be displaced by (-)-perhexiline and/or the metabolites of (-)-perhexiline. During incubation with human liver microsomes both enantiomers are highly bound to microsomes, although the binding did not appear to be enantioselective (Davies et al., 2007). Distribution of the individual enantiomers into intracellular organelles may also be responsible for the difference in accumulation. Other cationic amphiphilic drugs have been shown to accumulate up to 100- fold in organelles within the cell (Kornhuber et al., 2010), and similar accumulation has been reported for perhexiline in mitochondria and lysosomes (Deschamps et al., 1994), however, as previously discussed this process is unlikely to be enantioselective.

Finally, the mechanism of CPT inhibition suggests that (+)- perhexiline may potentiate its own accumulation in tissues, particularly liver where it causes significant lipid accumulation (Figure 3B). The octanol-water partition coefficient (LogP) of perhexiline is approximately 7, making it very lipophilic, allowing it to bind and diffuse into these lipid deposits and thereby increasing its intra-hepatic volume of distribution as a result of its inhibition on CPT-1. This would also explain the lower tissue:plasma concentration ratio of (-)- perhexiline as it does not induce an accumulation of lipids (Figure 3D), and the lack of enantioselectivity following
administration of the racemic formulation, as both enantiomers could distribute equally into lipid deposits.

In conclusion, the current study demonstrates that although chemically identical, the (+)- and (-)- enantiomers of perhexiline have different effects on hepatic energy metabolism and neuronal function. In DA rats, only (+)-perhexiline appeared to inhibit hepatic CPT1, causing hepatic steatosis and peripheral neuronal dysfunction, as observed in clinical cases of perhexiline toxicity. In contrast, (-)-perhexiline affected hepatic carbohydrate utilisation, causing accumulation of glycogen. All of the observed metabolic changes occurred at clinically relevant concentrations, and without significant changes in LFTs. Further studies are clearly required to investigate the effects of (+)- and (-)-perhexiline on myocardial energy metabolism and function, to determine whether an enantiomeric formulation of perhexiline may have improved efficacy and/or safety.
Figure Legends

**Figure 1:** Transmission electron micrographs of livers from DA rats treated for 8 weeks with A) vehicle, B) (+)-perhexiline, C) racemic perhexiline and D) (-)-perhexiline. Solid hatched arrows indicate glycogen stores and solid arrows indicate lipid vesicles.

**Figure 2:** A) hepatic lipid content, B) hepatic glycogen content and C) Von Frey paw withdrawal thresh holds in animals treated with vehicle, (+)- (n=3), (-)- (n=4) or (±)- (n=4) perhexiline (*P<0.05 compared to controls, (-) and (±)-perhexiline; #P<0.01 compared to controls; $P<0.001 compared to (-)-perhexiline).

**Figure 3:** Linear regression analyses of hepatic (+)-perhexiline concentration on A) lipid or B) glycogen content, and (-)-perhexiline concentration on C) glycogen content and D) lipid content.

**Figure 4:** (A) Dose-corrected plasma (▲), hepatic (■) and myocardial (■) perhexiline concentrations and (B) hepatic (■) and myocardial (■) tissue:plasma concentration ratios of perhexiline in DA rats administered (+)-, (-)- or (±)-perhexiline over a 2 month period. #P<0.05 c.f. (+)-PX in liver; *p<0.05 c.f. (+)-PX in heart; %p<0.05 c.f. R-(+)PX in liver; $p<0.05 c.f. R-(+)PX in plasma; @p<0.05 c.f. R (±)-Px in heart.

**Figure 5:** Cis- (■), Trans-1- (■) and Trans-2- (■) OH-perhexiline concentrations in A) liver and B) plasma, and C) liver:plasma concentration ratio in animals treated with vehicle, (+)-, (-)- or (±)-perhexiline. Comparison between enantiomers and metabolites were performed using a 2 way ANOVA (*P<0.05, ***P<0.01 compared to (+)- perhexiline). Racemic perhexiline data is included for visual reference only and not for statistical analysis.
Table 1: Plasma biochemistry results following 4 and 8 weeks of dosing with vehicle (control) or 200 mg/kg/day of racemic, (-)- or (+)- perhexiline in DA rats. (**P<0.01 c.f. control). Data are expressed as mean ±SEM.

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<tr>
<th></th>
<th>Control</th>
<th>Racemic</th>
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<th>(+)</th>
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<td>(±0.50)</td>
<td>(±0.96)</td>
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<td>1.75</td>
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<td>(±371.05)</td>
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<td>13.13</td>
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<td>(±5.77)</td>
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<td>2.23</td>
<td>3.78 **</td>
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<td>(±0.20)</td>
<td>(±0.22)</td>
<td>(±0.10)</td>
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</table>
Figure 1.
Figure 2.

A) 

% Lipid per Field

B) 

% Glycogen per Field

C) 

Pressure (g)
Figure 3.

A) (+)-Px Vs Lipid

B) (+)-Px Vs Glycogen

C) (-)-Px Vs Glycogen
Figure 4.

A) 

B)
Figure 5.

A) Liver OH-Px (mg/kg)

B) Plasma OH-Px (mg/mL)

C) Ratio OH-Px (Liver:Plasma)
Acknowledgments

John K Brealey, from the Electron Microscope Unit of The Queen Elizabeth Hospital, conducted the electron microscopy work in this project. John Pierides from the department of Surgical Pathology and Cytopathology carried out histological analysis for this project.
References


Chapter Six: General Discussion
The progressive advancement in medical care has led to an increase in the average life expectancy and ageing of the general population, leading to a spectrum and severity of diseases and outcomes that were much less common in the past. Acute coronary disease, angina and other cardiovascular diseases are therefore becoming more prominent in society. These cardiovascular disorders could be considered "rich man diseases", along with diabetes and metabolic syndrome X. In a world where life-threatening infectious diseases are now more associated with developing and third world countries, the rich man diseases are major causes of morbidity and mortality in developed countries.

As a result there is ever increasing pressure to develop new treatment modalities. Perhexiline is an old drug with a remarkable spectrum of clinical efficacy, which is limited by its toxicity, particularly in ‘at risk’ poor and intermediate metabolisers, and the need for ongoing therapeutic drug monitoring to individualise dosage. Perhexiline is marketed in Australia as a 50:50 racemic mixture of the (+)- and (-)- enantiomers, raising the potential for a future, enantiomerically-pure preparation that may avert some or all of the toxicity issues. However, the safety and efficacy of the individual enantiomers, to our knowledge, have never been described.

The main aim of my thesis was to investigate the pharmacodynamic properties of (+)- and (-)-perhexiline in vitro and in vivo to determine whether (+)- perhexilne or its optical antipode is responsible for the beneficial effects and toxicity seen in clinical practice.

I hypothesised that: the enantiomers of perhexilne may demonstrate enantioselectivity in the reduction of neutrophil NADPH oxidase induced superoxide formation; and that the enantiomers of perhexilne demonstrate enantioselectivity in hepato- and neuronal toxicity in the DA animal model.
The first experiments described in Chapter 3, were intended to investigate one of the potential beneficial effect of perhexiline and investigate the antioxidant potency of the enantiomers. There is evidence suggesting that oxidative stress plays a significant role in the development of acute myocardial diseases [114]. Reactive oxygen species (ROS) are major protagonists involved in the oxidative stress mechanism and O$_2^-$ undergoes a chemical reaction with nitric oxide resulting in the formation of the peroxynitrite radical, causing DNA and/or membrane damage, but it also reduces the bioavailability of nitric oxide resulting in endothelial dysfunction, and other diseases.

Kennedy et al [9] reported that, at a therapeutic concentration, perhexiline was able to inhibit O$_2^-$ generation via neutrophil NADPH oxidase by approximately 60% and thus demonstrated the versatility of this potent CPT-1 inhibitor. By inhibiting the production of O$_2^-$, perhexiline could theoretically increase nitric oxide availability and thus reduce the development of myocardial diseases such as endothelial dysfunction. These results strongly suggested that perhexiline may have more than one mechanism of action and its therapeutic benefits do not solely rely on inhibition of CPT-1.

My aim was to determine which of the perhexiline enantiomers inhibited neutrophil O$_2^-$ generation (as measured by superoxide lucigenin chemiluminescence) via NADPH oxidase. I hypothesised that inhibition of O$_2^-$ generation via neutrophil NADPH oxidase may be enantioselective and hence only one enantiomer may be responsible for the activity of the racemic mixture.

I have shown in Chapter 3 (manuscript 1), that the individual enantiomers of perhexiline are able to inhibit O$_2^-$ generation via neutrophil NADPH oxidase in a dose dependant manner and although statistically there was a difference in their potency with IC50 values of 1.19 and 1.64
µM for (-)- and (+)- perhexiline, respectively. Clinically this is unlikely to be significant as (-)- perhexiline is eliminated much more rapidly than (+)- perhexiline. IC50 values were similar in healthy volunteer and patients. Furthermore, at clinically relevant concentrations, both (+)- and (-)- perhexiline inhibited O$_2^*$ generation via neutrophil NADPH oxidase by 60%, similar to that previously reported by Kennedy et al [9]. This current study is the first to describe any potential therapeutic characteristics of the individual enantiomers of perhexiline. It therefore concurs that (-)- perhexiline may be a suitable replacement for racemic perhexiline as it does exhibit the classical toxic effects associated with perhexiline (see chapter 5) as well as having an equal potency towards its antioxidant effects.

In Chapter 4 (manuscript 2), I aimed to develop an animal model that would mimic the CYP2D6 catalysed metabolism of perhexiline in humans.

My hypothesis was that if the individual enantiomers displayed differing pharmacokinetic properties then these characteristics should also be present in my in vivo animal model to properly investigate the enantiomers’ pharmacodynamic properties. This hypothesis was tested by investigating the female SD animal as a model of CYP2D6 extensive metabolism and the DA rat as a model of CYP2D6 poor metabolism, as previously described [76].

Following the administration of racemic perhexiline to the DA rat, the higher systemic exposure to (+)- versus (-)- perhexiline was similar to that seen previously in humans [80]. In this animal model, I was able to achieve plasma concentrations within the therapeutic range and furthermore display the pattern of metabolite formation similar to that of humans. In the SD, it was difficult to attain perhexiline concentrations within the clinical therapeutic range. Based on the results from this study the female SD animal was not the most appropriate model to test for perhexiline enantiomer toxicity. Plasma perhexiline levels in this model were
well below the therapeutic perhexiline range and hydroxy metabolites were also much lower in those seen clinically in EMs; suggesting that this strain is very efficient in eliminating perhexiline from its system, which may protect the animal from perhexiline associated toxicity. On the other hand, our “poor metaboliser” model, the female DA rat strain, is not a true model of perhexiline poor metabolism due to the presence of cis-OH- perhexiline in both plasma and tissue, and a metabolic ratio that was similar to that of a human intermediate/extensive metabolism of CYP2D6. However, in this model not only was I able to achieve plasma perhexiline concentrations, which were within the plasma perhexiline therapeutic range, but I also demonstrated that this model does indeed demonstrate a pattern of metabolite formation which is similar to what we observe in humans. The results from this project suggest that the DA animal model would be an appropriate one to determine future toxicity and efficacy studies involving perhexiline and its enantiomers. Indeed, the female DA does develop perhexiline-induced hepatic steatosis and neuronal dysfunction, as can be seen in the electron micrograph images presented in Chapter 5, which resemble the perhexiline toxicity described in a clinical setting. These findings suggest that one must be careful when using the terms “Poor and Extensive CYP2D6 metaboliser” models, as the functional validity of these terms depends on the drug(s) being tested, rather than the animal model adopted. Therefore the female DA rat is an appropriate model to assess perhexiline toxicity in future studies, however there is a need to be cautious when associating perhexiline toxicity to metaboliser status when using this model.

In Chapter 5 (manuscript 3) my aim was to use the animal model developed in Chapter 4 to test the perhexiline enantiomers for toxicity, particularly the toxicity which is associated with perhexiline in a clinical setting. I hypothesised that one enantiomers may display the steatosis and neurotoxicity associated with perhexiline while the other may not.
This study, to the best of my knowledge, is the first to give insight into the pharmacokinetics and pharmacodynamics of the enantiomers of perhexiline, administered as an enantiomeric formulation, in an animal model with metabolism similar to humans. The results confirmed as per a previous human study by Davies et al [79], that the CYP2D enzymes involved in the metabolism of perhexilene are enantioselective for (-)-perhexiline. The data indicate that cis-hydroxy(-)-perhexiline is present at much higher concentrations in blood, hepatic and myocardial tissue than parent drug. In contrast, in the (+)-perhexiline-treated group, cis-hydroxy(+)perhexiline was present at much lower concentrations than the parent compound in all tissues. Furthermore it was observed that (+)-perhexiline was present at higher concentrations in all tissues analysed, when compared to its antipode. Similar results could be seen in the group administered racemic perhexiline, both in the enantioselectivity in metabolism as well as the distribution into tissues. There were a few surprising results in this study. The first when comparing the tissue and plasma concentrations of (+)-perhexiline administered as a racemic or enantiomeric formulation. When administered as an enantiomeric formulation, (+)-perhexiline showed a hepatic to plasma ratio of approximately 150:1, whereas administered as a racemic formulation, the ratio was 50:1. However, there was no significant difference in plasma concentrations whether (+)-perhexiline was administered in its enantiomeric or racemic formulation. These results may have unmasked an enantioselective transporter effect that seems to favour the uptake of (+)-perhexiline or, as reported in Chapter 5, (-)-perhexilene may play an inhibitory role in the uptake or efflux of (+)-perhexiline in or out of the cell, when administered as racemic formulation. These studies confirmed that the two enantiomers are pharmacokinetically different and there may be enantioselectivity, not only in metabolism, but also in transport in and out of tissues.

The other unexpected finding in this series of studies was that the pharmacodynamics of the two enantiomers differed significantly. It is known that perhexiline is a potent CPT-1 inhibitor
[69], and this is believed to be the mechanism of action that gives it its therapeutic benefits. However it is evident from the numerous clinical case reports, that once above the therapeutic plasma concentration range (>0.6 mg/L) for an extended period, perhexiline can cause hepat- and neuro- toxicities. The micro- and macro-scopic features associated with this toxicity are attributed to the increased lipid and phospholipid deposits within the hepatocyte as well as the neuron, suggesting that CPT-1 inhibition may also contribute to its toxic effects. The electron microscopy data from this hepatotoxicity study clearly demonstrated that, following oral administration of (+)-perhexiline, this group of DA rats displayed a significant accumulation of lipid within the hepatocyte compared to either the control or the (-)-perhexiline-treated groups. I also noted that the increase in lipid deposit occurred in a dose-dependent manner as explained in Chapter 5. Furthermore, neuro-toxicity, as assessed via Von frey filament analysis, was greatest for (+)-perhexiline. This was further established in electron micrographs of the dorsal root ganglia where Mallory bodies were only found in those rats treated with (+)-perhexiline, although this was only in 1% of cells. These results indicate that the steatosis seen in a clinical setting of perhexiline-induced toxicity may be attributed to the presence of (+)-perhexiline. Surprisingly, upon examination of the electron microscope images of hepatocytes from the (-)-perhexiline-treated group, I observed a different metabolic effect. Instead of an increase in lipid, I observed an increase in glycogen. There is evidence from various case reports that glycogen accumulates within the cell after administration of racemic perhexiline [115].

At the beginning of this project, the overall aim was to consider whether one could develop an enantiomerically pure perhexiline formulation that would be more efficacious and/or less toxic than the current racemic mixture. The main hurdle in determining this was (as mentioned in section 1.4 of this thesis) that perhexiline seems to have more than one
mechanism of therapeutic action and furthermore it is still not clear whether inhibition of CPT-1 is its primary therapeutic mechanism of action.

The experiments described in Chapter 3 were a replication and extension of the study of Kennedy et al [9] that showed both the enantiomers inhibited the production of superoxide via neutrophil NADPH oxidase with the same potency as the racemic counterpart, providing evidence that the perhexiline enantiomers both have therapeutic potential. In Chapter 4, I tested and refined a model to assess the toxicity of racemic, (+)- or (-)- perhexiline, and in Chapter 5, I tested these compounds and obtained good evidence that the enantiomers are different both in their pharmacokinetic and pharmacodynamic properties.

The major limitation throughout this project was the availability of the pure form of each enantiomer. The enantiomers were synthesised in house at the University of Adelaide chemistry department. This was an expensive process costing approximately $1000 per mg of enantiomers produced. As a result of this expensive process as well as the available funding at the time, I was restricted to treating only 4 rats with (+)- or (-)-perhexiline, although each enantiomer demonstrated different and significant effects in cellular energetics. If pure forms of (+)- and (-)- perhexiline had been readily available, there would have been an opportunity to establish dose-response relationships to determine if these effects were concentration dependent.

Although in Chapter 3 I determined that the enantiomers of perhexiline had effective anti-inflammatory properties as measured by superoxide inhibition, I was unable to investigate the major site of action and conclusively determine what effects perhexiline enantiomers had on the myocardium. I did not know whether myocardium accumulated glycogen or lipid after administration of (-)- or (+)- perhexiline, respectively, as in liver. I was unable to determine if
these effects were beneficial or detrimental to the myocardium. Hence future studies might consider these experiments. Furthermore, one might consider performing Langendorff heart studies where (±), (+)- or (-)-perhexiline could be perfused and compare any changes in myocardial contractile function and heart rate, while at the same time comparing effects on energetics, resulting in a more conclusive therapeutic profiling of the individual enantiomers.

In summary, in consideration of hypotheses set out in Chapter 1, I have concluded the following: hypothesis 1) is partially accepted as both (+) and (-)-perhexiline have an equally potent effect as assessed by IC50 values on superoxide inhibition via neutrophil NADPH oxidase. Hypothesis 2 can be accepted, as I was able to investigate the pharmacodynamic properties of both perhexiline enantiomers in a rat model of perhexiline toxicity, with plasma concentrations that are observed clinically.

In conclusion, with respect to the findings within this thesis, clinical administration of perhexiline is essentially the administration of two drugs with very different properties. It is evident from the data presented in this thesis that each enantiomer affects cellular metabolism in a different way. (+)-Perhexiline appears to impede lipid metabolism resulting in steatosis, a common effect seen in a clinical setting of perhexiline toxicity. In contrast, (-)-perhexiline causes an accumulation of glycogen, the implications of which are yet to be elucidated. Each enantiomer of perhexiline has an effect on the two cellular pathways that are responsible for the production of cellular energy required for cell function.

Further studies need to be conducted which would help address the gaps in our current knowledge. As we now have a toxic and partial therapeutic profile of the perhexiline enantiomers, future studies could consider: (a) the dose-response relationships for each enantiomer on the female DA rat, (b) the potency of the individual enantiomers for inhibition
of CPT-1, (c) what transporters are involved in both the uptake and efflux of perhexiline, (d) whether these transporters are enantioselective, and (e) the efficacy of the individual enantiomers in an animal model of angina or heart failure. Do both enantiomers display therapeutic effects? What is the effect of glycogen accumulation in the liver, heart and whole organism? Is glycogen accumulation therapeutic? Can perhexiline enantiomers be used to decrease risk of myocardial damage pre or post surgery? These are the kind of questions that need investigating and may one day lead to the development of a marketable enantiomerically-pure drug with decreased toxicity and increased efficacy for a variety of myocardial disorders.


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59. Kantor, P.F., et al., The antianginal drug trimetazidine shifts cardiac energy metabolism from fatty acid oxidation to glucose oxidation by inhibiting mitochondrial


