University of Alberta

The effect of oral lipids and lipoproteins on the biodistribution, metabolism and electrocardiographic side-effects of halofantrine

by

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Dedicated

To

My Loving Parents and Grandparents

Abstract

In the past, hyperlipidemia (HL) has been shown to affect the pharmacokinetic and pharmacodynamic properties of lipophilic drugs extensively bound to lipoproteins, including halofantrine (HF). The present thesis examined the effect of HL on the biodistribution, metabolism and electrocardiographic (ECG) effects of HF in the poloxamer 407 rat model of HL.

The HL state caused unexpected changes in the distribution of HF enantiomers. In contrast to plasma, concentrations of desbutyl-HF (DHF) were much higher in highly perfused tissues. Following in vitro incubation of racemic HF and DHF, HF and DHF enantiomers shifted from the lipoprotein deficient fraction to triglyceride-rich fractions in HL plasma. No significant differences were noted in HF metabolism between NL and HL liver microsomes. It appears that both reduced plasma unbound fraction and lipoprotein associated directed uptake of lipoprotein-bound drug by tissues play roles in enantiomer biodistribution.

In everted gut metabolism, formation of DHF enantiomers was inversely proportional to bile concentration whereas addition of lipids in the presence of bile caused a significant decrease in DHF:HF ratio of (-)-enantiomers. Pre-treatment of rats with peanut oil had no significant effect on DHF formation in the incubated sacs or microsomal preparations, nor did it affect the expression of intestinal CYP450. The above results were consistent with previous in vivo

evaluations showing that the DHF to HF ratio was decreased by the ingestion of a high fat meal.

In the ECG study, HL by itself had no effect on the ECG. In HL rats, plasma but not heart concentrations of the HF enantiomers were significantly higher compared to NL rats. DHF did not impart significant ECG prolonging effects after HF administration. The unbound fraction of HF was the controlling factor for drug uptake by the heart. Despite the lack of difference in HF heart concentrations, the QT and QTc intervals were significantly higher in HL compared to NL rats, suggesting a greater vulnerability towards HF induced QT interval prolongation in the HL state.

The HL serum resulted in decreased metabolism of HF enantiomers in the isolated primary rat hepatocytes. Studies with LLC PK1 and NRK 52E cells showed that HF is not a substrate of P-glycoprotein transporters.

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Table of contents

List of Tables

List of Figures

List of abbreviations and symbols

µL	Microliter
µM	Micromolar
~	Approximately
AA	Arachidonic acid
ATP	Adenosine triphosphate
ABC	ATP-Binding cassette
AM	Amiodarone
Apo	Apolipoprotein
AUC	Area under the concentration versus time curve
BSA	Bovine serum albumin
CAR	Constitutive androstane receptor
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
CHOL	Total cholesterol
CL	Total body Clearance
Clint	Intrinsic clearance
CM	Chylomicron
Cmax	Peak plasma drug concentration
CYA	Cyclosporine A
CYP450	Cytochrome P450
DATAAN	(+)-Di-O-acetyl-L-tartaric acid anhydride
DEA	Desethylamiodarone
DHF	Desbutylhalofantrine
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco’s Modified Eagle Medium

ECG	Electrocardiograms
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme linked Immunosorbent Assay
EPA	Eicosapentaenoic acid
F	Oral bioavailability
GAPDH	Glyceraldehyde-3 -phosphate dehydrogenase
GI	Gastrointestinal tract
HCl	Hydrochloric acid
HC	Higher cholesterol
HDL	High density lipoprotein
HEPES sodium	N-(2-hydroxy ethyl) piperazine-N'-2-ethane sulfonic acid sodium
HNF-4α	Hepatic nuclear factor-4α
HF	Halofantrine
HL	Hyperlipidemia (Hyperlipidemic)
HPLC	High performance liquid chromatography
h	Hours
i.p	Intraperitoneally
i.v	Intravenously
IDL	Intermediate density lipoprotein
IU	International unit
KCl	Potassium chloride
KH	Krebs-Heinseleit bicarbonate buffer
KH_2PO_4	Potassium dihydrogen phosphate
Km	Affinity constant
Kp	Tissue to plasma concentration
Kpu	Tissue to unbound plasma concentration
LCAT	Lecithin-cholesterol acyl transferase
LDH	Lactate dehydrogenase

LDL	Low density lipoprotein
Logp	Logarithm partition coefficient
LA	Linoleic acid
LC	Lower cholesterol
LN	Linolenic acid
LPDP	Lipoprotein deficient fraction
LPL	Lipoprotein lipase
LRP	LDL-receptor related protein receptors
MDR	Multi drug resistance associated protein
mg/dl	Milligram/deciliter
mg/kg	Milligram/kilogram
MUFA	Mono unsaturated fatty acids
NADPH	β-nicotinamide adenine dinucleotide phosphate tetrasodium
ng	Nanogram
NL	Normolipidemia (Normolipidemic)
P-407	Poloxamer 407
P-gp	P-glycoprotein
PLX	Poloxamer 407
PO	Peanut Oil
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty aids
PXR	Pregnane X receptor
QTc	QT intervals normalized to the heart rate
r^2	Correlation coefficient
RF	Rifampin
Rh-123	Rhodamine 123
SBS	Simulated rat bile solution
SD	Standard deviation

TG	Triglyceride
Vd	Volume of distribution
VLDL	Very low density lipoprotein
Vmax	Maximum rate of formation
Vmax/km	Intrinsic clearance
α	Level of significance

1 INTRODUCTION

1.1 Lipids

Lipids represent a major dietary source of energy to mammals and act as an essential structural component of the cell membrane, providing insulation to the cells and hence protection from physical injury and heat loss. Edible oils and animal fats constitute the major dietary source of lipids in humans. Apart from dietary sources, lipids which are used as a vehicle for lipophilic drugs in oral and parenteral formulations may also contribute to the exogenous pool of lipids.[1,2] Lipids are components of the mixed function oxidase system, where they play an important role in anchoring the CYP enzymes, facilitating substrate binding and transfer of electrons.[3] The activity of the CYP450 mixed function oxidase system is therefore dependent on the lipid content which includes their specific phospholipid matrix, fatty acid composition, cholesterol content and minor constituents such as vitamin E.[4] Apart from altering CYP function through changes in lipid microenvironment, fatty acids liberated on hydrolysis of triglycerides can also impart competitive or mechanism based inhibition of drug metabolism.

Due to their favourable properties of tissue penetration and potency, many existing and newly marketed drugs are lipophilic in nature.[5] Given their lipophilic nature, such drugs can associate with circulating lipid-containing particles, called lipoproteins, and with other plasma proteins which have shown to alter the pharmacokinetics/pharmacodynamic (PK/PD) properties of many lipophilic drugs

in the past.[6-13] Hyperlipidemia (HL) is a pathological state characterized by increases in serum lipoproteins, primarily of the low and very low density categories. These elevated levels of low density lipoproteins (LDL) can lead to atherosclerosis and an increased risk of serious cardiovascular diseases, including stroke and myocardial infarction.[14,15] Hyperlipidemia has a number of causes including heredity[16], lifestyle[17-19], metabolic disorders[20-25], and drug therapy (e.g. immunosuppressant therapy)[26]. This may potentially influence the PK/PD properties of the lipophilic drugs through various mechanisms, including alterations in the biodistribution and metabolism of lipophilic drugs.

1.1.1 Oral lipids

Any discussion of the effect of lipids on the PK/PD of a drug would be incomplete without consideration of the physiology of lipids. Dietary lipids are mainly composed of long chain triglycerides, with smaller proportions of phospholipids and cholesterols.[2] Under normal conditions, the small intestine can digest 95% of the triglycerides with only a small loss to the feces. Acylation of one, two or three fatty acid moieties on the glycerol backbone results in the formation of monoglycerides, diglycerides, or triglycerides respectively (Figure 1).[2,27] Fatty acids are generally classified based on their length (short, medium or long chain) and presence/or absence of double bonds (saturated or unsaturated) between carbon atoms. Digestion of these triglycerides into monoglycerides, diglycerides and free fatty acids by the action of lipases is an essential step for their absorption from the intestinal lumen; triglycerides are not directly absorbable by the enterocytes (Figure 2).[2,27]

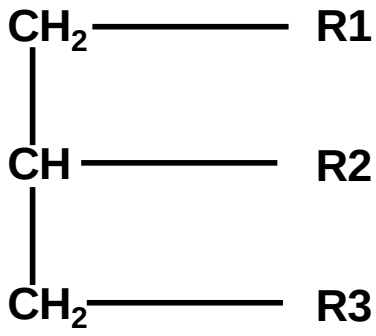

Figure 1: The stereochemistry of triglyceride (TG) structure where R1, R2 and R3 represent fatty acids acylated to the glycerol backbone at the sn-1, sn-2 or sn-3 position, respectively. Adapted from reference.[28]

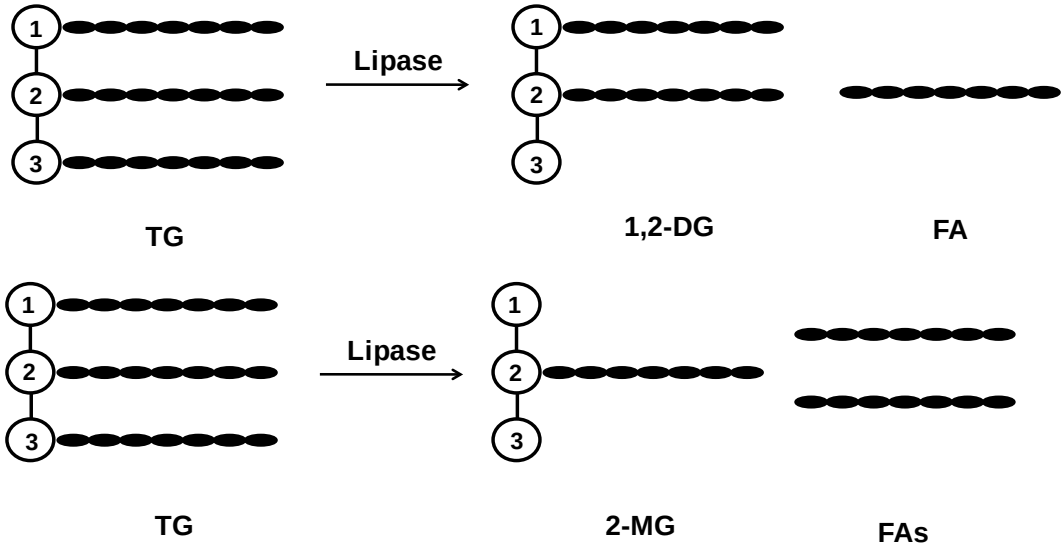

Figure 2: The cleavage of one or two fatty acids from the glycerol backbone of triglycerides (TG) by pancreatic lipase leads to the formation of monoglycerides (MG) or diglycerides (DG), respectively and free fatty acids.

Absorption of long chain triglycerides is facilitated through the collective action of bile salts, phospholipids, lipase (salivary, gastric and pancreatic) and colipase.[2,27] Upon absorption, the products of the lipid digestion are transferred by fatty acid binding protein from the brush border membrane to the smooth endoplasmic reticulum where CYP enzymes are expressed (Figure 3).[29] In the smooth endoplasmic reticulum, the re-esterification of free fatty acids and monoglycerides culminates in the intestinal formation of triglycerides. Similarly formation of cholesteryl ester and phosphatidylcholine takes place through acylation of free cholesterol and lysophosphatidylcholine.[2,30] These lipids migrate from the smooth endoplasmic reticulum to the rough endoplasmic reticulum where addition of apolipoproteins takes place to form nascent chylomicrons (CM).[2,31] These nascent CM are then transported to Golgi apparatus where final assembling takes place prior to their extrusion in to the intracellular space through the attachment of the Golgi vesicles containing CM to the plasma membrane (Figure 3).[32]

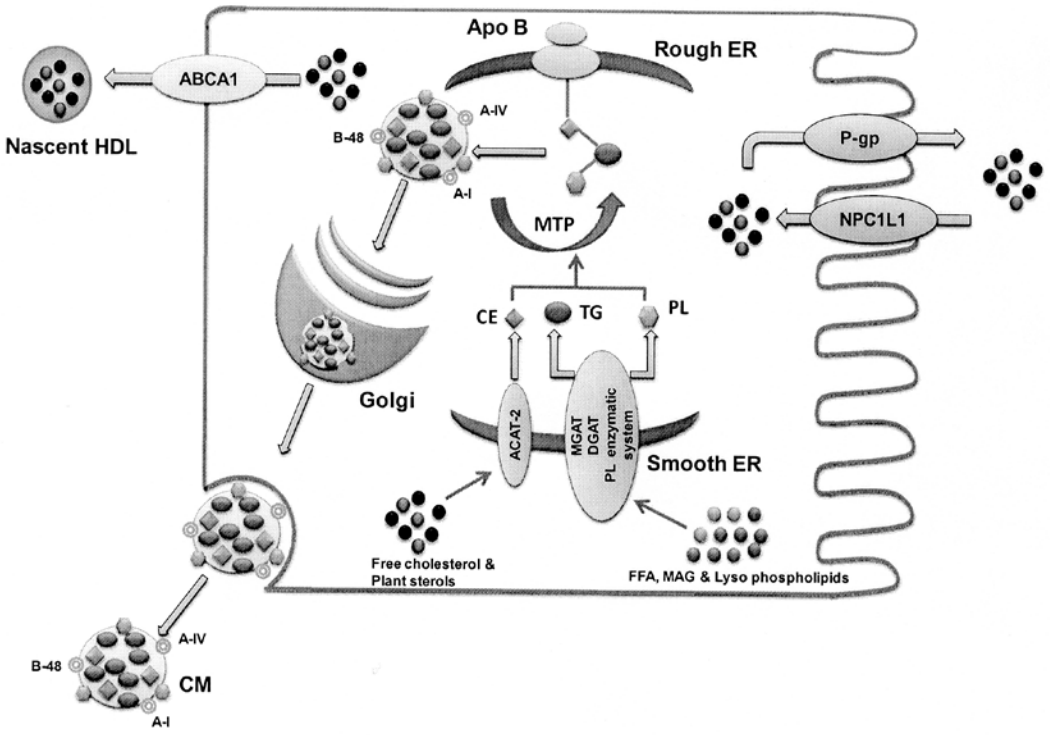

Figure 3: Schematic representation of the formation and release of chylomicrons (CM) in enterocytes following the absorption of lipid digestion products from brush border membrane of small intestine. Transport of cholesterol is regulated by both P-glycoprotein (P-gp) and NPC1L1 on the apical side of the enterocytes whereas ABCA1 is expressed on the basolateral side of the enterocytes. Free fatty acids (FFA), monoacylglycerol (MAG), lysophospholipids, cholesterol and plant cholesterol are transported to smooth endoplasmic reticulum (Smooth ER) where esterification of cholesterol to cholesteryl esters (CE) is catalyzed by acyl-CoA:cholesterol O-acyltransferase 2 (ACAT-2) whereas formation of triglycerides (TG) takes place through the action of a monoacylglycerol acyltransferase (MGAT)-diacylglycerol acyltransferase (DGAT) complex. Formation of phospholipids (PL) is catalyzed by phosphoglyceride-synthesizing enzymes. Formation of nascent CM, catalyzed by microsomal triglyceride transfer

protein (MTP), takes place through assembling of lipids (TG, PL and CE) with apo B-48 in rough endoplasmic reticulum (Rough ER). These nascent CM are then transferred from rough ER to Golgi apparatus where final assembling takes place before they are exocytosed into the intracellular space after the fusion of Golgi apparatus with basolateral membrane. Adapted from reference.[33] It is apparent that there is an important contribution of smooth endoplasmic reticulum to the metabolism of the lipid components, leaving open possible interferences with drug metabolism, which is largely facilitated by metabolizing enzymes such as CYP located within the same organelle.

1.1.2 Lipoproteins

Transport of triglycerides and cholesterol in the systemic circulation is restricted due to their aqueous insolubility. The transport of these essential nutrients in the aqueous environment of the body is accomplished via circulating lipoproteins (Figure 4).

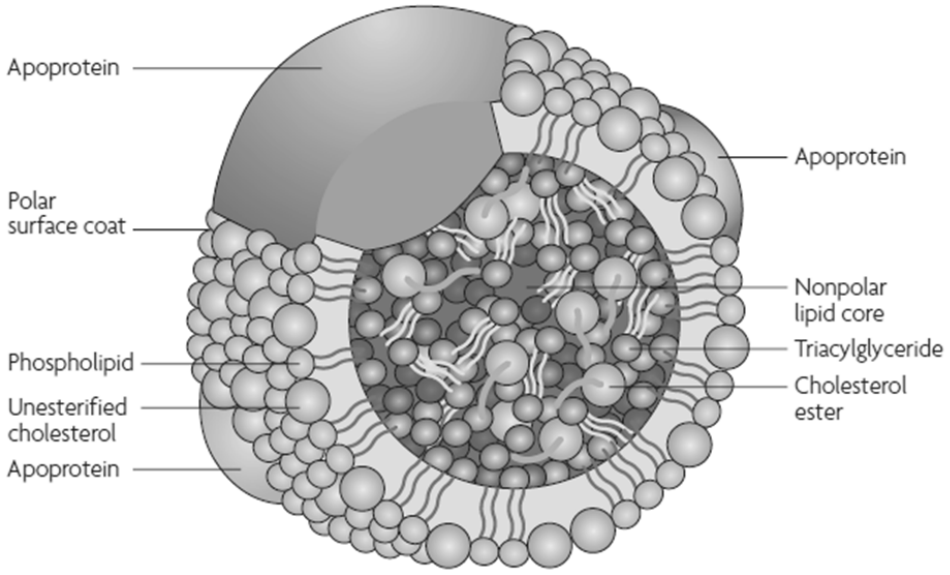

Figure 4: The general structure of lipoprotein. Adapted from reference.[34]

As shown in figure 4, lipoproteins are composed of an outer hydrophilic coat made up of amphipathic phospholipids and apolipoproteins and an inner hydrophobic core comprised of cholesteryl esters and triglycerides.[34-36] Apolipoproteins embedded on the outer coat of lipoproteins assist in maintenance of their structural integrity, facilitate enzymatic reactions involved in lipid metabolism, and serve as ligands for lipoprotein receptors on the cell surface of the tissues.[36] Lipoproteins, which differ in their protein and lipid contents are

classified into five groups based on their size, density and type of embedded apolipoproteins, in order from lowest to highest density: chylomicrons (CM), very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL).[34-36] For each of these lipoproteins, lower density is also associated with larger size.

The primary role of VLDL is to transport endogenously produced triglycerides from liver to extrahepatic tissues (Figure 5). Removal of triglycerides from VLDL and CM released from liver and small intestine, respectively, by lipoprotein lipase (LPL) on the vascular endothelium results into chylomicron remnants and IDL. Chylomicron remnants are taken up by the liver whereas IDL are converted further into smaller and more stable LDL. The LDL particles serve as a major carrier of cholesterol from liver to the peripheral tissues. HDL, commonly recognized as “good cholesterol”, is the smallest and densest of all lipoprotein classes with highest protein content. It serves as a major carrier of cholesterol from peripheral tissues to the liver.[34,35,37]

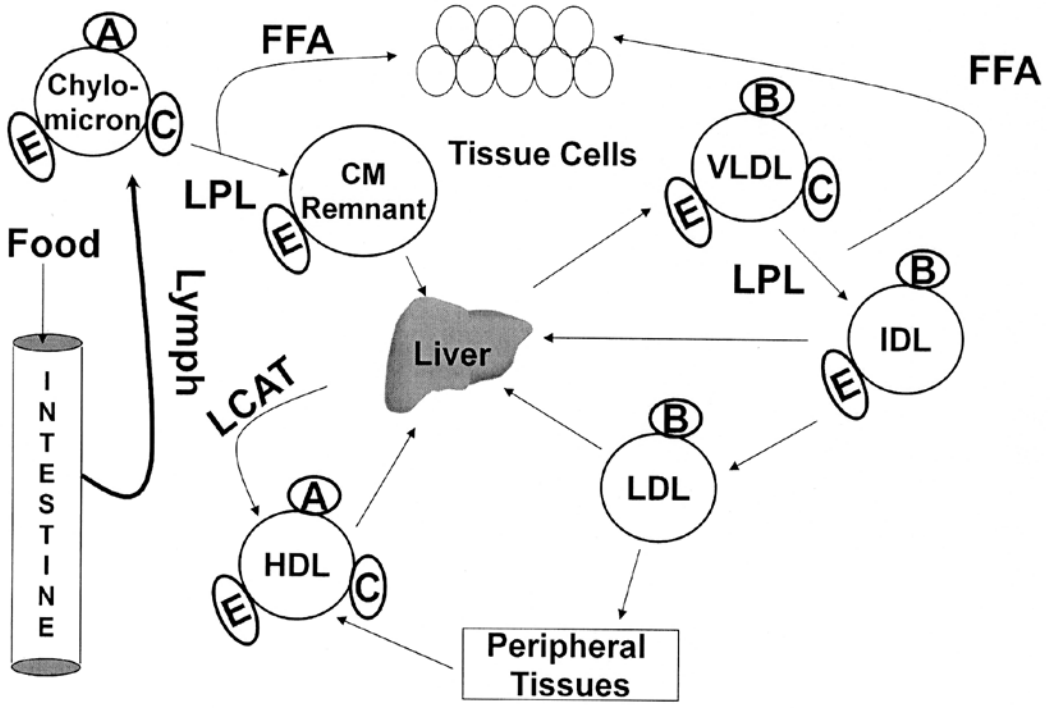

Figure 5: Schematic diagram of lipoprotein transport and receptor mediated pathways of lipoprotein metabolism. Following the ingestion of a high fat meal CM are assembled in enterocytes and secreted into lymph vessels which eventually empty into the systemic circulation via the thoracic duct. Removal of fatty acids from circulating CM by LPL bound blood capillaries results in CM remnants which are sequestered by the liver. VLDL, synthesized by the liver, transport endogenous TG to tissues. Over time, LPL reduces VLDL to IDL which in turn is transformed into LDL. An important role of LDL is to transport cholesterol to peripheral tissues. LDL is taken up by liver through binding of its constituent apoproteins to LDL receptors. HDL synthesized in liver transports cholesterol from peripheral tissues back to liver. Abbreviations: Free fatty acids (FFA), Chylomicrons (CM), Lipoprotein lipase (LPL), Very low-density lipoproteins (VLDL), Intermediate-density lipoproteins (IDL), Low-density

lipoproteins (LDL), High-density lipoproteins (HDL) and lecithin-cholesterol acyltransferase (LCAT). A, B, C and E represent types of apolipoproteins embedded within the lipoprotein outer core. Adapted from reference. [37]

1.2 Effect of lipids on drug metabolism

Drug metabolism is an important consideration in the drug discovery and development process. Metabolism of the drug following its administration is a major determinant of its duration and potency of pharmacological response. Drug metabolism in humans and other animal species is mediated through an array of Phase I and II enzymes located either in endoplasmic reticulum or cytosolic fraction of the cell.[3,38] Cytochromes P450 (CYP450) are the major enzymes involved in the metabolism of drugs and xenobiotics. At least 57 human CYP genes which belong to 18 families and 43 subfamilies have been identified and characterized.[3,38,39] Of these, the CYP1, CYP2 and CYP3 subfamilies are involved in the metabolism of drugs and xenobiotics.[3,38] The expression and activity of the CYP enzymes involved in drug metabolism are known to be modulated through variety of internal and external factors.[3]

Coingestion of food is capable of influencing the pharmacokinetics[7-9], including the metabolism, of drugs.[40-42] From the vantage of metabolism, the basis of food-drug interactions is most likely secondary to the ability of food components, including proteins and their constituent amino acids, carbohydrates, nucleic acids to competitively interact with the enzymes or cofactors necessary for metabolism of the coadministered drug.[41]

1.2.1 Oral lipids and drug metabolism

The small intestine serves as the major gateway of entry into the systemic circulation for many nutrients and xenobiotics including orally administered drugs. Upon their absorption, drugs may be metabolized through numerous pathways involving both phase I and II metabolic reactions. Major intestinal CYP enzymes involved in drug metabolism include CYP3A1, CYP2B1 and CYP1A1.[43]

To date, many studies have shown that bioavailability of lipophilic drugs increases on coadministration with high fat diet or fatty acids alone. High fat meals can affect the oral bioavailability of lipophilic drugs through increased solubilization of drug in the intestines, interference with drug transport protein activity[44-46] and hepatic metabolism[47], bypass of the liver first pass metabolism of drug associated with CM via lymphatics into systemic circulation [48] or combination of these events. Apart from dietary fatty acids, surfactants present along with lipids in pharmaceutical formulations can also inhibit the activity of CYP enzymes and transporters expressed in the small intestinal tissues. For instance, certain non-ionic surfactants belonging to the macrogolglyceride family such as Labrasol®, Gelucire® 44/14 and Labrafils® (mixture of mono-, di- and triglycerides and polyethylene glycol) can impart inhibitory effects on drug metabolizing enzymes as a result of fatty acid liberation after digestion by gastric and pancreatic lipases present in the GI tract.[49,50] Cremophor EL (polyethoxylated castor oil) used as a surfactant in many commercially available formulations has also been shown to liberate fatty acids upon digestion by pancreatic lipases.[51,52]

However, it should be noted that degree of effect is attributed to the type of fatty acids present in the surfactants. Effects of oral lipids on the metabolism of drugs is discussed below stratified by specific drug examples, and are also summarized in Table 1.

1.2.1.1 Specific drug examples

1.2.1.1.1 Halofantrine

Halofantrine (HF) is a chiral, highly lipophilic anti-malarial drug active against chloroquine-sensitive and -resistant strains of *P.falciparum*.[53] HF is metabolized to its active and chiral metabolite desbutylhalofantrine (DHF) through CYP3A1/2, 1A1, 2C11, 2C6, 2B1/2 and 2D1/2. Of these, CYP3A1/2 and CYP2C11 are primary isoenzymes involved in the metabolism of HF.[54]

Oral coadministration of lipids with racemic HF has been shown to increase the oral bioavailability of HF in humans and animal species.[8,55,56] For instance, administration of peanut oil 30 minutes prior to oral administration of HF HCl resulted in 2-3 fold increase in plasma AUC of HF enantiomers in the rat.[8] Intraduodenal infusion of HF formulated with oleic acid has shown to reduce the enterocyte based metabolism of HF in rats.[57] In humans, coadministration of HF HCl with high fat meal resulted in a 3-fold increase in the mean relative oral bioavailability of HF compared to the fasted state.[55] In fasted male beagle dogs, absolute oral bioavailability of HF HCl was low (<10%) but increased substantially (~12-fold) when administered post-prandially.[56] It was observed that

the increase in plasma AUC values of DHF in humans[55] and beagle dogs[56] was not in proportion to the increase in HF concentrations.

1.2.1.1.2 Amiodarone

Amiodarone (AM) is a highly lipophilic class III antiarrhythmic drug used in the treatment of life threatening ventricular and supraventricular arrhythmias.[58] Similar to HF, AM when coadministered with a high fat meal caused a greater increase in AM concentrations compared to its active metabolite desethylamiodarone (DEA) in healthy subjects.[59] Metabolism of AM in the rat small intestine is mediated through CYP1A1 and CYP3A1.[60,61] The reduction in the formation of DEA was not observed when AM was incubated with everted rat intestinal sacs in the presence of hydrolyzed soybean oil emulsion and simulated bile solution.[40] Amiodarone differs from HF because its dealkylation is facilitated by CYP1A1 to a much greater relative extent, with a relative rate of formation being ~2:1 for CYP3A1 compared to CYP1A1[61] (ratio for HF is ~ 24[54]). Therefore it is possible that competitive inhibition of CYP3A1 by polyunsaturated fatty acids (PUFAs) present in the soybean oil is compensated by CYP1A1 activity. In the everted gut sacs pretreated with oral peanut oil containing 1% cholesterol, a decrease in the formation of DEA was observed.[40] However, no change was observed in the formation of DEA on incubation of AM in microsomes prepared from peanut oil treated small intestine.[40] When microsomes are prepared, most cellular components, including most non-membrane bound lipids, are removed. Therefore decreased metabolism observed after peanut oil treatment was apparently due to the presence of the residual lipids in the intestinal

tissues after their oral absorption. It was felt that these residual lipids could have competitively inhibited the CYP enzymes involved in the metabolism of AM.

Apart from coadministration with a high fat meal, as was seen with AM,[6, 57] bioavailability of drugs may increase when coadministered with individual fatty acids.[62-64] Cis-4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA), an essential PUFA present in fish oil, breast milk, flaxseed oil, canola oil, walnuts, and phytoplankton,[65] is known to increase bioavailability of saquinavir,[62] cyclosporine A[63] and midazolam.[64] There was no significant effect of orally administered DHA on pharmacokinetic profiles of these drugs administered intravenously, suggesting that DHA increased their bioavailability through inhibition of presystemic metabolism of these drugs in the small intestine.[62-64]

1.2.1.1.3 Saquinavir

Saquinavir, a potent human immunodeficiency virus-protease inhibitor, has bioavailability of approximately 4% in the fed and 1% in the fasted state.[66] It has been reported that 90% of its metabolism takes place through hydroxylation via CYP3A isoenzymes.[66] Incubation of DHA with saquinavir in rat liver microsomes has been shown to inhibit the metabolism of saquinavir in a dose dependent manner. Bioavailability of saquinavir (50 mg/kg) was increased ~ 4-fold (from 6.5% to 26.4%) on oral coadministration with DHA (250 µg/kg) when compared to control (2% carboxy methyl cellulose sodium). Oral administration of DHA (200 µg/kg) did not affect the kinetic parameters of saquinavir (10 mg/kg) when administered intravenously, which indirectly suggested that DHA increased the bioavailability of saquinavir through its inhibitory effect on gut CYP3A.[62]

1.2.1.1.4 Cyclosporine A

Cyclosporine A (CyA), an immunosuppressive lipophilic agent widely used in prevention of organ graft rejection, is a substrate for CYP3A and P-glycoprotein. It has been reported previously that bioavailability of CyA was increased (~3.8-fold) with a high fat meal in healthy volunteers.[67,68] In plasma, CyA binds extensively to lipoprotein fractions. Based on typical binding of a low hepatic extraction ratio drug such as CyA to blood components, increased lipoprotein levels after ingestion of a high fat meal would be expected to result in a decreased unbound fraction and hence decreased clearance and volume of distribution of the drug. However, intravenous administration of CyA after a high fat meal to healthy volunteers behaved in a contrary fashion, and a marked increase in both the clearance (1.5-fold) and volume of distribution (1.6-fold) of CyA were observed.[67,68] This inverse relationship was attributed to the increased transport of CyA associated with lipoproteins across cell membranes through the action of lipoprotein receptors.[67,68] These results were somewhat mirrored in rats given oral doses of peanut oil 30 min prior and 2h after oral and intravenous administration of CyA, in which increases in area under the plasma concentration vs. time curve was expected. In contrast there was no significant change in AUC, and there seemed to be a trend towards a decrease in AUC.[7]

Some studies have also shown that fish oil and its components when used as a vehicle for CyA can increase bioavailability and limit its toxicity.[69,70] Coadministration of CyA (5 mg/kg) with DHA (50, 100 and 200 µg/kg) resulted

in marked dose-dependent increase in CyA blood concentration. DHA has been shown to inhibit the formation of 6β-hydroxytestosterone from testosterone mediated through activity of CYP3A enzymes in rat liver microsomes in a competitive manner.[63] A significant increase (130%) in the bioavailability of CyA on oral coadministration with DHA could therefore result from an inhibitory effect of DHA on the CYP3A isoenzymes involved in CyA metabolism. The lack of effect of DHA on basolateral to apical transport of radiolabelled CyA in a Caco-2 monolayer confirmed that DHA has no inhibitory effect on P-glycoprotein mediated efflux.[63]

1.2.1.1.5 Midazolam

Midazolam, a preoperative anesthetic agent, is extensively metabolized by gut CYP3A4 isoenzymes. Co-incubation of midazolam with DHA in the everted gut experiments resulted in a decrease in the extraction ratio of midazolam by intestinal tissue with increasing concentrations of DHA. In the same experiments it was also apparent that DHA by itself did not affect P-glycoprotein activity in the gut tissues. Formation of 1'-OH and 4-OH midazolam were decreased by the intestinal microsomes with increasing concentration of DHA. Lineweaver-Burk plots for the inhibition formation of both metabolites demonstrated that DHA caused a competitive inhibition of CYP3A activity in the small intestine. Significantly higher plasma AUC values of midazolam (10 mg/kg) were observed after its oral coadministration with DHA (100 mg/kg). However, there was no significant effect of oral DHA (100 mg/kg; administered 2 h before midazolam) on the AUC of midazolam (5 mg/kg) administered intravenously. Bioavailability

of midazolam was increased by 53% compared to control with DHA coadministration. Olive oil was used as a non DHA control lipid in this study.[64]

1.2.1.1.6 Simvastatin

In contrast to single dose studies with lipids or fatty acids, oral coadministration of prescription omega-3-acid ethyl esters 4 g (4 capsules) containing primarily eicosapentaenoic acid and DHA with 80 mg (1 tablet) simvastatin (CYP3A4 substrate) to healthy volunteers for 14 days showed no significant effect on steady state pharmacokinetics of simvastatin.[71]

1.2.1.1.7 Diosgenin

Oral administration of diosgenin (100 mg/kg in 0.5% carboxymethylcellulose) to hyperlipidemic (HL) rats (rendered HL by feeding high fat diet for 4 weeks) resulted in a 423% increase in AUC and 80% decrease in clearance of diosgenin which could be attributed to high fat-induced reduction in the first pass intestinal extraction or efflux transport of diosgenin .[72]

Table 1: Summary of the effect of oral lipids or lipoproteins on the metabolism and/or pharmacokinetics of drugs discussed above. Abbreviations: docosahexaenoic acid (DHA), low density lipoprotein (LDL), high density lipoprotein (HDL), peroxisome proliferator-activated receptor (PPAR) and area under the curve (AUC).

Drugs	Treatment/Disease	Species	Effect
Halofantrine	High fat meal	Humans	3-fold increase in mean relative oral bioavailability[55]
	High fat meal	Beagle dogs	12-fold increase in absolute oral bioavailability[56]
	Oral peanut oil	Rats	2-3-fold increase in plasma AUC[8]
	High fat meal	Beagle dogs	Decreased clearance and volume of distribution[73]
Amiodarone	High fat meal	Humans	Increased bioavailability[59]
	Oral peanut oil	Rats	1.83-fold increase in plasma AUC[9]
	Oral peanut oil	Rat everted small intestine	Decreased intestinal metabolism[40]
	Hyperlipidemia	Rat liver microsomes	Decreased hepatic metabolism[74]
Saquinavir	DHA	Rats	4-fold increase in bioavailability[62]
Cyclosporine	High fat meal	Humans	3.8-fold increase in bioavailability[67,68]
	DHA	Rats	2.2-fold increase in bioavailability[63]
	Coincubation with oleic acid, human LDL and HDL	Rat hepatocytes	Decreased uptake and metabolism[75]
	Coincubation with human LDL perfusate	Rat isolated liver perfusion	Decreased uptake and metabolism[76]
Midazolam	DHA	Rats	1.5-fold increase in bioavailability[64]
Diosgenin	Hyperlipidemia	Rats	Decreased clearance[72]
PPAR agonist MRL-I and II	-	Obese Zucker rats	Decreased glucuronidation[77]

1.2.2 Effect of fatty acids on CYP isoenzymes

The inhibition of liver CYP isoenzymes by fatty acids present in dietary oils (Table 2) is mainly dependent on the degree of unsaturation and length of the fatty acid chains.[78] The order of inhibition for fatty acids is usually saturated < monounsaturated < polyunsaturated fatty acids.[78] For instance, saturated fatty acids have weak inhibitory effect on CYP3A mediated metabolism of testosterone 6β-hydroxylation in rat liver microsomes. On contrary, unsaturated fatty acids potently inhibited hepatic CYP3A mediated metabolism.[63,78] Saturated fatty acids were devoid of inhibitory effects on activity of human CYP isoenzymes (CYP1A2, 2C9, 2C19, 2D6, 2E1 and 3A4) at concentrations up to 200 µM. PUFA had potent inhibitory effect on the activity of CYP2C9 and 2C19 but less potent effects on CYP1A2, 2E1 and 3A4 (Figure 6).[79] Arachidonic acid, the main product of α-linolenic acid, had a significant inhibitory effect on CYP1A1, 1A2, 2C8 and 2C19 in human liver microsomal preparations.[80] In another study, oleic acid, linoleic acid, linolenic acid and arachidonic acid each inhibited the rat liver microsomal mediated formation of the O-demethylated metabolite of p-nitroanisole. Similar results were observed with hexobarbitone in the same study. Metabolism of both p-nitroanisole and hexobarbitone was strongly inhibited by arachidonic acid, the most unsaturated of all fatty acids used in this study.[81] All of the above results were from acute in vitro incubations of fatty acids which resulted into competitive inhibition of metabolic reactions mediated through CYP isoenzymes.

Table 2: Fatty acid composition of common dietary oils (wt %)

Oils	Saturated, %	Monounsaturated, %	Polyunsaturated, %
Corn[4]	15.0	27.5	57.4
Olive[4]	17.3	77.4	8.50
Hazelnut[4]	8.30	69.7	20.2
Fish[4]	35.7	28.8	34.2
Peanut[82]	12.9	81.5	5.66
Soybean[83]	9.0	28.0	60.0

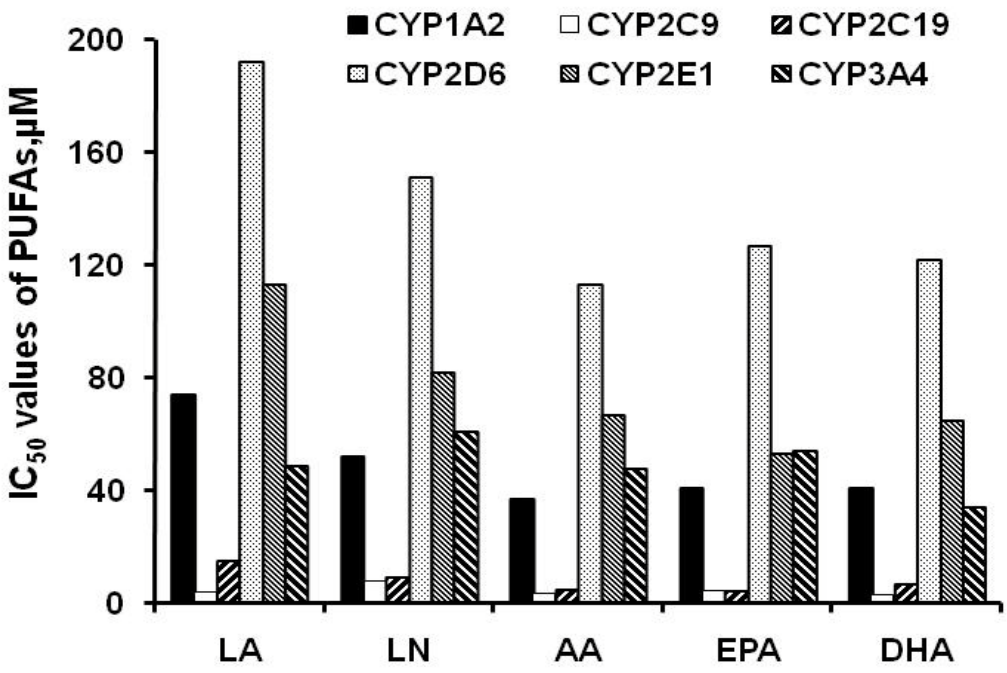

Figure 6: Inhibitory effect of PUFA (μM) on the metabolic reactions catalyzed by human CYP isoenzymes. Abbreviations: Polyunsaturated fatty acids (PUFAs), linoleic acid (LA), linolenic acid (LN), arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Figure constructed based on data presented in Table 2 of reference[79].

Long term treatment with oils has resulted in induction or suppression of some drug metabolizing enzymes (Table 3). For instance, oral administration of 1mL/kg/d corn, olive, sesame and soybean oil for 7 days to Sprague-Dawley rats resulted in a significant induction of hepatic CYP3A2 by soybean oil, but inhibition of CYP2C11 by corn and olive oil.[83] In another instance, CYP2E1 activity was induced in rat after treatment with high PUFA containing corn and menhaden oil (5 and 20% dietary composition each) for 4 days compared to lard and olive oil, which are high in saturated fatty acids and monounsaturated fatty acids.[84] Hepatic CYP450s (CYP1A2, 2B2, 2E1 and 3A) were induced and pulmonary CYP2B1 was suppressed following treatment with 20% corn oil for 4 days.[85]

It is possible that long term treatment with oils could have resulted from altered regulation of drug metabolizing enzymes. At the molecular level, gene expression of various drug metabolizing enzymes is regulated via interaction of xenobiotics or nutrients with nuclear receptors. For instance, the expression of CYP1 genes are regulated by the aryl hydrocarbon receptor whereas expression of CYP2B and CYP3A genes are regulated by constitutive androstane (CAR) and pregnane X receptors (PXR) belonging to a steroid family of orphan receptors.[86] Recently, arachidonic acid, linoleic acid, eicosapentaenoic acid and DHA at a concentration of 100 μM were shown to down-regulate the phenobarbital induced expression of the CYP2B1 gene in rat primary hepatocytes. The effect was most pronounced with arachidonic acid and DHA treatments.[87] In another study, DHA has shown to

down-regulate the phenobarbital-induced expression of the CYP2B1 gene in rat primary hepatocytes, through its inhibition of CAR translocation from cytosol to nucleus dose-dependently.[88]

In human hepatocytes, hepatic nuclear factor-4α (HNF-4α) appears to play a major role in the expression of transcription factors such as CAR and PXR, drug-metabolizing enzymes (CYP2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, uridinediphosphate-glucuronosyltransferase 1A1, 1A9 and sulphotransferase 2A1) and transporters (ATP-binding cassette (ABC) transporter B1, B11, C2, organic anion transport protein 1B1 and organic cation transport protein 1).[89] The fatty acyl-CoA thioesters (long hydrocarbon chains between C12 and C20 linked via thioester to coenzyme A) have been reported to be the ligands of HNF-4α.[90] Induction or inhibition of hepatic nuclear factor-4α is mainly dependent on the chain length and the degree of saturation of fatty acyl-CoA ligands. For instance, saturated fatty acyl-CoA (chain length of 14 and 16 carbon atoms) are agonists of HNF-4α whereas ω-3 and ω-6 polyunsaturated fatty acyl-CoAs and saturated (C18:0)-CoA have antagonistic effects on HNF-4α expression.[90] Thus it can be concluded that fatty acids can modulate the metabolism of a drug through direct competition with drug molecules or indirectly through modulation of expression of drug metabolizing enzymes.

Table 3: Effect of long-term consumption of common dietary oils on CYP enzymes involved in the metabolism of drugs and xenobiotics.

Oils	Dose	Treatment duration (days)	Effect on CYP450 isoenzymes	
			Induction	**Inhibition**
Corn[83-85]	1mL/kg/d	7	-	2C11 (Hepatic)
	5%	4	2E1 (Hepatic)	-
	20%	4	2E1, 1A2, 2B2 and 3A (Hepatic)	2B1 (lung)
Menhaden[84]	5%	4	2E1 (Hepatic)	-
	20%	4	2E1 (Hepatic)	-
Olive[83]	1mL/kg/d	7	-	2C11 (Hepatic)
Sesame[83]	1mL/kg/d	7	-	-
Soybean[83]	1mL/kg/d	7	3A2 (Hepatic)	-

1.2.3 Lipoproteins and drug metabolism

Drugs in the systemic circulation undergo binding with plasma proteins such as albumin, α-1 acid glycoproteins and globulins. Apart from these plasma proteins, lipophilic drugs are also known to associate with circulating lipoproteins in the plasma.[8,91,92] In some ways it is difficult to separate the effects of oral lipids from those of lipoproteins on drug metabolism, because upon their oral administration the absorbed lipids are repackaged in the enterocytes into CM, and eventually into other lipoprotein classes by the liver. Hence, in the post-prandial state, and in hyperlipidemia (HL) where baseline lipoprotein levels are intrinsically higher than normal conditions, association of drugs with circulating lipoproteins can result in decreases in their unbound fraction.[7-9,13,93,94] It is typically thought that only the unbound drug has the ability to traverse the cell membrane and reach the primary intracellular sites of drug metabolism or pharmacological effect. Therefore, a decreased unbound fraction of the drug can result in restriction of the drug in the blood and reduction in metabolism within hepatic and extra-hepatic tissues possessing metabolizing enzymes. On the contrary, it is also possible that lipoprotein receptors expressed in the liver and other organs[95] could facilitate increased uptake of lipoprotein-bound drug through interactions between the receptors and apo-proteins embedded within the outer coat of the lipoprotein particles.

Metabolism of lipoprotein-bound drug may be dependent on the type of lipoproteins to which it is bound, and because lipoprotein concentrations are

always in state of flux in the systemic circulation, affected by dietary intake and redistribution, liver, where the majority of drug metabolizing enzymes are expressed, is considered as a terminal site of catabolism for lipoproteins containing ApoB (CM, LDL and VLDL) through their recognition via LDL receptors.[95,96] Liberation of fatty acids following the catabolism of lipoproteins in the hepatocytes could directly reduce the metabolism of drug bound to the lipoproteins.[51, 61, 71] Thus lipoproteins can decrease the metabolism of drug through modulation of CYP expression or through decreased unbound fraction which in turn results in decreased load of drug into tissues. Both of these possibilities can result in a net reduction in the metabolism of drug in the presence of lipoproteins.

HL, like any other disease, can potentially affect the expression of drug metabolizing enzymes in the liver and other tissues. In the poloxamer 407 rat model of HL, it was observed that total CYP content in the liver microsomal protein was 1.94-fold lower compared to normolipidemic (NL) Sprague-Dawley rats.[74] Western blot analysis of these samples demonstrated significant decreases in the expression of CYP2C11, CYP3A1 and CYP3A2. Expression of CYP1A2, 2B1/2, 2C6 and 2D1 did not change.[74] This was not a poloxamer specific change, however, as in another study involving obese hyperlipidemic Zucker rats significant decreases were likewise observed in the expression of a number of drug metabolizing enzymes such as CYP3A1, CYP2C11, uridinediphosphate-glucuronosyltransferases 1A1, 1A6 and 2B1, glutathione S-transferase A2 and

quinine reductase. Significant reduction in the expression of transport proteins such as multidrug resistance-associated protein 2 and organic anion transport protein 2 were also observed in obese Zucker rats compared to lean Sprague-Dawley rats[77] In the Zucker obese rats, expression of the CYP3A regulator, CAR, was significantly lower than in control lean rats.[97] In addition, down-regulation of hepatic CYP3A expression after high fat intake was attributed to reduced expression of CAR and retinoid X receptor.[47]

Both CYP2C11 and CYP3A1/2 are regulated by the transcription factors such as CAR and pregnane X receptor.[98] Therefore, it can be postulated that down regulation of CYP2C11 and CYP3A1/2 in HL could have resulted from decreased expression of both transcription factors. Unlike Sprague-Dawley rats, however, Wistar rats rendered HL by poloxamer 407 did not show changes in their CYP3A expression in liver or intestine.[13] This could be possible due to apparent differences observed in the expression of hepatic CYP450 enzymes between Sprague-Dawley rats and Wistar rats. For instance, high mRNA expressions of CYP1A2 (3-fold) and CYP3A2 (2-fold) were observed in Wistar rats compared to Sprague-Dawley rats. There were no significant differences noted in mRNA levels of other CYP isoenzymes (CYP2B1/2, 2C6, 2D2, 3A1 and 4A1) tested between Wistar and Sprague-Dawley rats.[99] Effects of lipoproteins on the metabolism of specific drug examples discussed below are summarized in table 1 above.

1.2.3.1 Specific drug examples

1.2.3.1.1 Amiodarone

A significant increase (~2.5-fold) in the liver concentrations of AM were noted in HL compared to NL rats.[74] When incubated with liver microsomes prepared from NL and HL rats, the formation of DEA from AM fitted best to a sigmoidal Michaelis-Menten model with one enzyme. Rates of DEA formation were higher in NL than in HL. There were no significant differences observed between affinity constant (K_m) and V_{max} of NL and HL rat liver microsomes. However, clearance via DEA formation in HL livers was significantly lower than in NL livers. Addition of poloxamer 407 directly to the microsomal incubates resulted in no significant impact on the formation of DEA from AM, suggesting that poloxamer 407 by itself does not have any effect on the CYP450 enzymes and that the decrease in metabolism was due to the HL caused by the agent.[74]

1.2.3.1.2 Cyclosporine A

CyA when incubated with suspensions of rat hepatocytes in the presence of oleic acid, LDL and HDL resulted in a significant decrease in the uptake and metabolism of CyA. In this experiment, the effect of human LDL was found to be more pronounced than oleic acid and human HDL.[75] Another study with isolated rat liver perfusion also showed that uptake and metabolism of CyA was decreased when perfused with LDL.[76] Together these results suggest that association of CyA with the LDL particles restricts the amount of CyA accessible to drug metabolizing enzymes in the liver, thus resulting in decreased metabolism of

CyA. These conclusions were at odds with the increase in the clearance observed by the same group in healthy volunteers given high fat meals.[67,68] They are also inconsistent with data from in vivo administration of CyA to HL rats, in which a trend towards increased clearance was apparent, and where hepatic uptake was increased in HL compared to NL rats.[6] One possible confounding aspect of these in vitro studies involving rat liver[75,76] is that human LDL and HDL rather than rat lipoproteins were used. Although lipid from human LDL is taken up by rat liver, the uptake is less rapid and efficient than with rat LDL.[100]

1.2.3.1.3 Peroxisome proliferator-activated receptor (PPAR) agonists

Intravenous administration of the peroxisome proliferator-activated receptor (PPAR) agonists MRL-I and MRL-II to obese Zucker and lean Sprague-Dawley rats resulted in a significant decrease in clearance of MRL-I (~5-fold) and MRL-II (~2-fold) in obese Zucker rats compared to lean Sprague-Dawley rats.[77] There was no significant change observed in volume of distribution between two strains of rats. When kinetic parameters for metabolism of MRL-I and MRL-II were evaluated using liver microsomes from both strains of rats, significant decreases in V_{max} (~ 1.5-fold) and CL_{int} (~2-fold) of the formation of acyl glucuronide of MRL-I and MRL-II were observed in obese Zucker rats compared to lean Sprague-Dawley rats. K_m values did not differ between two strains. These results were in line with observed decreases in the expression of drug metabolizing enzymes in the obese Zucker rats.[77]

1.3 Effect of hyperlipidemia on the tissue distribution of lipophilic drugs

Distribution of the drugs to the tissues is dependent on many factors including physicochemical properties of drugs (lipophilicity, ionic state, molecular weight etc.), membrane permeability, influx and efflux transporters, blood perfusion rate and plasma protein binding.[101] As described above, pharmacokinetics of lipophilic drugs is known to be altered by HL conditions. The association of the lipophilic drugs with plasma lipoproteins in HL state has resulted in decreased unbound concentration of the drugs which is the major determinant of the drug distribution to the tissues. Apart from the unbound fraction, drug distribution to the tissues as a result of the lipoprotein receptor (present in various tissues)[95] mediated uptake of drug bound to the lipoproteins has been shown to cause unexpected changes in the tissue distribution of the lipophilic drugs AM[74] and CYA[6] which are discussed below.

Amiodarone

A single i.v administration of AM (25mg/kg) to HL rats demonstrated selective increase, decrease or no change in tissues compared to NL rats. For instance, the AUC of AM was increased significantly in heart (2-fold), liver (2.5-fold) and spleen (5.6-fold) tissues. A significant decrease in the AUC of AM was noted in brain (94%), kidney (50%) and lung (47%) tissues. No changes were observed in fat and thyroid tissues. The calculated Kp (tissue to plasma concentration ratio) from AM AUC was lower in HL compared to NL tissue whereas mean Kpu

(tissue to unbound plasma concentration) values were significantly higher in HL compared to NL tissues except for brain. There was no significant increase noted in the AUC of DEA in HL tissues. However, significant decreases were noted in AUC of DEA in kidney (67%), lung (47%) and spleen (97%).[74]

Cyclosporine A

In contrast to AM, following a single i.v dose of CYA (5mg/kg), an immunosuppressant which is extensively bound to lipoproteins, a decrease in the AUC of CYA was noted in heart (1.5-fold) and spleen (1.7-fold) tissues whereas higher AUC values were observed in kidney (1.5-fold) and liver (1.6-fold) tissues.[6]

1.4 Poloxamer 407 hyperlipidemic rat model

According to the Fredrickson-Levy-Lees classification, there are six categories of lipoprotein disorders namely, Type I, IIA, IIB, III, IV and V, which are commonly used as phenotypic descriptors of HL.[102] Type I HL is characterized by elevated levels of TG. The LDL and HDL are often low whereas VLDL is slightly elevated. Type IIA HL is associated with increased levels of plasma CHOL and LDL. Type IIB HL is characterized by elevated levels of total CHOL, TG, LDL and VLDL. In Type III HL there are increased concentration of both plasma CHOL and TG and an abnormal LDL. Type IV HL, also known as endogenous HL, has elevated TG and VLDL with moderately elevated levels of total CHOL. Type V is distinguished by elevated VLDL and CM in the plasma of fasting subjects on a regular diet.

Depending on the type of disorder, animal models such as obese Zucker rat model, genetically modified knockout mice (e.g. lipoprotein receptor or apolipoprotein E knockout mice) and chronic cholesterol fed laboratory animals are used.[103] In the present thesis, the poloxamer 407 (P407) rat model, most closely resembling type IV hyperlipidemia in humans, was used.[104] The use of i.p. P407 in rodents, first described by Johnston et al., provides an attractive model of HL due to its rapid onset and sustained yet reversible nature.[105,104] Repeated doses of P407 can result in atherosclerosis in rodents,[106,107] something that is difficult to achieve in those species.

P407 (Pluronic F-127), which is widely used in controlled drug delivery applications, is a block copolymer made up of repeated polyoxyethylene and polyoxypropylene units.[108] P407 undergoes reverse thermal gelation which means that the compound is a mobile viscous liquid at reduced temperatures and forms a semi-solid gel matrix at physiologic temperature. The P407 acts by inhibition of LPL responsible for TG degradation through hydrolysis.[105] The P407 has half-life of 20.9 h and at 96-120 h post-dosing when the plasma lipid concentrations return to normal levels, only 3% of the administered dose is predicted to remain in the circulation.[104,108]

Injection of P407 provides for a large increase in cholesterol and triglyceride plasma concentrations which can be used to evaluate the effects of HL on the pharmacokinetics of drugs, without any known adverse effects on the animals

given the agent.[104] On the negative side, the increases in lipoprotein levels are relatively extreme, which means that the results obtained must be viewed with caution when extrapolating to the human population.

P407 in vitro inflammation studies showed that P407 did not affect the production of Interleukin (IL)-6 and IL-8 by human umbilical vein endothelial cells and nitric oxide production by macrophages over the concentration range 0-40 μM.[109] In a recently published study, it was demonstrated that HL by itself do not produce proinflammatory factors such as IL-1, tumor necrosis factor alpha (TNF-α) and cyclooxygenase-2 (COX-2).[110]

1.5 Halofantrine

Halofantrine (HF) was identified as a potential antimalarial agent by the World War II Malaria Chemotherapy program. However, further research was not done by the US Army Medical Research and Development Command (Walter Reed Army Institute of Research) until the growing concerns of drug resistance were identified against *P.falciparum* during 1960s together with potential risk of malaria to the US troops in the Vietnam War. Out of ~250,000 compounds tested as a part of this program, halofantrine emerged as potential antimalarial agent effective against chloroquine-resistant and -sensitive strains of *P.falciparum*.[111-113] Halofantrine [1-(1,3-dichloro-6-trifluoromethyl-9-phenanthryl)-3-N,N-dibutylaminopropanol-hydrochloride] is a highly lipophilic (log P ~ 8.5)[114], 9-

phenanthrenemethanol derivative, one of the 3 classes of arylaminoalcohols.

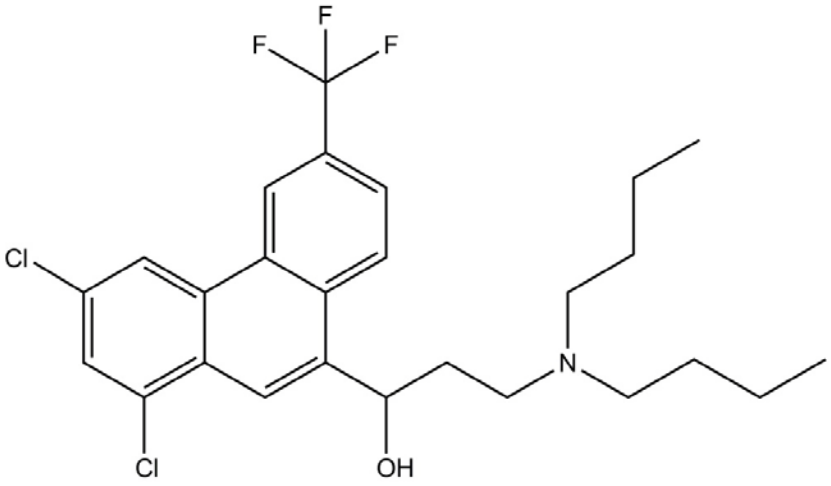

Figure 7: Structure of halofantrine.

1.5.1 Pharmacology and Pharmacodynamics

In humans, malaria is caused by the transmission of a protozoan parasite called *Plasmodium* by the bite of female Anopheles mosquito or by inoculation with infected blood. There are four species of *Plasmodium* namely *Plasmodium vivax, Plasmodium malariae, Plasmodium ovale* and *Plasmodium falciparum*. After transmission, the parasites multiply in liver and infect red blood cells.[113]

In the red blood cells, malarial parasites undergo digestion of host haemoglobin to form ferriprotoporphyrin IX[115] which in turn is converted into haemozoin, a crystalline cyclic dimer of ferriprotoporphyrin IX.[116] Halofantrine, which acts selectively during intraerythrocytic stages of Plasmodium species,[117] accumulates within the parasite food acid vacuoles and forms a halofantrine-ferriprotoporphyrin IX complex which results into the inhibition of haemozoin

formation.[118] The accumulation of toxic ferriprotoporphyrin IX-halofantrine complex in the digestive vacuoles results into the death of malarial parasites.

Both enantiomers of HF are equipotent in their antimalarial activity *in vitro*.[119,120] For instance, *in vitro* IC_{50} values of (+)-HF against chloroquine-resistant and -sensitive strains of *P.falciparum* are 2.8 ± 0.4 and 6.2 ± 0.2 nmol/L whereas for (-)-HF they are 3.0 ± 0.4 and 6.1 ± 0.2 nmol/L, respectively.[120]

1.5.2 Pharmacokinetics

HF is administered orally in 3 divided doses of 500 mg every 6 hours. The clinical use of HF is problematic because no clear relationship exists between HF dose and peak serum concentration (Cmax) or area under the plasma concentration–time curve (AUC) of HF due to its erratic and variable absorption after oral administration.[55,113] This is attributed to poor solubility of HF in water (<0.01%) at high pH. The poor solubility of HF is also the major limiting factor for the development of an intravenous formulation of HF.

Halofantrine is chiral and is administered orally as the racemate.[8,121] When administered to humans and rats, HF exhibits stereoselectivity in its pharmacokinetics.[121,122] After oral and iv dosing of HF, (+)-HF attains higher AUC values compared to its antipode. Compared to iv dosing, significantly higher stereoselectivity was noted in AUC values after oral dosing. The clearance and volume of distribution of (+)-HF is lower compared to its antipode. After oral dosing, significantly higher C_{max} values were noted for (+)-HF compared to (-)-

HF whereas t_{max} values do not differ between HF enantiomers. The half life of HF was not significantly different between enantiomers or between the routes of administration. The estimated oral bioavailability (F) of (+)-HF is 0.26 whereas for (-)-HF it is 0.16. The F of (±)-HF in rats is 0.23 which is in close agreement with estimated values in humans (F=0.31). HF is a low extraction ratio drug. Therefore low F of HF enantiomers is mainly attributed to the incomplete absorption of HF after oral dosing. Less than 1% of the administered drug is excreted in the urine.[122] In isolated feline myocytes, (+)-HF showed more potent inhibition of potassium channels than its antipode, suggesting stereoselectivity in its electrocardiac effects.[123]

Halofantrine is relatively slowly but extensively metabolized in rats and humans.[54,111] One of the major sites of metabolism of HF is at the tertiary amine within the alkyl chain extending from the phenanthrene ring system. The main product of this biotransformation is N-desbutylhalofantrine (DHF), which is also the major circulating metabolite of HF in humans.[113] Using recombinant rat CYP isoenzymes, it has been shown that HF is a substrate of CYP3A1, CYP2C11, CYP1A1, CYP2C6 and CYP2D1.[54] Stereoselectivity in the formation of DHF enantiomers was noted, with CYP3A1, CYP1A1 and CYP2D1 contributing more towards formation of (-)-DHF compared to its antipode whereas CYP2C11 caused a greater formation of (+)-DHF than (-)-DHF.[54]

Both HF and DHF possess similar pharmacokinetic profiles, although DHF exhibits higher stereoselectivity in its plasma concentrations than HF when given as the preformed metabolite.[124] Stereoselectivity was observed in the plasma protein binding of DHF but not HF enantiomers.[125] Both DHF and HF are equipotent in their antimalarial activities in vitro, but data pertaining to relative effects on cardiac electrophysiology are conflicting.[123,126]

1.6 Rationale and hypotheses

1.6.1 Rationale

Hyperlipidemia has the ability to change the pharmacokinetic and pharmacodynamic properties of lipoprotein-bound drugs, [6,7,9-12,34,127] one example of which is the antimalarial drug (±)-halofantrine.[128] The pharmacokinetics of HF are markedly affected by elevations in plasma lipoprotein concentrations, due to decreases in CL and Vd.[8,73] When HF was spiked with fasted rat, human and dog plasma in vitro, (-)- and (+)-HF showed higher affinity towards the lipoprotein-deficient and lipoprotein-rich fractions, respectively, suggesting some stereoselectivity in its lipoprotein association.[92] In addition to changes in pharmacokinetics, increased lipoproteins might influence drug effect. For example, an increase in the IC_{50} of HF was observed when post-prandial serum was incubated with *P. falciparum* culture in vitro.[129] In another study, a decrease in HF induced QTc prolongation was observed in hyperlipidemic (HL) compared to fasted rabbits.[130]

For low extraction ratio drug such as HF, the expected decrease in unbound fraction in HL would normally be accompanied by decreases in clearance and volume of distribution, leading to no net change in unbound plasma or total tissue concentrations. However, it is known that lipoprotein remnants can be actively taken up by certain tissues via the actions of tissue lipoprotein receptors which recognize specific apoproteins embedded in the outer core of the lipoprotein particles.[34] We have studied this phenomenon using two other lipoprotein-bound model drugs, AM[74] and CYA,[6] in a HL rat model. In those studies we observed that elevated lipoproteins can cause changes in pharmacokinetics, tissue distribution and metabolism, with possible ramifications on drug effect or toxicity.

High fat meals can affect oral bioavailability of lipophilic drugs by increased solubilization of drug in the intestinal milieu, thereby assembling the drug with chylomicrons in the enterocytes.[131] As discussed above (Section 1.2.1.1.1 under Oral lipids and drug metabolism), oral coadministration of lipids with racemic HF is known to increase its oral bioavailability.[8,55,56,111] However, after oral coadministration of HF with high a fat meal to humans[55] and beagle dogs[56] it was noticed that the plasma AUC values of DHF in humans[55] and beagle dogs[56] did not increase in proportion to the increase in HF concentrations. These less than proportional increases in the DHF AUC values were thought to be due to the transport of the drug from the enterocytes to the systemic circulation via the lymphatic vessels, allowing the drug to bypass a hepatic first pass effect.

However, HF possesses a low hepatic extraction ratio, and as such lymphatic shunting does not fully explain the discordance in metabolite to drug AUC ratio in the fatty meal state. Even if the drug entered the portal vein directly instead of being shunted into the mesenteric lymphatic drainage, most of the drug would still gain access to the hepatic vein circulation as unmetabolized drug. Unless the fatty meal markedly reduced the metabolic capability of the liver, the most likely candidate explanation for the lower than expected metabolite to drug ratio in the postprandial state is an inhibition of HF metabolism presystemically within the gastrointestinal tract. On the basis of its low extraction ratio, any food-related increases in hepatic blood flow would not be expected to cause a change in bioavailability.

As mentioned above in section 1.3, the altered lipoprotein levels have caused unexpected changes to the distribution of lipophilic drugs in the liver, which is a major site of metabolism for most drugs. In the HL state, although significantly lower plasma unbound concentrations may occur, internalization of drug-lipoprotein complex via the action of liver LDL and VLDL receptors can increase the concentrations of drug in the liver. This phenomenon has been previously noted for the lipophilic drug AM whose Kpu values were high in HL compared to NL liver despite the decreased unbound fraction in the plasma.[132] Similar results were also noted for HF enantiomers as shown in Figure 10. In the face of a significantly decreased expression of CYP3A1/2 and CYP2C11 in the HL state,[132] both of which are implicated in the metabolism of HF enantiomers, higher than

expected unbound liver concentrations in HL livers could still contribute towards increased metabolism of HF enantiomers in the HL state.

P-glycoprotein efflux transporters which are expressed on the plasma membrane of many tissues are known to limit the tissue accumulation of many drug substrates. In the *P. falciparum* malarial parasite, it was noted that accumulation of HF was altered by the P-glycoprotein homologue Pgh-1 encoded by the *pfmdr*1 gene.[133] However, there is no direct evidence published to date that HF is a substrate of mammalian P-gp, which is known to limit drug uptake into the brain.[134] Therefore, LLC PK1 and NRK 52E cell lines were used as a model to characterize the transport of HF enantiomers by P-gp efflux transporters.

After oral doses, the absorption of HF is erratic. This is of potential concern because the drug can cause life threatening cardiac arrhythmias in susceptible patients; if bioavailability is high in some patients they may be more at risk of this side effect.[135,136] The malarial patients treated with three oral doses of HF (500mg every 6 hours) had AUC and C_{max} values of 43-61 mg·h/L and 0.9-1.2 mg/L, respectively. The maximum QT interval prolongation was observed at plasma concentrations of ~1400 ng/mL.[111] Although QT prolongation was observed after HF treatments, ventricular cardiac arrhythmias were reported only in the patients with underlying cardiac disease or pretreatment with other QT prolonging drugs or improper dosing of HF.[137] The cardiac toxicity of HF is attributed to its concentration dependent prolongation of QT interval mediated *via* inhibition of

potassium channels encoded by the human ether-a-go-go-related gene.[138,139] Although both enantiomers are equipotent in their antimalarial activities *in vitro,* [120] (+)-HF exhibits more potent prolongation of QT interval compared to its antipode.[123] DHF is equipotent to HF in its antimalarial effect,[140] and like HF can prolong the QT interval; there is some debate, however, over whether the metabolite is less, equal or more active than the parent drug.[123,126]

For most drugs, it is assumed that only unbound drug in plasma can traverse tissue cell membranes and elicit pharmacological response, when the target receptors lie within tissue cells. However, in HL, tissue accumulation of drug could be enhanced due to selective uptake of the drug-lipoprotein complex by lipoprotein receptors present in various tissues.[36,141] For instance, AM uptake in heart, a tissue replete with very low density lipoprotein (VLDL) receptors, was increased more than expected in the HL compared to the NL state, even though the plasma unbound fraction was lower.[142]

Given the potential seriousness of QT prolongation in HF use, it is particularly important to identify factors which might influence its ability to prolong the QT interval. Thus far there has been one attempt made to study the influence of HL on HF cardiotoxicity.[130] It was reported that while HF caused prolongation of the QT interval in NL rabbits, very little increase occurred in HL animals. The study may not have been conclusive, however, due to the experimental design utilized. For example, the electrocardiograms (ECG) were obtained under conditions of

relatively long term anesthesia (at least 75 min, not including surgery) and mechanical ventilation, both of which could have influenced the QT interval.[143,144] Although stereoselectivity in cardiac effects were known,[123] a nonstereospecific assay was used to assay HF; more importantly, drug and metabolite concentrations in the heart, the target organ for the toxicity, were not assessed.

Patients infected with *P. falciparum* have been shown to possess elevated plasma TG, the levels of which were positively associated with the severity of the infection.[145] Similarly, another study showed that malaria resulted in significantly higher plasma levels of total CHOL, TG, LDL and VLDL and lower levels of high density lipoproteins.[146] Taken together along with the results from previous studies in rats, where HL was shown to affect the pharmacokinetics of HF, it is possible that HL conditions in malarial patients could affect the pharmacokinetic and pharmacodynamic properties of HF.

1.6.2 Hypotheses

On the basis of the line of thought provided in the rationale, my hypotheses are as follows:

1. There will be no marked changes in the uptake of HF enantiomers in HL compared to NL tissues. In the HL state, lipoprotein binding of the HF and DHF enantiomers will be altered. A decreased metabolism of HF will be seen in the HL compared to the NL state.

2. The postprandial intestinal components will inhibit the metabolism of HF enantiomers in the intestinal segments.
3. The metabolism of HF enantiomers will increase in the presence of HL serum in the primary rat hepatocytes.
4. The intracellular concentration of HF enantiomers will decrease in rifampin (RF) induced compared to uninduced LLC PK1 cells.
5. Repeated dosing of HF will result into higher concentration of HF enantiomers in HL compared to NL hearts. The higher concentration of HF enantiomers will result in increased prolongation of QT-intervals in HL rats.

1.7 Objective

Based on the above mentioned rationale, the general objective of the thesis was to examine the effect of oral lipids and serum lipoproteins on the biodistribution, metabolism and cardiac toxicity of halofantrine.

To accomplish the above mentioned general objective, our sub-objectives were as follows:

1. **To assess the effect of hyperlipidemia on the stereoselective tissue distribution, lipoprotein association and microsomal metabolism of (±)-halofantrine**
2. **To assess the effect of bile and lipids on the stereoselective metabolism of halofantrine by rat everted-intestinal sacs**
3. **To examine the influence of hyperlipidemia on the metabolism of HF enantiomers in primary rat hepatocytes**
4. **To determine the possible involvement of P-glycoprotein on the intracellular accumulation of HF enantiomers and Rh-123 in LLC PK1 and NRK 52E cell lines.**
5. **To investigate the effect of hyperlipidemia on the electrocardiographic effects of repeated doses of halofantrine**

2 EXPERIMENTAL

2.1 Materials

(±)-HF HCl and (±)-DHF HCl were gifts from SmithKline Beecham Pharmaceuticals (Worthing, UK). (+)-Di-O-acetyl-L-tartaric acid anhydride was purchased from Fluka (Ronkonkoma, NY, USA). Imipramine HCl, quinidine HCl, poloxamer 407, β-nicotinamide adenine dinucleotide phosphate tetrasodium (NADPH), cholesterol, peanut oil, bile extract porcine, L-α-phosphatidylcholine type XVI-E, porcine pancreatic lipase type II, sodium potassium tartarate, cupric sulfate anhydrous, bovine serum albumin (BSA), Folin-Phenol reagent, trypan blue solution (0.4%), new born calf serum heat inactivated, recombinant human insulin, collagenase, HEPES sodium salt, sodium dodecyl sulphate, ethylene glycol tetraacetic acid, trypsin inhibitor, Percoll, collagen from rat tail, medium 199 powder, fetal bovine serum (without heat inactivation) and rifampin were each obtained from Sigma (St. Louis, MO, USA).

Methanol, acetonitrile and hexane [all high performance liquid chromatography (HPLC) grades], triethylamine, sulfuric acid, glacial acetic acid, ammonium hydroxide, hydrochloric acid, sodium chloride, potassium chloride, sodium hydroxide, potassium dihydrogen phosphate, sodium bicarbonate, sodium dihydrogen phosphate, disodium hydrogen phosphate and ethylene diamine tetraacetic acid (EDTA) were purchased from Caledon Laboratories Ltd. (Georgetown, ON, Canada). Dimethyl sulfoxide (DMSO), magnesium sulphate, magnesium chloride and calcium chloride were obtained from Fisher Scientific

(Fair Lawn, NJ, USA). Tween-20 and D-glucose were obtained from BDH (Toronto, Canada). Primary antibodies for rat CYP1A1, CYP2B1/2, CYP3A1, secondary IgG with horseradish peroxidase and low range markers were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Soybean oil emulsion was obtained from Baxter (Mississauga, ON) as Intralipid. Heparin sodium injection, 1000 and 10,000 U/mL, (Leo Pharma, Thornhill, Ontario, Canada), dexamethasone sodium phosphate injection, USP (Sabex, Boucherville, Quebec, Canada) and cefazolin (Novopharm, Toronto, Ontario, Canada) were purchased from the University of Alberta Hospitals. Normal saline sodium chloride (9 mg/mL) was obtained from Hospira Healthcare Corporation (Montreal, Quebec, Canada). Isoflurane USP was purchased from Halocarbon Products Corporation (River Edge, NJ). Total serum cholesterol and triglyceride assay kits were purchased from Diagnostic Chemicals Limited (Charlottetown, PE, Canada). LDH kit (lactate dehydrogenase) used for the assessment of intestinal tissue viability was purchased from Sigma-Aldrich, Canada (Oakville, ON, Canada).

Penicillin-streptomycin and Dulbecco's Modified Eagle Medium (DMEM) were obtained from GIBCO, Invitrogen Corporation (Carlsbad, CA, USA). Glycine, acrylamide, N,N-bis-methylene-acrylamide, ammonium persulphate, beta-mercaptoethanol and TEMED were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Tris hydrochloride and sodium azide were purchased from EM Science (Gibbstown, NJ, USA). Skim milk was obtained from DIFCO

laboratories (Detroit, MI, USA). Chemiluminescence Western blotting reagent detection kit was purchased from GE Healthcare Life Sciences (Piscataway, NJ, USA). LLC PK1 and NRK 52 E cells were purchased from American type culture collection (ATCC: Manassas, VA, USA).

2.2 Methods

2.2.1 Assays

2.2.1.1 Instrumentation

A High Performance Liquid Chromatography (HPLC) system comprising a Waters 600 multisolvent delivery system pump, a Waters ™ 717 plus autosampler and a Waters ™ 486 tunable absorbance detector was used for analysis of HF and DHF enantiomers. Data collection was performed using a CR501 Chromatopack Schimadzu integrator or EZ-Chrom software (Alltech, Pleasanthon, CA, USA).

The chromatographic separation of HF and DHF enantiomers and their internal standards was carried out using a respective 4.6mm×250mm and a 4.6mm×150mm C18 reversed phase Ultrasphere® ODS analytical column (Beckman Coulter, Inc. Brea CA, USA). The analytical column was protected from strongly retained impurities by serial placement of a guard-Pak pre-column Module (Waters, Milford MA, USA) containing an ODS cartridge insert before the analytical column.

2.2.1.2 Preparation of standard and stock solutions for drug analysis

The stock solution of HF for drug analysis was prepared by dissolving 10.7 mg of HF HC1 in 100 mL of acetonitrile (equivalent to 100 µg/mL of HF base). The stock solution of DHF for drug analysis was prepared by dissolving 10.3 mg of DHF HCl in 95 mL of 50% acetonitrile in HPLC water (equivalent to 100 µg/mL of DHF base). The working standard solutions were prepared daily by sequential dilution of stock solutions in the respective vehicle of the stocks. The stock solution of quinidine as an internal standard for DHF was prepared by dissolving 5.83 mg of quinidine HCl in 100 mL of HPLC water (equivalent to 50 µg/ml of quinidine base). The stock solution of imipramine (100 µg/mL) as an internal standard for HF was prepared by dissolving 11.6 mg imipramine HCl in 116 mL of methanol. All stock solutions were kept in amber colour bottles at 4°C and remained stable for more than 6 months.

2.2.2 Sample Extraction technique

Established stereospecific HPLC assays involving precolumn derivatization of the enantiomers with (+)-di-O-acetyl-L-tartaric acid anhydride (DATAAN) were used to measure HF and DHF enantiomer concentrations in the specimens.[122,147,148] Quality control samples were included in all analytical runs as a check for assay validation. In all specimens there was no interference of endogenous components with drug or internal standard peaks.

A 0.25 M solution of DATAAN was freshly prepared in acetic acid: dichloromethane (1:4, v/v) for derivatization of HF and DHF enantiomers.

2.2.2.1 Plasma or serum

All collected plasma samples were allowed to thaw before analysis. To assay HF enantiomers in 100 µL plasma, 30 µL of imipramine (100 µg/mL) was added as an internal standard followed by 300 µL of acetonitrile for precipitation of proteins. All the tubes were vortex mixed for 3 seconds and centrifuged at 2500 g for 3 min. The supernatant was transferred to disposable glass tubes (13×100mm) followed by sequential addition of 200 µL of ammonium hydroxide and 3.5 mL of hexane: tert-butyl methyl ether (50:50). The tubes were vortex-mixed for 90 seconds, centrifuged at 2500 g for 5 min and the organic layers were transferred to clean tubes. The extracts were dried under vacuum by a thermosavant (Savant Speedvac, Colin Drive, Holbrook, NY, USA). To the dried extract was added 300 µL of DATAAN solution and the tubes were placed in an oven at 45^{o}C for 30 min. The reaction was terminated by addition of 50 µL of methanol and solvent was dried under vacuum. The dried derivatized samples were reconstituted in 170 µL of mobile phase and appropriate volumes (50-100 µL) were injected on the HPLC column.

For assay of DHF enantiomers, 50 µL of quinidine and 300 µL of acetonitrile were added to 100 µL of plasma. The tubes were vortex mixed for 3 seconds and centrifuged at 2500 g for 3 min. The protein-free supernatant was transferred to clean glass tubes containing 300 µL of Sorensen phosphate buffer (pH 8, non-

isotonic). To each tube was added 4 mL of hexane, vortex mixed for 60 seconds and centrifuged at 2500 g for 3 min. The organic layer (supernatant) was transferred to new tubes and evaporated to dryness under vacuum. After drying, 300 μL of DATAAN solution was added to each tube and incubated at 4^{o}C for 5 min. To stop the reaction, 50 μL of methanol in water (1:1) was added and the tubes were vortex mixed for 3 seconds. After evaporating the solvents under vacuum, the dried extract was reconstituted with 150 μL of mobile phase and aliquots of 50-100 μL were injected into the HPLC.

2.2.2.2 Tissues

At the time of analysis, each tissue sample was thawed, blotted with paper to remove blood, weighed and homogenized in Sorenson's phosphate buffer (pH 8) with a buffer to tissue mass ratio of 3:1.

To assay HF enantiomers in the tissue homogenates, some minor modifications were required. Briefly, for assay of HF 400 μL of tissue homogenate (equivalent to 100 mg tissue) were transferred to clean glass test tubes. To this 30 μL imipramine HCl (internal standard) and 1.5 mL acetonitrile was added to each tube, which was vortex mixed for 3s and centrifuged at 2500 g for 3 min. After transferring the protein free supernatant to new tubes, 0.5 mL of ammonium hydroxide was added followed by 7 mL of hexane: tert butyl-methyl ether (50:50). All the samples were vortex mixed for 90 sec, centrifuged at 2500 g for 3 min and the organic layers were transferred to clean tubes. The extracts were dried

under vacuum and derivatized and subjected to chromatography as described for plasma.

For assay of DHF enantiomers in tissue homogenate, 150 µL of quinidine HCl (internal standard) in water and 1.5 mL of acetonitrile were added to 400 µL of tissue homogenate. The tubes were vortex mixed for 3 s and centrifuge at 2500 g for 3 min. Protein free supernatant was transferred to clean glass tubes and 7 mL of hexane was added to each tube. The samples were vortex mixed for 60 s, centrifuged at 2500 g for 3 min and organic layer (supernatant) were transferred to new tubes. The supernatants were then dried under vacuum and the samples were derivatized and subjected to chromatography as described for plasma.

2.2.3 Analytical techniques

2.2.3.1 Halofantrine

For stereospecific analysis of HF, the mobile phase consisted of acetonitrile: [25 mM KH_2PO_4: 3 mM sulphuric acid: 3.6 mM triethylamine] in a combination of 53.5:46.5 (v/v) containing 1.5 g/L of sodium dodecyl sulphate. The mobile phase was degassed by passing through a 0.45 µm filter and pumped at an isocratic flow rate of 1.2 mL/min for chromatographic separation of peaks at room temperature. A UV wavelength of 254 nm was used for detection of HF and imipramine.

2.2.3.2 Desbutylhalofantrine

The mobile phase for stereospecific analysis of DHF consisted of acetonitrile: [25 mM KH2PO4: 3 mM sulphuric acid: 3.6 mM triethylamine] in a combination of

47:53 V/V containing 1.5 g/L of sodium dodecyl sulphate. The mobile phase was degassed by passing through a 0.45 µm filter. An isocratic flow rate of 1.2 mL/min was used for chromatographic separation of peaks at room temperature. The UV detection wavelength for DHF and quinidine was 254 nm.

2.2.4 Standard Curves

2.2.4.1 Distribution of HF and DHF in lipoprotein fractions of NL and HL plasma

For the quantitation of HF and DHF enantiomers in separated plasma lipoprotein fractions, a dedicated standard curve (50-2500 ng/mL) was generated for each individual fraction (LPDP, TRL, LDL and HDL).

2.2.4.2 Microsomal study

For quantitation of DHF enantiomers in microsomal incubations, a standard curve was prepared in 500 µL of microsomal incubation mixture which consisted of a mixture of phosphate buffer solution (pH=7.4) containing 5mM of magnesium chloride hexahydrate and microsomal protein from gut or liver (1 mg/ml). The calibration samples ranging from 50-10,000 ng/mL were prepared by adding desired amounts of each working standard solutions of DHF and extraction was carried out as mentioned above by the DHF HPLC method.

2.2.4.3 Tissue distribution, everted gut metabolism and ECG study

For assay of HF and DHF enantiomers in tissues, standard curves ranging from 12.5-30,000 ng/mL were prepared by spiking the desired amount of each working standard solutions of HF and DHF to 400 μL (~100 mg) of drug-free tissue homogenates and extraction was done according to above described method.

2.2.4.4 Hepatocyte incubation

For assay of HF enantiomers remaining to be metabolized after HF incubation with NL and HL hepatocytes, standard curves ranging from 25-500 ng/mL were prepared by spiking the desired amount of each working standard solution of HF to 0.25 million/0.5 mL of blank cells in media with additive followed by the addition of 0.5mL of 1N NaOH and 1mL of acetonitrile. The extraction was done according to above described method for HF analysis.

2.2.4.5 LLC-PK1 incubation

For assay of intracellular concentrations of HF and DHF enantiomers in uninduced and rifampin (RF) induced LLC-PK1, standard curves ranging from 25-3000ng/mL and 12.5-1000 ng/mL respectively, were prepared by spiking the desired amount of working standard solution of HF and DHF to 0.25mL of blank cell suspension from respective treatments in 1X PBS followed by 0.25mL of 1N NaOH and 1mL of acetonitrile. Extraction was carried out according to the above mentioned method for HF and DHF analysis.

For measurement of the intracellular accumulation of rhodamine 123 (Rh-123)[149] in uninduced and RF induced LLC-PK1 and NRK 52E cells, a standard curve ranging from 0.1-0.8µg/mL was prepared by spiking the desired amount of working standard solution of Rh-123 (12.1µM or 4.6 µg/mL) into 0.5 mL of a mixture of 0.3M NaOH and 0.3M HCl (1:1). The fluorescence was measured at an excitation and emission wavelength of 498 and 525 nm, respectively.

2.2.5 Quantification of HF and DHF enantiomers

Both HF and DHF enantiomers were quantified based on standard curves constructed using either peak height or peak area ratios of each enantiomer of parent drug or metabolite to internal standard versus nominal parent drug or metabolite enantiomer concentration.

2.2.6 Experimental animals

All experimental protocols involving animals were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. Male Sprague-Dawley rats (Charles River, CRC, Quebec, Canada) were used in all of the tissue distribution, ECG, everted gut metabolism, and hepatocyte studies of HF and DHF. Body weight ranged from 250-350 g and all of the rats were housed in temperature controlled rooms with a 12 h light per day. The animals were fed a standard rodent chow containing 4.5% fat (Lab Diet ® 5001, PMI nutrition LLC, Brentwood, USA). Free access to food and water was permitted prior to experimentation.

2.2.7 The effect of HL on stereoselective tissue distribution of HF and DHF enantiomers

For biodistribution assessments, 56 rats were divided into normolipidemic (NL; n=28) and HL (n=28) groups. Rats were rendered HL by single intraperitoneal (i.p.) administration of 1g/kg P407 dissolved in normal saline (0.13 g/mL). Light isoflurane/O_2 anesthesia administered by anesthetic machine was used to ensure proper i.p. administration of P407 to rats. Rats in the NL groups received the same volume of the normal saline vehicle i.p.

2.2.7.1 Preparation of dosing solutions

All of the dosage solutions were prepared fresh on the day of study. The (±)-HF HCl dosing solution (5 mg/mL) was prepared in N,N-dimethylacetamide: polyethylene glycol 400: 5% dextrose in water: acetic acid (16:24:160:1).[125] At 36 h after administration of P407, 2 mg/kg HF HCl dosing solution was administered to each rat via the tail vein under light anesthesia.

Poloxamer 407 (P-407) solution was prepared by dissolving 2.05 g of pluronic F-127 in 13.7 ml of normal saline. The mixture was kept in a fridge overnight to complete the solubility.

2.2.7.2 Sample collection

At approximately 0.16, 1, 3, 6, 12, 24 and 48 h after dosing, the rats (n=4 from each time point) were anesthetized and exsanguinated by withdrawal of blood by cardiac puncture. The blood was centrifuged at 2500 *g* for 15 min to permit

separation of plasma. Heart, lung, liver, kidney, spleen, fat and brain specimens were excised at the same time and stored at -20^{o}C until assayed for HF and DHF enantiomers.

2.2.8 The effect of bile and lipids on the stereoselective metabolism of HF by rat everted intestinal sacs

2.2.8.1 Preparation of simulated bile solution

Simulated rat bile solution (SBS) was prepared to mimic the in vivo conditions of the rat GI tract that might be present in the absence of or after ingestion of a high fat meal. The SBS was prepared as a mixture of 10 mmol/liter bile extract (4.9 g/liter), 12.5 mg/liter lipase, 0.6 g/liter cholesterol and 0.8 g/liter lecithin in Krebs-Henseleit (KH) bicarbonate buffer.[150] To permit solubilization of the components, the mixture was sonicated for ~5 h at 37ºC. Lipase was added to permit partial hydrolysis of the triglycerides present in the soybean oil constituents of 20% soybean oil emulsion (Intralipid), such as would occur in the intestinal tract in vivo. To facilitate the formation of micelles from fat and bile salts, 5% hydrolyzed intralipid emulsion and HF was mixed with SBS and vortex mixed for 30 min. To evaluate the effect of higher concentrations of cholesterol (HC), SBS was prepared with high cholesterol concentrations (3.86 g/liter).

2.2.8.2 Everted gut metabolism

All rats received rodent chow up to the time of experimentation, although one subgroup of animals (n=4) were pretreated with two doses of 1% cholesterol in

peanut oil w/v (2 ml/kg) given 5 h and 1 h before obtaining the intestinal segments (PO) .

A single vertical incision was made under anesthesia along the midline of the abdomen to gain access to the intestines. The bile duct of each rat was closed using a single thread knot. Distal to the pyloric sphincter and the ligament of Treitz, the intestinal tissue was cut into 5 segments of approximately 10 cm long each. Segments were sequentially labeled G1 to G5. The bile duct was ligated with suture in Segment G1, the most proximal segment, representing mostly the duodenum. Segments G2 to G5, extending towards the ileum, represented a continuum of intestinal composition, ranging in series, from jejunum to ileum. Each segment was rinsed with ice cold KH (pH 7.5). Each segment was everted using a glass rod. One end of the everted gut sac was ligated with a silk thread and tied. The other end was left open for filling with KH (37ºC) and then tied with silk thread to form the sac. Each segment was then placed into a jacketed tissue bath containing 10µg/ml HF HCl in a total incubation media volume of 40 ml. HF HCl was prepared by dissolving 0.0052 g HF in 200 µL ethanol and 16 µL (400µg HF HCl) of this stock was added to each chamber.

Everted intestinal sacs were incubated in the presence of varying compositions of buffer, bile and lipid. Sacs were allocated to one of three major groups: i) KH (n=6), ii) KH + 5, 10 or 25% SBS (n=4 each) and iii) KH + 5% soybean oil emulsion (Intralipid) + 5, 10 or 25% SBS (n=4 each). The intestinal segments

from the rats pre-treated with PO were incubated with KH+5% SBS (n=4). Simulated bile solutions having high cholesterol (HC) had KH + 5, 10 or 25% SBS (n=4 each) and KH + 5% soybean oil emulsion (Intralipid) + 5 or 25% SBS (n=4 each) groups.

Each chamber was continuously oxygenated during the incubations by bubbling a mixture of oxygen and carbon dioxide (95:5) through the solutions at 37ºC for 4 h. The time duration of incubations was based on preliminary assessments of DHF formation, in which consistent increases in tissue DHF were noted for 4 h. At the end of the incubation period, each sac was removed from the chamber and washed with ice-cold saline to remove any excess drug on the surface of the tissues. An aliquot of the solution inside the sac was collected using a syringe for analysis of HF and DHF enantiomers on the serosal side of the gut. Each sac was blotted dry, weighed and homogenized with Sorenson's phosphate buffer (pH 8) with a buffer to tissue mass ratio of 3:1. Homogenates were stored at -20^{o}C until analyzed with stereospecific HPLC assay for HF and DHF enantiomers.

2.2.9 Effect of hyperlipidemia on halofantrine metabolism using rat hepatocytes in vitro

The two step liver perfusion method described previously by Seglen et al. was utilized for the isolation of hepatocytes after minor modifications.[151]

2.2.9.1 Preparation of perfusion solutions

For the isolation of hepatocytes, stock solutions of A_1 (prepared with 115 mM sodium chloride, 5 mM potassium chloride and 1 mM potassium dihydrogen phosphate in 500 mL of water), A_2 (25 mM Hepes sodium salt dissolved in 100 mL of deionized water), A_3 (0.5 mM ethylene glycol tetraacetic acid (EGTA) dissolved in 50 mL of deionized water plus 1 crystal of NaOH to dissolve EGTA), 1 mM calcium chloride solution and 1.2 mM magnesium sulphate solution, were prepared one day before the experiment. On the day of experiment, the fresh working solutions A*, A, B and C were prepared with appropriate proportions of stock solutions as follows: Solution A* was prepared by mixing 100 mL of solution A1, 100 mL of solution A2, 0.8 g of glucose and 0.8 mL of heparin in a final volume made up with 500 mL of autoclaved water. Solution A was prepared by mixing 250 mL of solution A* and 50 mL of solution A3 and completed to 400 mL with autoclaved water. Solution B was prepared by mixing 203 mL of solution A*, 325 µL of 1 mM calcium chloride, 162.5 µL of trypsin inhibitor and 162.5 mg of collagenase (collagenase was added just before perfusion) and completed to 325 mL with autoclaved water. Solution C consisted of 25 mL of solution B supplemented with 100 µL of 1.2 mM magnesium sulphate and 75 mL of DME media. All the solutions were adjusted to pH 7.4 (by using 1N HC1 or 1N NaOH) and filtered through a 22 µm membrane prior to use.

2.2.9.2 Isolation of rat hepatocytes

At 36 h before experiment, rats were rendered NL or HL by intraperitoneal injection of saline or poloxamer 407 (1g/kg) under light isoflurane anaesthesia. On the day of experiment, a midline laparatomy was performed under isoflurane anaesthesia for the cannulation of the portal vein and the suprahepatic inferior venacava. During the course of experiment, all tubing and solutions were maintained at 37°C and saturated with 100% O_2. Following the cannulation, solution A was perfused through portal vein into the liver at a flow rate of 35mL/min for 8-10 min using a peristaltic pump till all the blood was out. Immediately after the perfusion of solution A, the pump was switched to solution B with a flow rate of 30 mL/min for 10-15 min until the liver appeared completely blanched and softened. The liver was externally washed with normal saline (0.9% sodium chloride solution) during the entire period of isolation. After perfusion with solution B, the liver was carefully excised and placed in a Petri dish containing 100 mL of solution C and the capsule was stripped away from one side of the liver and cells dissociated by brushing the liver with a plastic comb. The cells were then filtered through a 253 µm nylon filter into a funnel and kept in a shaking water bath for 5 min at 37°C and supplemented with 100% O_2 with gentle shaking. After 5 min incubation, the cell suspension was filtered again through a 100 µm filter into 50mL sterile cell culture plastic tubes (VWR International, Mississauga, Ontario, Canada) and placed on ice until it was 4° C. Thereafter, the cells were centrifuged at 1000 rpm for 5 min at 4° C and the supernatant was aspirated and cells were resuspended in the DME media (additive-free). This step

was repeated twice, after which the supernatant was aspirated and the cells were suspended into DME media. This cell suspension was added to the Percoll gradient (prepared by adding 6.75 mL of 2X phosphate buffer saline, 8.25 mL of lx phosphate buffer saline and 10 mL of Percoll) and centrifuged at 4000 rpm for 10 min at 4° C. Finally, the supernatant was aspirated and cells were resuspended in DME media containing additives and the cells were adjusted to 0.5 million/mL.

2.2.9.3 Determining the viability of hepatocytes

The cell viability was determined by using the trypan blue exclusion method. For this purpose, 50 µL of cell suspension in DME media with additives was added to 50 µL of 0.2% trypan blue solution after which the cells were counted in 16 microscopic squares. The viable cells were expressed in million/mL excluding the dead cells using the following equation:

Cells in millions/mL = Cells in 16 microscopic squares × Dilution factor × 10,000

2.2.9.4 Preparation of primary cultures

The hepatocytes were seeded on a 24-well plastic culture plates (VWR International: Mississauga, Ontario, Canada) precoated with collagen (50µg/mL) on the day before the experiment. Collagen stock solution was prepared by dissolving 25 mg collagen in 12.5 mL of 100 µL acetic acid in 87 mL of autoclaved water. Out of this 1 mL of collagen stock was diluted to 40mL with 100 µL acetic acid in 87 mL of autoclaved water and 0.5 mL was added to each well of 24 well plates. After viability assessments, the cell suspension was diluted to 0.5 million/mL with DME media supplemented with 10% fetal calf serum and

antibiotics penicillin/streptomycin (1%). 0.5 mL cell suspension containing 0.25 million cells were added per well and the plates were incubated for 6 h at 37°C in a humidifier with 95% O_2 and 5% CO_2. After 6 h, media containing the dead cells was removed and cells were treated with drug in the presence or absence of 5% NL and HL serum in media.

2.2.9.5 Drug treatment to cells

All experimental groups were treated with (±)-HF (500ng/mL) from 0-72 h (n=6 wells for each time point). In serum treatment groups, cells were pre-incubated or co-incubated with NL or HL serum. In serum pre-incubation groups (n=3 rats), NL hepatocytes were preincubated for 24 h at 37^{o}C with medium or 5% NL or HL serum in medium. After 24 h, medium containing serum was removed and treatment was initiated with drug incubated with media alone. For serum co-incubation groups (n=3 NL and n=3 HL rats), (±)-HF was pre-incubated with NL or HL rat serum for 1 h at 37^{o}C in a shaking water bath to facilitate the association of HF enantiomers with serum lipoproteins. This preincubated mixture of drug and serum was diluted to 5% with medium and added to the wells containing NL and HL hepatocytes. In the group containing no serum (n=6 NL and n=6 HL rats), (±)-HF in medium (500ng/mL) was co-incubated with NL or HL hepatocytes. At various time points from 0-72 h after drug treatment, each experiment was terminated by addition of 0.5mL 1N NaOH to each well, samples collected in Eppendorf tubes and stored at -30^{o}C until analyzed for the concentration of HF enantiomers remaining.

2.2.9.6 Media and drug preparation

a) Media preparation

DME media with additive was prepared by adding 10% fetal calf serum, 1% penicillin/streptomycin antibiotic, 0.0057 mg/mL insulin, and 1M dexamethasone. The pH was adjusted to 7.4 using 1N HCl or 10N NaOH before adding insulin and penicillin/streptomycin antibiotic and DME media was filtered through bottle top filters after which fetal calf serum was added.

b) Drug preparation

Stock solutions

For control and serum preincubation groups, a 2.5 mg/mL stock was prepared by dissolving 10 mg HF HCl powder in 4mL of DMSO solution. For serum coincubation groups, a 10 mg/mL stock was prepared by dissolving 20 mg of HF HCl powder in 2mL of methanol.

Working solutions

Control and serum preincubation groups

10 µL of HF HCl (2.5 mg/mL) in DMSO was dissolved in 50 mL medium plus additive to a final concentration of 500 ng/mL.

Serum coincubation groups

2.5 µL of HF HCl (10 mg/mL) in methanol was added to 2.5 ml of NL or HL serum to have a final concentration of 10 µg/mL and the solution vortex mixed for 30s and incubated in a shaking water bath for 1h at 37^{o}C to facilitate the association of HF enantiomers with serum lipoproteins. The amount of methanol

added to the serum did not exceed 0.1%. Afterwards the 2.5 ml of NL or HL serum containing drug was added to 50 mL media so that the final drug and serum concentration in media were 500 ng/mL and 5%, respectively.

2.2.9.7 Collection of NL and HL serum

At 36 h prior to the collection of NL and HL serum, rats were administered either l g/kg P407 (0.13 g/mL in normal saline) or normal saline intraperitoneally. The rats were allowed free access to water and food for these 36 h. Blood was collected from NL and HL rats by cardiac puncture in glass test tubes without heparin. Immediately, the blood was kept at 4^{o}C for 30 min after which serum was separated from blood cells by centrifugation for 10 min at 2500g and stored at -20°C until used.

2.2.10 LLC PK1 and NRK 52E cell incubations

2.2.10.1 Media Preparation

LLC PK1 and NRK 52E cell lines were used as a model to assess the effect of P-glycoprotein efflux transport on the accumulation of HF enantiomers in brain tissue. In contrast to NRK 52E cells, LLC PK1 cells do not express P-gp as was evident by immunoblot analysis in the presence of C219 monoclonal antibody of P-gp.[152] Therefore, rifampin, a known inducer of P-gp, was used in the present study to induce the expression of P-gp in LLC PK1 cell lines.[153]

LLC PK1 cells were grown in medium 199 supplemented with 10% FBS and 1% penicillin (100 IU/mL) and streptomycin (100 µg/mL). Medium 199 was prepared

by dissolving 9.5 g dry powder of M199 in 1L of autoclaved water followed by addition of $NaHCO_3$ (2.2g/L). The pH was adjusted to 7.2 by using 1N NaOH or 1N HCl and the medium was filtered through steritop filters. To the filtered medium was added 10% FBS (without heat deactivation) and a 1% mixture of penicillin/streptomycin (10,000 U/mL penicillin + 10,000 µg/mL streptomycin). The medium containing 25µM rifampin was prepared by addition of 0.0216g of rifampin powder dissolved in 1.5 mL of methanol in 1L of medium 199 plus additive.

NRK 52E cells were grown in DME medium supplemented with additives 10% fetal bovine serum and 1% penicillin (100 IU/mL) and streptomycin (100 ng/mL).

2.2.10.2 Stock solutions

The HF stock solution (2.5mg/mL) was prepared by dissolving 10mg of HF in 4mL of DMSO. The 100mM verapamil stock solution was prepared by dissolving 0.049 g of verapamil HCl in 1mL of DMSO. A stock solution of 10mM CYA was prepared by dissolving 12.026 mg CYA in 1 mL of DMSO. A 50mM stock solution of AM was prepared by dissolving 34.105mg of AM HCl in 1 mL DMSO. A 121µM stock solution of Rh-123 was prepared in the dark by dissolving 4.6 mg of Rh-123 powder in 230 µL of methanol and completing to the final concentration of 121µM with autoclaved water.

2.2.10.3 Cell culture and growth conditions

To remove DMSO from the cells, the entire stock of the cells was mixed with 9 mL of complete growth medium and centrifuge at 125*g* (1000rpm) for 5-7 minutes. After removing the supernatant, cell pellet was re-suspended in 10 mL of respective medium supplemented with additives and the cells were seeded in 75cm^2 cell culture flask at 37^{o}C in 5% CO_2 humidified incubator. The cell growth was checked after 4 h and medium was replaced every 48 h until confluent growth was achieved in the flask. Once the confluent growth was achieved in the flask, cell splitting was carried out on the basis of a 1:3 ratio. Before splitting the cells, old medium was discarded and the cells were washed with 5mL of 1X PBS containing EDTA. After this 5mL of trypsin EDTA (prepared with 90mL of PBS EDTA and 10 mL of 2.5% trypsin) were added and the mixture left into the incubator at 37^{o}C for less than 10 minutes. The cells were gently flushed to disperse them homogenously and divided among 3 flasks containing respective medium supplemented with additives. This process was repeated until sufficient cell number was achieved for experiments.

2.2.10.4 Study groups and drug treatment

To achieve sufficient induction of P-glycoprotein in LLC PK1 cells, cells were divided into uninduced without RF and RF induced cells. The cells were plated into 24-well plastic culture plates (VWR International: Mississauga, Ontario, Canada) after splitting into a 1:6 ratio. After 6h of platting, medium was discarded and the cells were divided into two groups (uninduced and RF induced) by adding

respective medium with or without RF. The media were changed every 48 h and the cells were grown for 1 week to achieve sufficient expression of P-glycoprotein in RF treated cells. On the 7^{th} day of incubations, the media were removed and treatment was initiated with control media containing HF (3000ng/mL). The final concentration of DMSO in each well was 0.12%. At various time points from 0, 1, 4,8, 24, 48, 72 and 96 h media containing HF were removed and the reaction was terminated by washing the cells three times with 0.5mL ice cold PBS (1X). After this 0.5mL NaOH (1N) was added into each well containing 0.5mL PBS (1X) and samples were collected and analyzed as described above for the intracellular accumulation of HF.

To assess the intracellular accumulation of Rh-123 in LLC PK1 and NRK 52E cells, the cells were preincubated with or without verapamil (1, 10 and 100µM), CYA (0.1, 1 and 10µM) and AM (1, 10 and 50µM) for 1h. After 1h, treatment was initiated with Rh-123 (10µM) for 2h in the presence of inhibitors. After 2h, the media were removed and the cells were washed three times with ice cold PBS (1X). The cells were lysed with 0.3M NaOH (0.250mL) followed by the addition of 0.3M HCl (0.250mL). A 200µL aliquot from this was added to 96 well plates and fluorescence was measured at an excitation wavelength of 485 nm and emission wavelength of 535 nm.

2.2.11 Distribution of HF and DHF in rat plasma lipid fractions

2.2.11.1 Treatment of plasma samples

Aliquots (3 mL) of pooled rat plasma were spiked with 10 µL of (±)-HF (1000 ng/mL) and (±)-DHF (250 ng/mL) methanolic solutions each. The addition of this volume of methanol was known not to modify plasma lipoprotein-lipid composition.[154] The samples were vortex mixed for 30 s and incubation was allowed to proceed for 1 h at 37^{o}C prior to separation of lipoprotein fractions. The plasma was separated by step-gradient ultracentrifugation into its high (HDL) and low (LDL) density lipoprotein, its triglyceride-rich lipoprotein (TRL), which consists of very-low density lipoproteins (VLDL) and chylomicrons, and its lipoprotein deficient (LPDP) fractions as previously described.[154]

2.2.11.2 Lipid measurement

Enzymatic assay kits were used to determine total cholesterol and triglyceride concentration according to the manufacturer's directions using an ultraviolet spectrophotometer. For each fraction assay of aliquots was performed in triplicate.

To determine total CHOL and TG concentrations, peroxidase enzymatic cholesterol and triglyceride assay kits were utilized.

Principle for total CHOL determination: The CHOL esters are hydrolyzed to free CHOL by the action of CHOL esterase. The free CHOL is then oxidized by CHOL oxidase to cholesten-3-one with simultaneous production of hydrogen peroxide. The hydrogen peroxide then binds with 4-aminoantipyrine and phenol,

in the presence of peroxidase, to yield a chromogen with maximum absorbance at 505 nm.

Principle for TG determination: Serum TG are hydrolyzed to glycerol and free fatty acids by the action of lipase. The glycerol is converted to glycerol-1-phosphate in the presence of ATP and glycerol kinase. The glycerol-1-phosphate is then oxidized by glycerol phosphate oxidase to yield hydrogen peroxide. The coupling of hydrogen peroxide with 3,5-dichloro-2-hydroxy-benzenesulfonic acid and 4-aminoantipyrine in the presence of peroxidase produces a red coloured quinoneimine dye with maximum absorbance at 515 nm.

For CHOL and TG determination in HL plasma, a 1/10 fold dilution of HL plasma samples was carried out. For determination of TG, 2mL of TG reagent was added to 10µL of NL or HL plasma samples whereas 2.5mL of CHOL reagent was added to 25 µL of NL or HL plasma samples for CHOL determination. Tubes were incubated at 37°C for 5 or 10 min and scanned at 505 or 515 nm, respectively, using a colorimetric spectrophotometer.[127] The CHOL and TG concentrations of <200 mg/dL were defined as normolipidemic.

2.2.12 Microsomal studies

Microsomal studies were conducted to study the effect of oral lipids or HL on metabolism of HF to DHF enantiomers. In group one, the effect of HL on hepatic metabolism of HF to DHF enantiomers was studied. In group two, the effect of high fat meal on intestinal metabolism of HF to DHF enantiomers was studied. A total of 8 male Sprague-Dawley rats were used in each group. To study the effect

of HL on hepatic metabolism of HF to DHF enantiomers, rats were rendered NL (n=4) or HL (n=4) by intraperitoneal administration of saline or P407 (1g/kg) at 36 hours before the onset of the study. To mimic the effect of oral fat on intestinal metabolism of HF to DHF enantiomers, two doses of 1% cholesterol in peanut oil (2 mL/kg; w/v) were administered 5 h and 1 h before obtaining the whole length of small intestine.

2.2.12.1 Microsomal preparation

For preparation of microsomes, both livers and small intestine were excised from rats under isoflurane/O_2 anesthesia. Both tissues were washed in ice-cold KCl (1.15% w/v), cut into pieces and homogenized separately in cold sucrose solution (5 g of tissue in 25 mL of sucrose 0.25 M). Microsomal protein from homogenized tissues was separated by differential ultracentrifugation as follows: The homogenate was centrifuge at 600 G or 2230 rpm for 8 min after which the supernatant was transferred to Ti 50.2 tubes and centrifuged at 12,000 G or 14,000 rpm for 10 minutes. The supernatant was transferred to a new tube, mixed with 1M $CaCl_2.2H_2O$ (10µL per 1 mL of the supernatant) and centrifuge at 27,000 G or 21,500 rpm for 15 minutes. The cytosole present in the supernatant was transferred to 2 mL microcentrifuge tubes and stored at -80 C. The pellet was resuspended in 1.15% KCl solution and centrifuged at 27,000 G or 21,500 rpm for 15 minutes after which supernatant was thrown. The final pellet was suspended in 0.25M sucrose solution and stored at -80 C until used. [155] Separate microsomes were prepared from each rat to characterize the kinetics of the *in vitro* formation of DHF from HF and the effect of HL on the DHF formation. The Lowry method

was used to measure the total protein concentration in each microsomal preparation.[156]

2.2.12.2 Lowry assay method for protein concentration in microsomal preparations

The serial standard solution of bovine serum albumin (BSA) was used for the quantification of unknown protein concentrations in microsomal preparations. For the determination of protein concentrations in liver and gut microsomal preparations, Reagent A consisting of 1mL of cupric sulfate 1% in distilled water, 1mL of Na.K.tartarate (sodium and potassium tartarate) 2% in distilled water, 20 mL of 10% Na_2CO_3 anhydrous in 0.5 M sodium hydroxide and reagent B comprising of 1/10 diluted solution of Folin-Phenol reagent in distilled water were used. The BSA stock solution of 500 µg/mL (50 mg/100H_2O) was used for preparing the working standard solutions (0, 100, 200, 300, 400 and 500 µg/ml) of BSA in distilled water.

For the determination of unknown protein concentrations, 250 µL of reagent A was added to the mixture of 10 µL of microsomal preparation and 240 mL of distilled water (unknown concentration of protein) or 250 µL of each standard solution. This reaction mixture was incubated at room temperature for 10 minutes after which 750 µL of reagent B was added to each of the test tubes under continuous vortex mixing, and samples incubated at 50 °C for 10 more minutes.

At the end of the reaction, 200 µL of each mix was transferred to the ELISA plate and analyzed using an ELISA reader at 600 nm.[156]

2.2.12.3 Total CYP 450 measurement

The presence and amount of hepatic microsomal CYP content was measured spectrophotometrically by the method of Omura and Sato.[157] Briefly, 1 mg of microsomal protein, 200 µL of 0.5 M potassium phosphate buffer and sufficient distilled water was mixed in spectrophotometer cuvette to reach the final concentration of 1mg/mL. The top of the cuvette was covered with a small piece of parafilm and the cuvette was inverted gently to mix. A few milligrams of solid sodium dithionite ($Na_2S_2O_4$) was added to reduce the carbon monooxide- CYP complex, gently mixed by inversion and a background absorbance scan was recorded between 400 and 500 nm (reference scan). The samples were then gently bubbled with carbon monooxide for 30-60 s. Invert the samples to mix and gently tap to remove the bubbles after which a final scan was performed between 400-500 nm (sample scan). CYP content was calculated by the following equation:

CYP content (nmoL/mg protein) = $(\mathrm{OD450} - \mathrm{OD490})/91 \times 1000$

2.2.12.4 Microsomal incubation studies

Separated rat liver and intestinal microsomal preparations were used to study DHF formation kinetics. Incubation was performed by addition of 0.5 mL incubate containing 1 mg/mL protein to 0.5 M potassium phosphate buffer (pH 7.4) containing 5 mM of magnesium chloride hexahydrate and (±)-HF HCl (1.9-186 µM for liver and 70 µM for intestine) dissolved in methanol. A total volume

of 0.8% v/v methanol was added to each incubation through addition of (±)-HF HCl solution in methanol. Preincubation was performed in a 37^{o}C water bath for 15 min. After preincubation, reactions were initiated by addition of 1 mM NADPH to each tube. After 15 min the reactions were stopped by addition of 3 volumes of acetonitrile. After stopping the reactions, samples were frozen at -20^{o}C until assayed for DHF enantiomers.

2.2.12.5 Western blot analysis

Western blot analysis of gut microsomes from control and peanut oil treated rats was undertaken to assess the effect of lipids on gut CYP1A1, CYP2B1 and CYP3A1. Briefly, 100 µg microsomal proteins from each rat gut was diluted in loading buffer and boiled in a water bath for 5 min at 100^{o}C. Each denatured sample was loaded onto a 10% SDS-polyacrylamide gel and electrophoresed at 120 V for 2 h. The separated proteins were transferred to Trans-Blot nitrocellulose membrane (0.45 µm) in a buffer containing 25 mM Tri–HCl, 192 mM glycine, and 20% (v/v) methanol. Protein blots were blocked overnight at 4°C in a solution containing 5% skim milk powder, 2% bovine serum albumin and 0.5% Tween-20 in Tris-buffered saline buffer. Thereafter, the blocking solution was removed and the blots were rinsed three times in a wash buffer (0.1% Tween-20 in Tris-buffered saline). Membranes were incubated with primary antibodies (1/500 or 1/1000 dilution in Tris-buffered saline containing 0.01% sodium azide) to either rat CYP1A1, CYP2B1/2 and CYP3A1 overnight at 4 °C. The primary antibody solution was removed and blots were rinsed thrice with a wash buffer, followed by incubation with horseradish peroxidase conjugated with rabbit anti-goat or

goat anti-mouse IgG secondary antibody (1/5000 dilution) for 2 h at room temperature and washed as previously described.[74,158] Immunoactive proteins were detected using the enhanced chemiluminescence method (Amersham, Arlington Heights, IL) and the intensity of different CYP protein bands were quantified, relative to the signals obtained from non-specific bindings, using ImageJ®. Non-specific binding was used to normalize protein bands instead of loading controls such as GAPDH or β-actin. GAPDH was not detected while β-actin was not expressed uniformly in intestinal microsomal samples.

2.2.13 The effect of HL on the electrocardiographic (ECG) effects of repeated dose HF

Rats were rendered either NL or HL through intraperitoneal injection of saline or 1g/kg poloxamer 407 (P407) (0.13 g/mL solution in normal saline), respectively, given under light isoflurane anesthesia. A total of two doses of P407 or saline were administered to the rats. The next day after the first dose of P407 or saline, Silastic (Dow Corning, Midland, MI) cannulas were implanted into the right jugular veins under isoflurane/O_2 anesthesia. After surgery, 100 U/mL heparin in 0.9% saline was filled into the implanted cannulas and rats were transferred to regular holding cages where free access to water was permitted. The next morning, rats were transferred to plastic metabolic cages for dosing and blood sample collection.

To provide for a wide range of HF concentrations, NL and HL rats received discrete HF HCl doses of 4, 10, 20, 30 and 40 mg/kg/day. The main comparison dose group was the 20 mg/kg dose level, in which 8 rats were present in each of the NL and HL groups. To gain insight into the dose vs. concentration and ECG relationships, other smaller groups of animals received doses of 4, 10, 30 or 40 mg/kg/day (2 to 3 rats per dose). Drugfree vehicle NL and HL treated animals were included (n=4 each).

2.2.13.1 HF Dosing, ECG measurements and sample collection

A dosing solution of (±)-HF HCl (5 mg/mL) was prepared in N,N-dimethylacetamide: polyethylene glycol 400: 5% dextrose in water: acetic acid (16:24:160:1).[8] At approximately 36 h after first i.p dose of saline or P407, a total of 4 discrete doses of HF HCl or vehicle were administered to NL and HL rats for duration of 2 days with 12 h interval between each dose. The drug solution was administered slowly over a time period of 60 sec through an implanted right jugular vein cannula immediately followed by the administration of 0.5mL of 0.9% NaCl for injection to flush the drug solution remaining in the cannula. A solution consisting of 25% heparin 1000 U/mL, 55% polyethylene glycol 400 and 20% cefazolin 100 mg/mL was injected in the implanted cannulae to prevent the cannulae from becoming occluded between dosing and sampling. Based on the pharmacokinetic profile and the time of duration of P407 effect,[108] HL was maintained during the course of experiment by administration of the second dose of P407 just after the second dose of HF HCl. For NL rats, an equivalent dose of normal saline was injected at the same time.

To obtain the ECG readings, animals were briefly and lightly anesthetized under isoflurane anesthesia administered by an anesthetic machine. After placement of stainless steel subdermal needle electrodes, 12 s ECG recordings were made from all rats. The total duration of anesthesia prior to successful recording of the ECG (including induction) varied from 3 to 5 min. Recordings were made using a P55 general purpose AC preamplifier and the PolyVIEW data acquisition and analysis system (Grass Instrument Division, Astro-Med, Inc, West Warwick, RI). ECG recordings were taken at baseline conditions (before first dose of intraperitoneal injection of saline or P407 and before right jugular vein cannulation) and 12 h after the 1^{st} and 4^{th} doses of HF.

In order to correlate the ECG measurements with plasma concentrations of HF and DHF enantiomers, blood samples were collected into heparinized tubes immediately following the ECG recordings at 12 h after the first dose (~0.5 mL blood through tail vein) and the last dose (through cardiac puncture) of HF. All blood samples were centrifuged at 2500 g for 15 min to separate plasma from the blood cells. Blood collected at the end of the study was also used to measure the total cholesterol (CHOL) and triglyceride (TG) in the plasma. After cardiac puncture, hearts were harvested and blotted on the tissue paper to remove excess blood. All plasma and tissue samples were stored at $-30^{o}C$ until assayed for drug and metabolite.

2.3 Data and Statistical analysis

2.3.1 The effect of HL on stereoselective tissue distribution of HF and DHF enantiomers

The area under the concentration vs. time curves (AUC) from time of dosing to last time point (48 h) was measured for both plasma and tissues using the linear trapezoidal rule. The tissue to plasma concentration ratios of total (Kp) were calculated based on the post-distributive phase data. Using the unbound fractions of HF previously reported in NL and HL plasma,[8] the tissue concentration to unbound plasma concentration ratios (Kpu) were estimated.

All compiled data were expressed as mean±SD unless otherwise indicated. In the tissue distribution study, $AUC_{0\text{-}48}$ values for HF and DHF were determined for each specimen. The SD of partial AUC values was estimated to assess significance of differences. In this test, α was 0.05, the critical value of Z (Z_{crit}) for the 2–sided test after Bonferroni adjustment was 2.24, and the observed value for Z (Z_{obs}) was calculated as previously described.[159]

2.3.2 The effect of bile and lipids on the stereoselective metabolism of HF by rat everted intestinal sacs

Compiled data are expressed as mean±SD unless otherwise indicated. ANOVA followed by Duncan's Multiple Range post hoc test and Student unpaired t-tests were used as appropriate to assess the significance of differences between groups. Microsoft Excel (Microsoft, Redmond WA) or SPSS version 12 (SPSS Inc.,

Chicago, IL) were used for statistical analysis of data. The level of significance was set at $p<0.05$.

2.3.3 Effect of HL on the metabolism of HF enantiomers by primary rat hepatocytes *in vitro*

In hepatocyte incubations, the compiled data were expressed as mean ± SD of % (+)- and (-)-HF remaining to be metabolized by cultured primary rat hepatocytes. Duncan's post-hoc test was used to assess the significance of differences within treatment groups at each time points. Bailer's method as described under section 2.2.9.1 was used to assess the significant differences between AUC values calculated for the percentage of HF remaining to be metabolized.

2.3.4 Intracellular accumulation of HF enantiomers and Rh-123 in LLC PK1 and NRK 52E cell lines

All data are expressed as mean ± SD of the HF enantiomers and Rh-123 accumulated in LLC PK1 or NRK52E cell lines. In the presence of inhibitors, HF enantiomers and Rh-123 accumulation data (mean ± SD) are expressed as % of control. Level of significance was assessed using a t-test ($p<0.05$).

2.3.5 Microsomal studies

For microsomal metabolism, DHF formation kinetics were measured by plotting the rate of DHF formation vs. substrate concentration. Both one and two enzyme models were fitted to the formation rate versus substrate concentration data using non-linear curve fitting routines:[54]

$$\textit{Rate of DHF formation} = \frac{V_{max,1} \times [HF]}{k_{m_1} + [HF]} + CL_{int,2} \times [HF] \qquad \ldots (1)$$

Where $V_{max,1}$ and k_{m1} and the ratio of $V_{max,1}$ to k_{m1} $(CL_{int,1})$ represented maximal rate of formation of DHF enantiomer, affinity constant of HF enantiomer and intrinsic formation clearance of DHF enantiomer, respectively, for the high affinity saturable enzyme. The $CL_{int,2}$ represented the intrinsic formation clearance of the low affinity non-saturable enzyme. For the one enzyme system, the product of $CL_{int,2} \times [HF]$ was removed from the above equation. The best fitted enzyme model was judged based on residual sum of squares and Akaike Information Criterion.

Student's paired or unpaired t-tests were used as appropriate to assess the significance of differences between groups. Microsoft Excel was used in statistical analysis of data. The level of significance was set at $p < 0.05$.

2.3.6 Lipoprotein fraction distribution studies

For lipoprotein fractions, compiled data were expressed as mean ± SD of percentage of total recovery of HF and DHF. Duncan's post-hoc test was used to assess the significance of differences within lipoprotein fractions of each group. Student's paired or unpaired t-tests were used for statistical comparison between NL and HL groups and between enantiomers within each group. The level of significance was set at $p < 0.05$ for all comparisons.

2.3.7 Western blot analysis

Student's paired or unpaired t-tests were used as appropriate to assess the significance of differences between groups. Microsoft Excel was used in statistical analysis of data. The level of significance was set at $p< 0.05$.

2.3.8 The effect of HL on the electrocardiographic (ECG) effects of repeated dose HF

The accumulation factors for HF enantiomers in plasma were determined as the quotient of the last measured plasma concentration to those 12 hours after the first dose. A random coding was assigned to the recorded ECG strips to ensure that assessor was not aware of the type of treatment or time of ECG recordings while measuring ECG parameters (PR, RR and QT intervals). The QT intervals were also normalized to the heart rate (QTc) using the Fridericia [$QTc = QT/ (RR)^{1/3}$] formula.[10]

All data are reported as mean ± SD. The comparison of means were done using Student unpaired or paired t-tests as appropriate (Microsoft Excel 2003; Microsoft, Redmond, WA). Linear regression was used to characterize the relationships between various measures, using the correlation coefficient (r^2) as a measure of the strength of relationship. In all figures, absence of regression line denotes non-significant correlation between tested parameters. The level of significance in all statistical testing was set at $\alpha = 0.05$.

3 RESULTS

3.1 The effect of HL on stereoselective tissue distribution of HF and DHF enantiomers

3.1.1 Biodistribution of halofantrine (HF) enantiomers

Consistent with previous studies[8,54,122,124,125], the plasma concentration vs. time profiles of HF enantiomers displayed a multicompartmental nature (Figure 8 and Figure 9). In plasma the concentrations of the (+) enantiomers exceeded those of antipode in both NL and HL rats. In addition, as expected, the plasma concentrations of both enantiomers in HL exceeded those in NL rats by 11-15-fold.[8] In the NL animals concentrations were much higher in tissues than in plasma.

Brain had the lowest uptake of any of the HF enantiomers in all rats. In general, the AUC of HF enantiomers were highest in the lung, liver and spleen, for both of the NL and HL groups (Figure 8 and Figure 9; Table 4). Compared to NL AUC, higher tissue uptakes of (+)-HF were noted in HL liver (118% of NL) spleen (148% of NL) and brain (231% of NL) whereas significantly lower values were present in lung (64%), heart (66%) and fat (41%). For the (-) enantiomer, AUC was significantly lower in HL kidney (84% of NL), lung (53%), brain (31%) and fat (39%) but higher in spleen (147%) than in NL rats. Unlike (+)-HF, no differences were seen for heart and liver. No significant changes were seen in the

AUC of kidney for the (+)-enantiomer. When compared to NL, only HL heart tissues were found to have a numerically lower (+):(-)-HF ratio.

Using the AUC data in Table 4, the tissue to plasma concentration ratios (Kp) in HL (range 0.03 to 1.78) were consistently lower in all tissues compared to NL (range 0.21 to 32). Using known mean values of unbound fraction for the (+) enantiomer of 0.064 and 0.0070%, and 0.062 and 0.0077% for the (-) enantiomer, in NL and HL plasma, respectively[8], the tissue to plasma unbound concentrations (Kpu) were estimated in the post distributive phases from 12 to 48 h postdose (Figure 10). Compared to NL, the Kpu values for (+)-HF in HL rats was significantly higher in the lung, liver, kidney and spleen. In contrast, the heart showed a significantly lower value in HL. In contrast, except for brain and fat, significant differences in Kpu were seen for (-)-HF in all tissues where Kpu in HL was greater than NL (Figure 10).

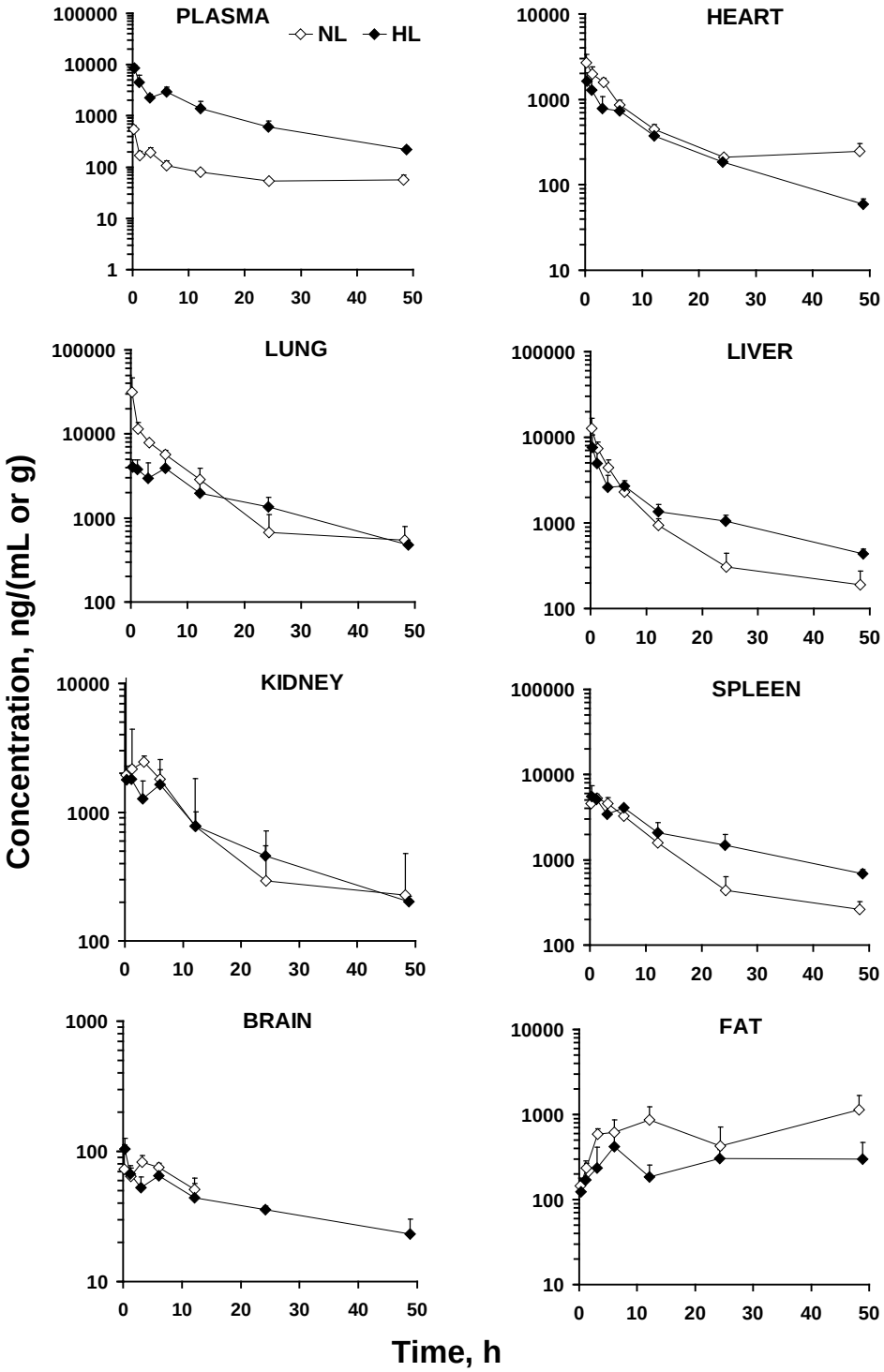

Figure 8: Mean concentration (±SD) versus time curves of (+)-HF from time 0 to 48 hours in NL (open diamond) and HL (closed diamond) rat plasma and different tissues after single iv dose of 2mg/kg (±)-HF HCl.

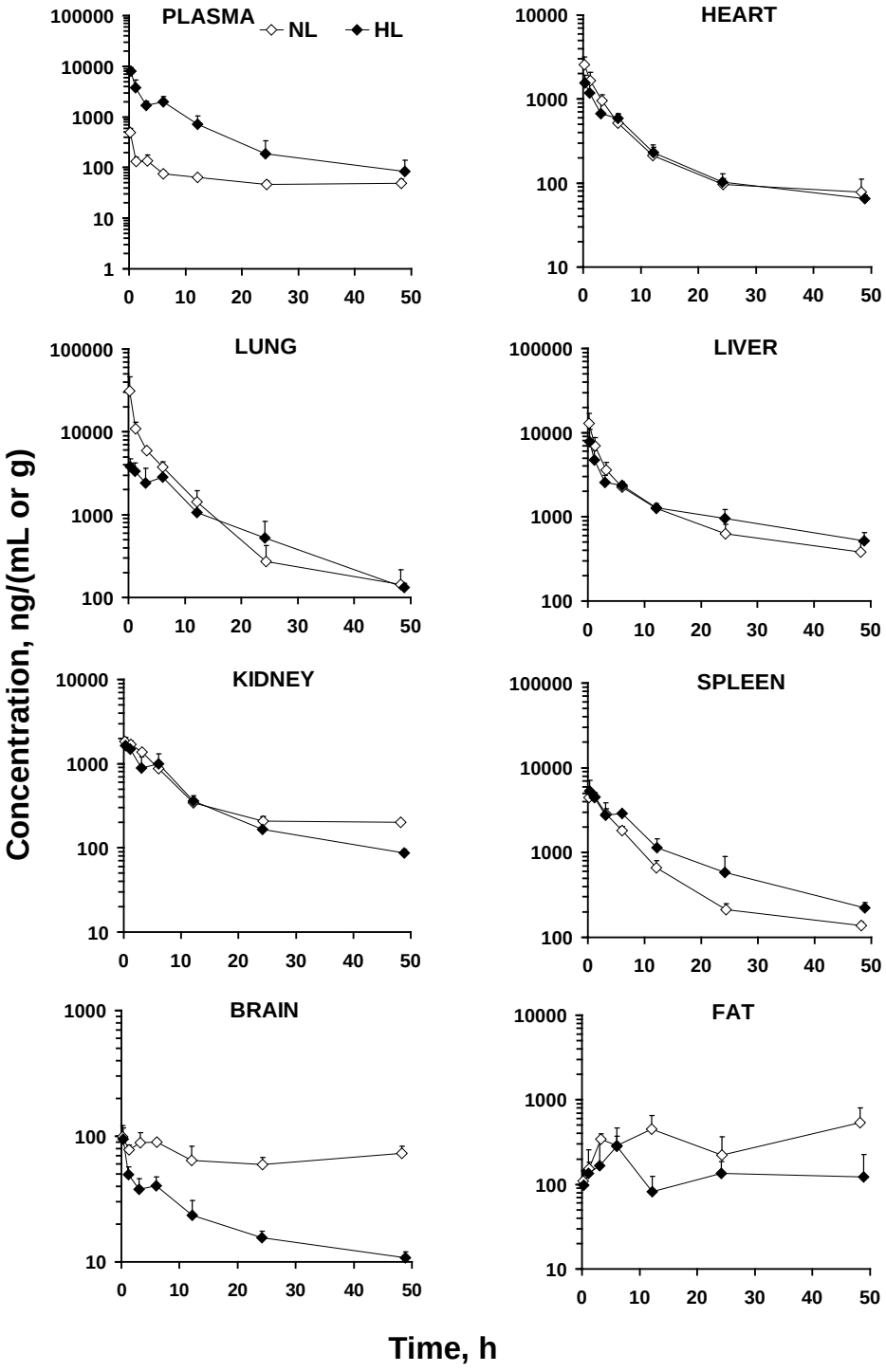

Figure 9: Mean concentration (±SD) versus time curves of (-)-HF from time 0 to 48 hours in NL (open diamond) and HL (closed diamond) rat plasma and different tissues after single iv dose of 2mg/kg (±)-HF HCl.

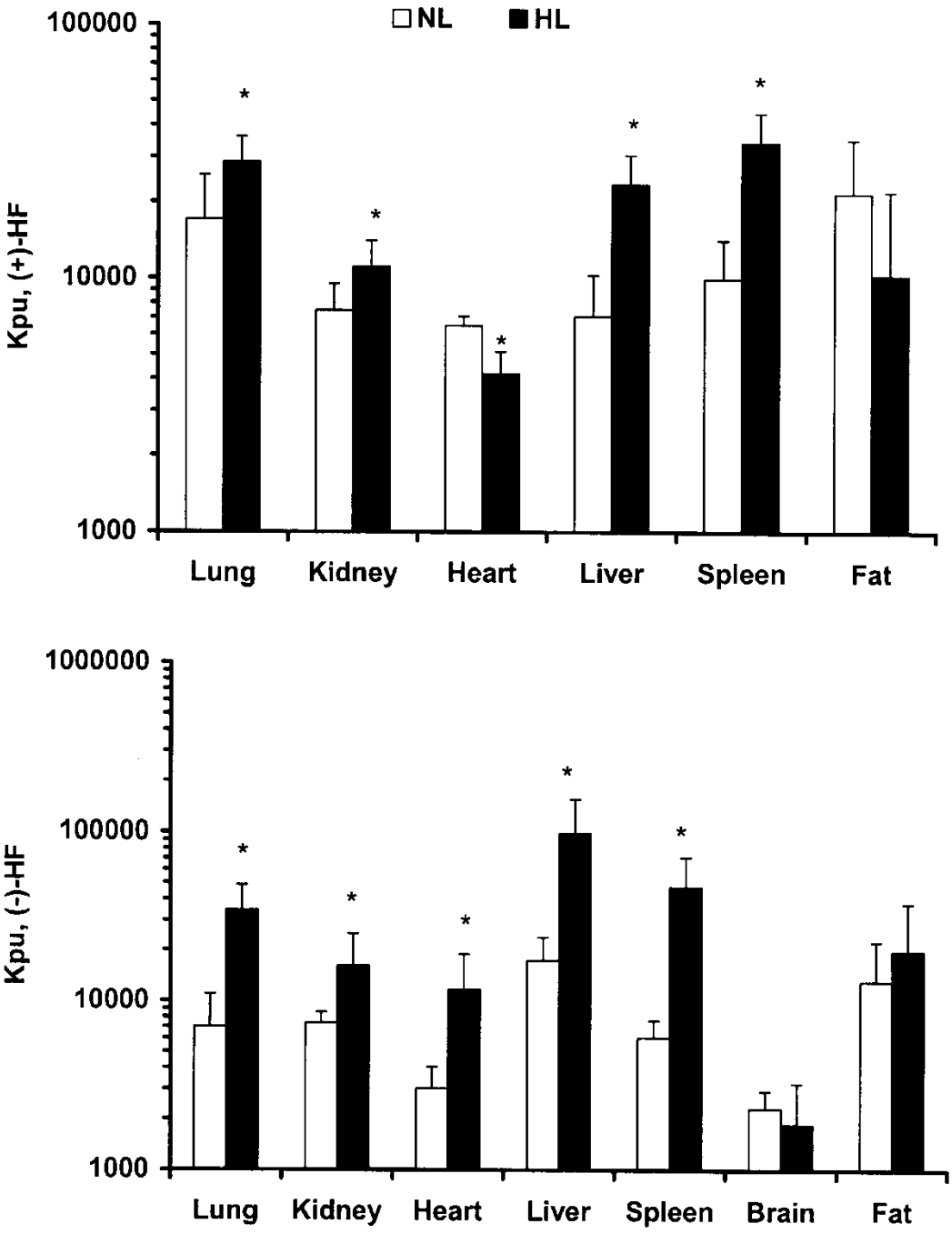

Figure 10: Mean±SD of tissue to unbound plasma concentration ratios (Kpu) of (+)- and (-)-HF in NL and HL tissues during the post-distributive phase (12 hour to 48 h post dose) after single iv dose of 2mg/kg (±)-HF HCl. * represents significant ($p < 0.05$) difference from NL groups.

Table 4: Tissue uptake of HF enantiomers in NL and HL rats given 2 mg/kg iv bolus doses of HF HCl. The mean±SD of the AUC0-48 (µg·h/[mL or g]) of (+)-HF and (-)-HF are shown. Asterisks represent significant differences in AUC from NL (Bailer's method, $p<0.05$).

Tissues	AUC_{0-48}					
	(+)-HF			(-)-HF		
	NL	HL	HL:NL ratio	NL	HL	HL:NL ratio
Plasma	3.94±0.14	58.1±3.58*	14.8	3.22±0.12	35.7±2.73*	11.1
Heart	23.2±0.73	15.4±0.78*	0.67	13.4±0.78	11.7±0.60	0.87
Lung	127±8.25	81.5±6.28*	0.64	87.1±5.92	46.5±4.41*	0.53
Liver	56.5±2.77	66.7±3.00*	1.18	64.8±3.03	63.7±3.03	0.98
Kidney	33.6±1.46	32.1±1.94	0.96	20.3±0.45	17.1±1.17*	0.84
Spleen	62.0±3.20	91.6±5.81*	1.48	36.2±1.21	53.1±3.62*	1.47
Brain	0.83±0.058[a]	1.92±0.067	2.31	3.32±0.13	1.03±0.044*	0.31
Fat	33.7±4.50	13.7±1.14*	0.41	17.0±2.25	6.66±0.97*	0.39

[a]AUC was measured till 12h after which no drug was detected; statistics were not performed on these data.

3.1.2 Biodistribution of desbutylhalofantrine (DHF) enantiomers

Although DHF enantiomers were below the lower limit of quantitation in many of the plasma samples, DHF enantiomers were measured in each of the studied highly perfused tissues (Figure 11 and Figure 12; Table 5). Similar to HF, tissue levels were lowest in brain but highest in lung, spleen and liver. The rank order of AUC was the same for both enantiomers, with highest to lowest being lung>spleen>liver>kidney>heart>brain (Table 5). The AUC ratio of (+):(-)-DHF was consistently low in all HL tissues. In HL, (+)-DHF concentrations were significantly lower than NL only in lung (85%) and heart (61%) tissues (Table 5). For (-)-DHF in HL, concentrations were significantly higher than in NL liver (153%) and spleen (160%), but lower than lung (92%) (Table 5).

The calculated DHF:HF AUC ratios for both enantiomers were higher in some of the HL tissues compared to NL (Table 5). For HL, the mean (+)-DHF:HF ratios were numerically higher in lung and liver but lower in kidney, heart and spleen tissues compared to NL. For (-)-DHF:HF ratios HL was associated with higher values in lung, kidney, liver and spleen but lower values in heart tissues compared to the NL group.

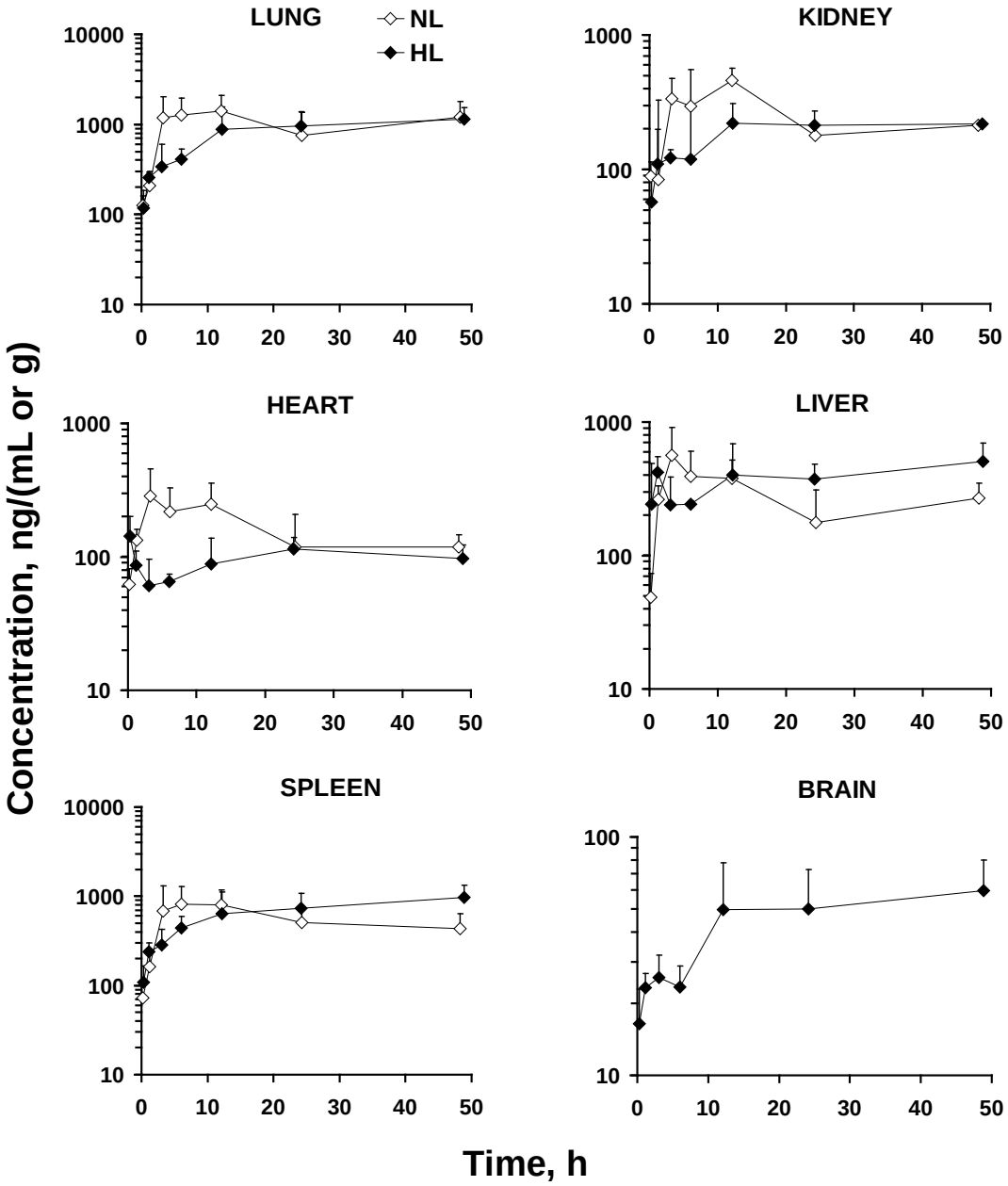

Figure 11: Mean concentration (±SD) versus time curves of (+)-DHF from time 0 to 48 hours in NL (open diamond) and HL (closed diamond) rat plasma and different tissues after single iv dose of 2mg/kg (±)-HF HCl.

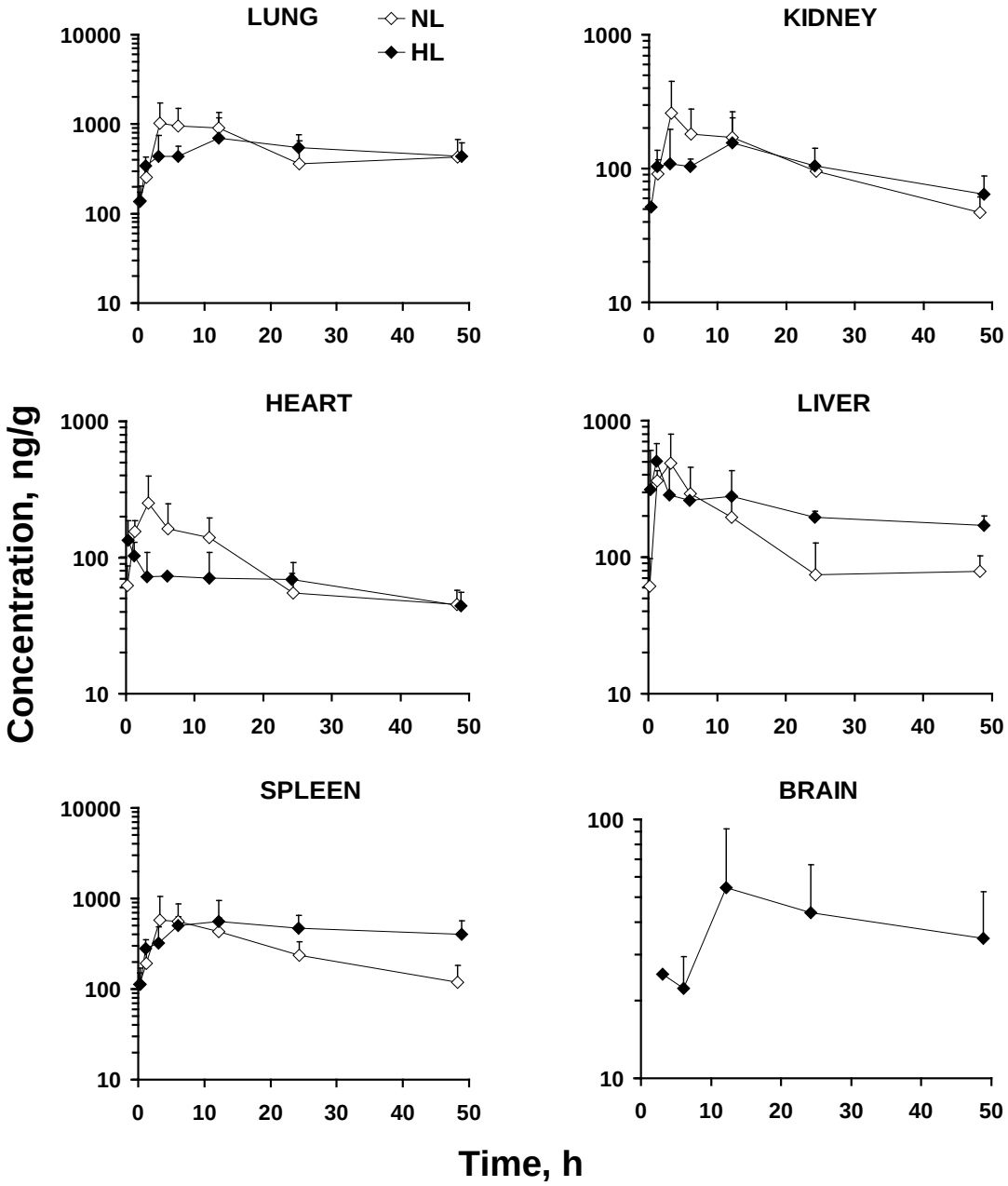

Figure 12: Mean concentration (±SD) versus time curves of (-)-DHF from time 0 to 48 hours in NL (open diamond) and HL (closed diamond) rat plasma and different tissues after single iv dose of 2mg/kg (±)-HF HCl.

Table 5: Tissue uptake of DHF enantiomers in NL and HL rats given 2 mg/kg iv bolus doses of HF HCl. The mean±SD of the AUC0-48 (µg·h/[mL or g]) of DHF enantiomers are shown. DHF to HF ratios for both enantiomers based on AUC values are shown. Asterisks represent significant differences in AUC from NL (Bailer's method, $p<0.05$). Blank cells represent values either could not be measured or calculated.

Tissues	AUC_{0-48}						DHF:HF ratio			
	(+)-DHF			(-)-DHF			(+)-DHF:HF		(-)-DHF:HF	
	NL	HL	HL:NL ratio	NL	HL	HL:NL ratio	NL	HL	NL	HL
Heart	7.72±1.19	4.74±0.39*	0.61	4.43±0.58	3.18±0.21	0.72	0.33	0.31	0.33	0.27
Lung	49.9±7.38	42.4±5.59*	0.85	27.0±3.98	24.8±3.22*	0.92	0.39	0.52	0.31	0.53
Liver	13.4±1.56	19.1±2.03	1.4	7.16±0.79	11.0±0.78*	1.5	0.24	0.29	0.11	0.17
Kidney	12.2±1.60	9.60±1.23	0.79	5.40±0.52	5.03±0.66	0.93	0.36	0.30	0.27	0.29
Spleen	27.4±3.43	34.3±4.56	1.3	13.9±1.63	22.2±2.67*	1.6	0.44	0.37	0.38	0.42
Brain	-	2.3 ± 0.25	-	-	1.9 ± 0.31	-	-	1.20	-	1.8

3.1.3 Distribution within plasma separated by lipoprotein class

The total CHOL and TG values in the NL plasma prior to fractionation for lipoprotein association were 62.1±0.26 and 179±4.86 mg/dL, respectively. In contrast the HL plasma had CHOL and TG levels of 1562±21.3 and 5477±182 mg/dL, respectively.

Of the total incubated HF (1000 ng/mL), more than 90% and 75% of the HF was recovered from the NL and HL plasma lipoprotein fractions respectively. After incubation, both HF and DHF enantiomers were generally measurable within lipoprotein-containing fractions (Table 6). Significant differences were noted in association of HF and DHF within lipoprotein fractions in NL and HL treatments (Table 6). In NL, most of the drug was found associated with the LPDP fraction whereas in HL plasma a significant amount of HF was found in TRL fractions. In NL, association of (+)-HF was more uniformly distributed between the fractions whereas (-)-HF displayed preferential binding with the lipoprotein-deficient fraction of the plasma. In the presence of HL, both enantiomers exhibited a marked shift to the TRL fraction.

A similar trend to HF was seen for DHF enantiomers in association with lipoprotein fractions of NL plasma except that there was less difference noted between enantiomers (Table 6). In the NL plasma both DHF enantiomers were mostly found in the LPDP fractions of NL, whereas in HL most was found in the TRL fractions. The level of DHF in the HL HDL fraction was too low to measure.

The rank order for the ratio of (+) to (-) enantiomer of HF in NL and HL lipoprotein fractions was LPDP < [LDL=TRL] < HDL and HDL < LPDP < TRL < LDL, respectively. For DHF in NL lipoprotein fractions TRL had the highest (+) to (-) ratio whereas in HL plasma it was HDL < TRL < [LPDP=LDL] respectively (Table 6).

Table 6: Association of HF and DHF enantiomers within plasma fractionated according to lipoprotein content. Plasma was incubated with (±)-HF (1000 ng/mL) and (±)-DHF (250 ng/mL) for 1 h at 37°C in plasma from fed normolipidemic and P-407-treated hyperlipidemic rats. Data are expressed as percent of total recovery of HF and DHF. Between fraction ranking and significance ($p<0.05$) is depicted in ascending order under the respective values.

	Normolipidemia				**Hyperlipidemia**			
	HDL	**TRL**	**LDL**	**LPDP**	**HDL**	**TRL**	**LDL**	**LPDP**
				Halofantrine				
(+)	29.1±3.8†	24.1±2.3†	14.7±0.95†	32.1±0.92†	ND	73.2±2.6*	14.2±1.6	12.6±1.6*†
	LDL < TRL < HDL = LPDP				HDL < LPDP = LDL < TRL			
(-)	16.4±1.7	17.6±0.76	10.9±0.75	55.1±2.6	ND	72.0±1.3*	11.7±1.1	16.4±1.1*
	LDL < HDL = TRL < LPDP				HDL < LDL = LPDP < TRL			
				Desbutylhalofantrine				
(+)	27.8±4.7	7.29±0.46	12.3±1.1	52.6±4.7	ND	68.8±1.8*	16.9±2.0†	14.3±0.45*
	TRL = LDL < HDL < LPDP				HDL < LPDP = LDL < TRL			
(-)	27.4±4.1	6.40±0.69	12.3±0.40	53.9±3.5	ND	73.7±3.9*	13.7±1.3	12.6±3.0*
	TRL < LDL < HDL < LPDP				HDL < LPDP = LDL < TRL			

HDL, High density lipoprotein fraction; TRL: Triglyceride rich fraction; LDL, Low density lipoprotein fraction; LDPD, Lipoprotein deficient fraction; ND, below the lower limit of quantitation
*difference in the specified enantiomer between NL and HL
†difference between the (+) and (-) enantiomer in this lipoprotein fraction.

3.1.4 DHF to HF formation in hepatic microsomes

The two enzyme model fitted best to the DHF formation data in all rats (Figure 13). There were no significant differences noted in any of the kinetic parameters between NL and HL rats (Figure 13; Table 7). There were differences between enantiomers, however, within NL and HL rats. Significantly higher V_{max} and k_m were observed for the (-)-enantiomer compared to its antipode for NL microsomes. Also compared to its antipode, the $CL_{int,2}$ of the (-)-enantiomer was significantly higher in HL microsomes (Table 7).

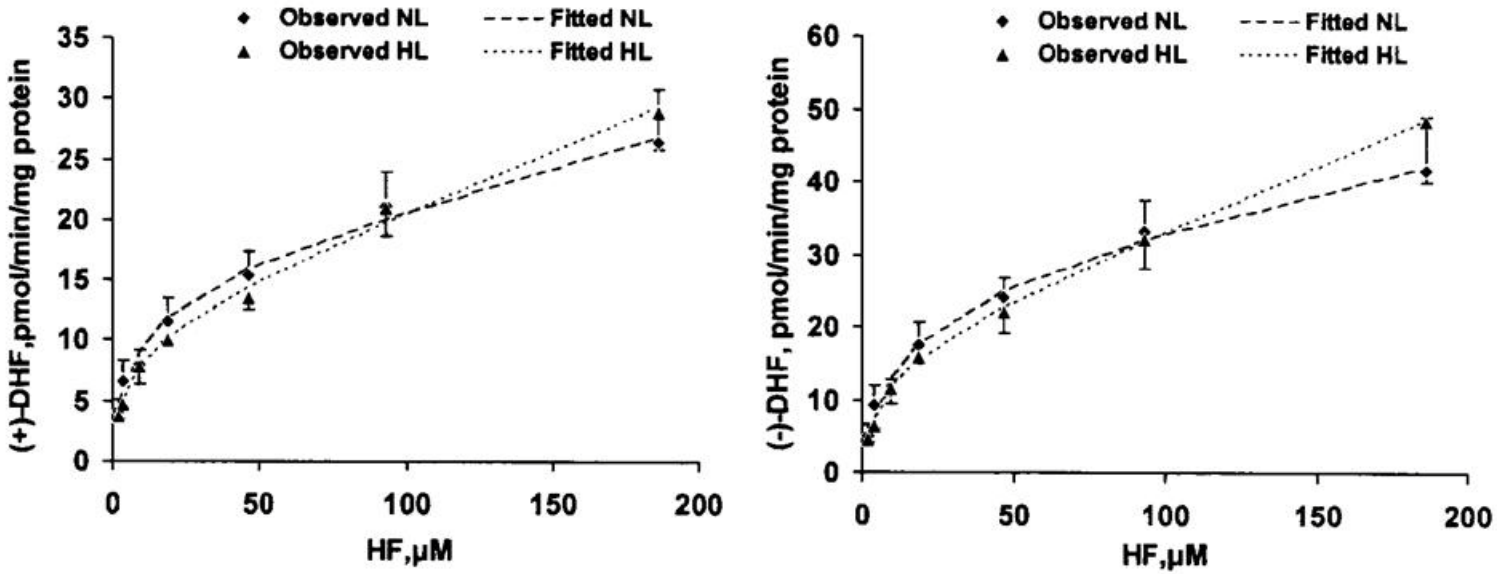

Figure 13: Mean±SD of rate of (+)- and (-)-DHF formation in NL and HL liver microsomes (n= 4 each group) after incubation of (±)-HF HCl (1.9-186 µM) at 37°C in a water bath. Both the observed (symbols) and fitted lines are shown.

Table 7: Enzyme kinetic parameters (Mean±SD; Range in parentheses) for desbutylhalofantrine enantiomer formation from racemate halofantrine by rat NL and HL liver microsomes from four individual rats, based on results of fit of eq. 1.

	NL		HL	
	(+)	**(-)**	**(+)**	**(-)**
V_{max} (pmol/min/mg protein)	15.1±4.13 (9.8–19.4)*	26.5±8.36 (15.0–34.5)	11.4±3.11 (8.93–15.9)	16.4±1.55 (15.2–18.6)
k_m (HF enantiomer, µM)	3.92±2.59 (1.21–7.35)*	6.10±3.82 (1.46–10.6)	3.25±2.65 (1.69–7.20)	3.09±0.61 (2.61–3.88)
$CL_{int,1}$ (µL/min/mg protein)	4.98±2.46 (2.64-8.08)	5.78±3.21 (3.26-10.3)	4.42±1.63 (2.21-6.13)	5.41±0.73 (4.78-6.23)
$CL_{int,2}$ (µL/min/mg protein)	0.13±0.072 (0.043–0.22)	0.19±0.14 (0.030–0.37)	0.20±0.046 (0.16–0.26)*	0.35±0.094 (0.25–0.48)

V_{max}, k_m and $CL_{int}'_1$ are for the high efficiency enzyme. $CL_{int}'_2$ is for the low efficiency enzyme.

*difference in the specified enantiomer between NL and HL ($p<0.05$)

†difference between the (+) and (-) enantiomer in respective lipemic state (NL or HL) ($p<0.05$)

3.2 The effect of bile and lipids on the stereoselective metabolism of HF by rat everted intestinal sacs

After placing the everted intestinal sacs in the chambers, no increases in lactate dehydrogenase (LDH) activity were noted in the serosal and mucosal fluids over the time course of the incubations, suggesting that tissues were viable during 4 h incubation period. We also noted that there was continual production of DHF from HF from 0 to 4 h in preliminary timed formation studies. The HF and DHF enantiomers were not detected in the serosal fluid (inner part of the sacs), but were detected in all everted sacs, thus indicating the ability of the intestine to absorb and metabolize the drug.

3.2.1 Effect of intestinal components on uptake of HF enantiomers

Although some differences were noticed, in general there was little variation between segments, within treatments, in the uptake of HF enantiomers when Krebs-Henseleit (KH) was incubated alone or with simulated rat bile solution (SBS) (Figure 14, upper panels). In the presence of 5% soybean oil emulsion there were no differences noted between segments in the uptake of HF enantiomers (Figure 14, upper panels). Within segments, the differences noted between treatments were greater in number and more pronounced than between segments. In particular, the inclusion of 5% soybean oil emulsion in the incubations was associated with more consistent reductions in the intestinal uptake of both HF enantiomers compared to incubations without the added lipids (Figure 14, lower panels).

Because there was little difference between segments in uptake of HF (Figure 14, upper panels), for each treatment all segments were combined for further analysis (Figure 15A-15C). There was no significant difference in HF concentration in the everted intestinal sacs between KH only and the addition of SBS (Figure 15A). Significant reductions (~50-80%) in HF enantiomer concentrations, however, were seen in the SBS-5% soybean oil emulsion groups compared with groups similarly treated with SBS in the absence of the lipid (Figure 15B and 15C). Pretreatment with peanut oil had a similar effect (~30% decrease) in HF enantiomer concentrations in the everted gut sacs (Figure 15B).

Increasing the cholesterol concentration in the SBS resulted in significantly higher everted gut sac uptake of both HF enantiomers (~46-106%) compared to SBS containing normal cholesterol. When soybean oil emulsion was added to the 5 and 25% SBS with high cholesterol concentration, however, the relative uptake of the HF enantiomers was either significantly decreased (~50-90%) or unchanged (Figure 16A and 16B).

3.2.2 Intestinal metabolism of HF enantiomers

The enantiomers of DHF were detected in all incubations except for 25% SBS plus 5% soybean oil emulsion, where neither enantiomer was measurable, or 10% SBS plus 5% soybean oil emulsion, where only (+)-DHF was detected (Figure 15D-2F). The addition of SBS and lipid in the incubation media resulted in a decreased formation of both DHF enantiomers compared with KH alone. With respect to SBS, the formation of DHF enantiomers seemed to be inversely

proportional to SBS concentrations (upto ~65-95% decrease was noted with increase in bile concentration) (Figure 15D). The DHF:HF ratio in 25% SBS was significantly lower (~90-95% for each enantiomer) than in all other treatments (Figure 15D). The addition of 5% soybean oil emulsion in the 5% SBS containing media caused a significant reduction (~71%) in the formation of (-)-DHF, but not (+)-DHF, in the everted gut sacs (Figure 15E); in 10% SBS there was a complete absence of metabolism to the (-) enantiomer (Figure 15F). Pretreatment with peanut oil caused no apparent change in the formation of DHF enantiomers (Figure 15E).

Similar to HF, DHF concentrations were greater (~115-137% for each enantiomer) in the high cholesterol-containing 5% SBS compared to 5% SBS with lower cholesterol (Figure 16C and 16D). The addition of increased concentrations of SBS tended to lower the mean concentrations of both DHF enantiomers in the presence of both high and low cholesterol concentrations (Figure 16C and 16D). The addition of 5% soybean oil emulsion caused decreases (~70%) in the DHF enantiomers concentrations in both lower and higher cholesterol-containing SBS (Figure 16C and 16D).

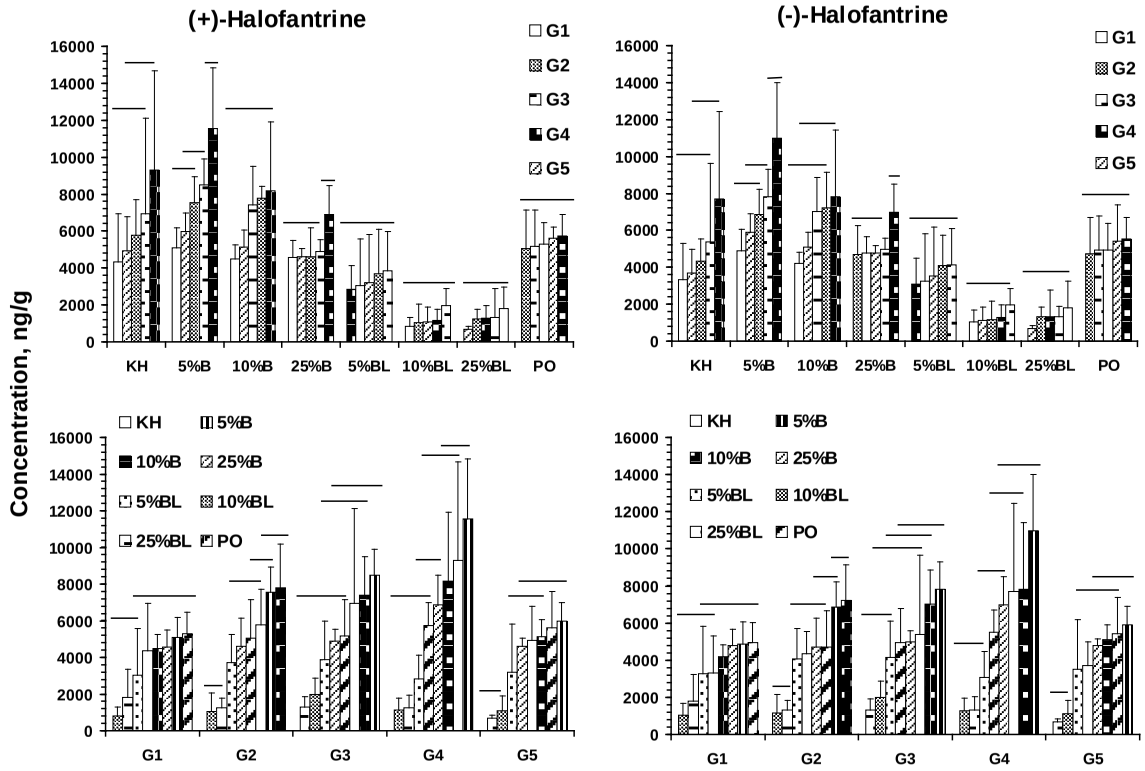

Figure 14: The mean±SD (n=4-6) concentration of HF enantiomers in individual segments incubated with KH buffer alone or in combination of bile or lipids. G1–G5 represent intestinal segments (~10cm each) starting from duodenum to ileum. The abbreviations 5%B, 10%B and 25%B represent Krebs-Henseleit (KH) buffer with added simulated bile solution concentrations of 5, 10 and 25%, respectively. The descriptors 5%BL, 10%BL and 25%BL represent KH + simulated bile solutions of 5, 10 and 25% with added 5% soybean oil emulsion. The abbreviation PO refers to rats pretreated with two doses of oral peanut oil within 6 h of the first dose; in the everted-gut experiment those guts were incubated with 5%B. For those groups where differences were not significant, a continuous horizontal line is drawn over the bars. Bars not encompassed by the same line are significantly different.

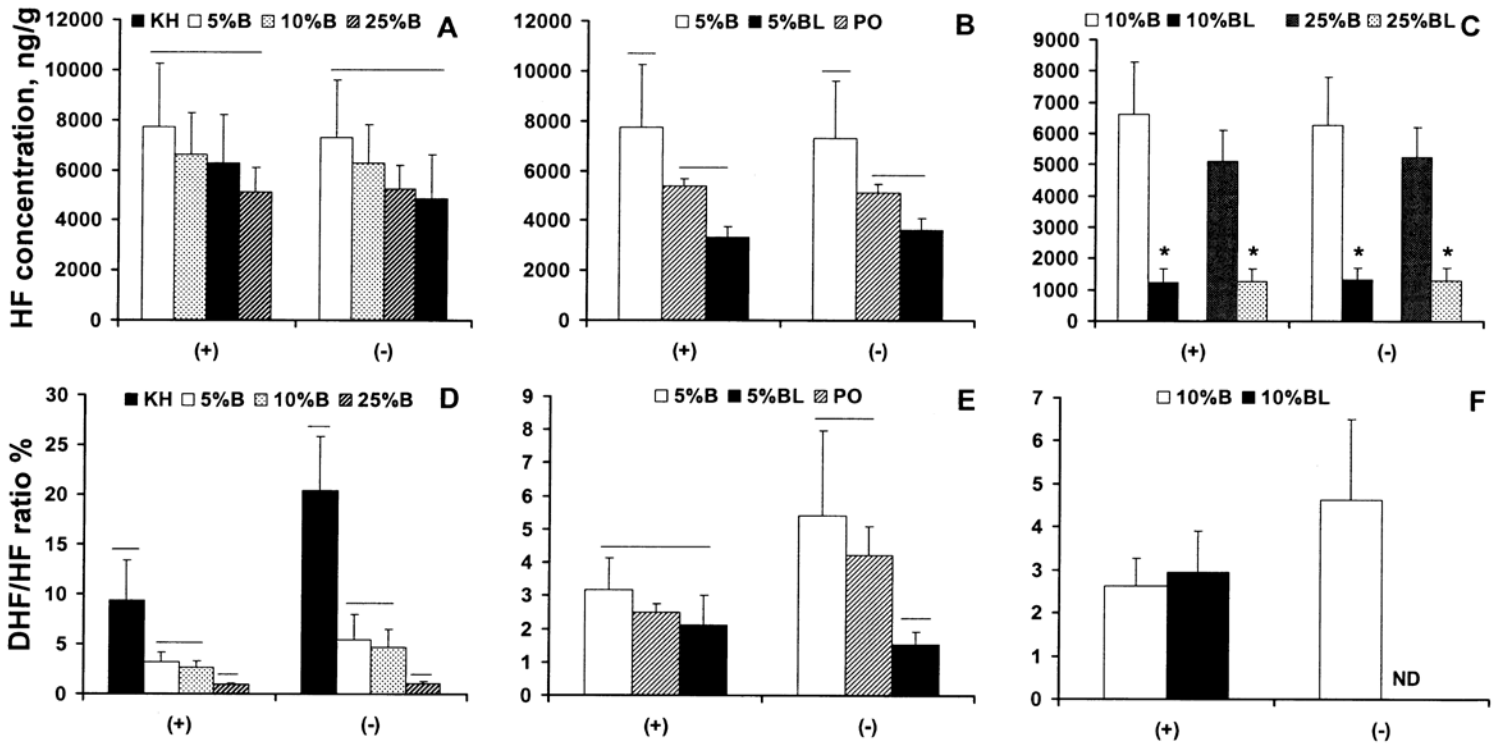

Figure 15: The mean±SD (n=4-6) concentration of HF enantiomers (Panel A, B and C) and DHF/HF ratio % (Panel D, E and F) on incubation of intestinal segments with KH buffer alone or in combination of bile or lipids. ND denoted not detectable, or below lower limit of quantitation. The DHF enantiomers were not detected in 25%BL incubation. See Figure 14 for abbreviations and meaning of horizontal lines. *denotes significant difference between bile incubations with and without lipids.

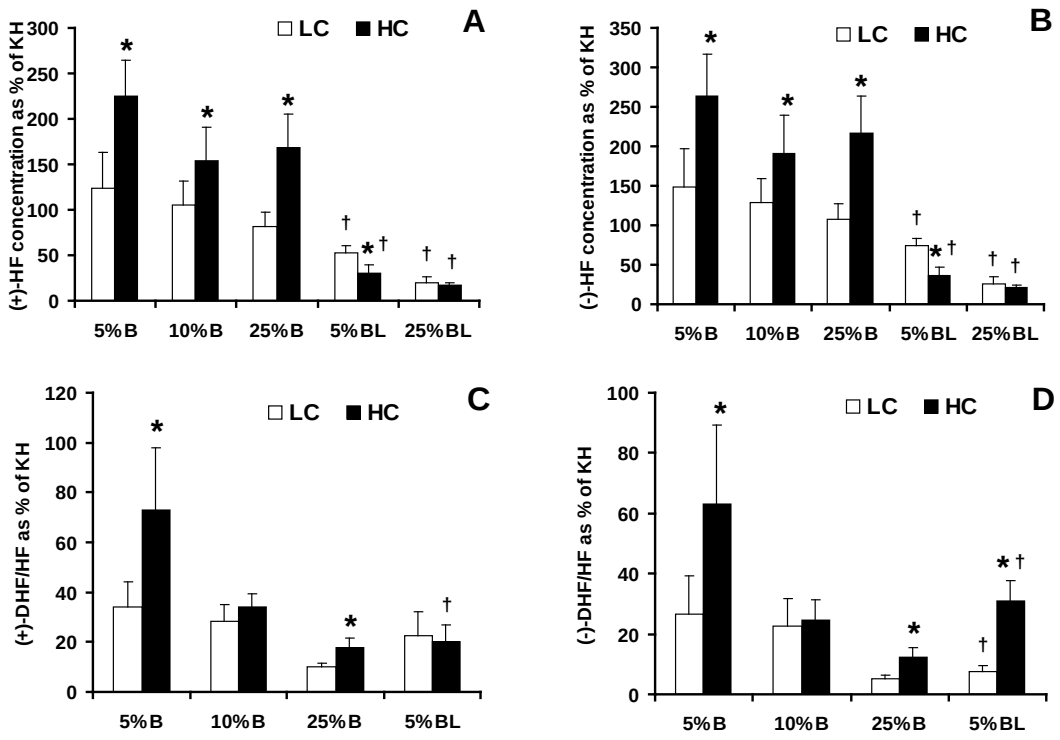

Figure 16: Mean±SD (n=4-6) concentrations of HF enantiomers (Panel A and B) and DHF enantiomers (Panel C and D) in intestinal segments incubated with lower cholesterol (LC) and higher cholesterol (HC) in the presence of simulated bile solutions with or without lipids. See Figure 14 for group abbreviations. The DHF enantiomers were not detected in 25%BL incubation. *Significant difference between LC and HC groups. †Significant difference between with and without lipid groups in the presence of low and high cholesterol in the incubations.

3.2.3 Stereoselectivity

Stereoselectivity was noted in the uptake and formation, respectively, of HF or DHF enantiomers by intestinal segments following the various incubation conditions (Table 8). With respect to HF, the highest (+):(-) ratios (~1.3) were noted in the KH group. All of the other groups had a ratio near 1, but it was of note that addition of soybean oil emulsion resulted in the lowest ratios (as low as 0.9). For DHF in general there was more stereoselectivity noted in the intestinal concentrations than was seen for HF.

Compared to KH, significant decreases in the stereoselectivity of HF uptake were noticed in the SBS-containing incubation media (Table 8). There appeared to be a trend towards decreases (~19-25%) in stereoselectivity of HF with increasing bile concentrations (Table 8). In contrast, for DHF as the SBS concentrations increased the level of stereoselectivity appeared to increase (~35-103%) (Table 8). In general the (+):(-) ratios of HF were opposite those of DHF. For example, KH had the highest (+):(-) ratio of HF, whereas it had the lowest ratio for DHF. The (-) enantiomer of DHF was not measurable in the 10% and 25% SBS groups with added soybean emulsion, so a stereoselectivity index could not be assessed for those groups.

Table 8: Stereoselectivity in mean±SD (+):(-) ratios of HF and DHF enantiomers in the intestinal segments incubated with KH alone or in combination with bile or lipids. Statistical results of post hoc ranking of the ratios are provided below the categories of HF and DHF, respectively. See Figure 14 for definition of abbreviations.

Mean	KH	5%B	10%B	25%B	5%BL	10%BL	25%BL	PO
HF								
(+):(-)	1.30±0.046	1.06±0.036	1.05±0.027	0.97±0.015	0.89±0.032	0.89±0.072	0.98±0.022	1.05±0.017
KH > [5%B = PO = 10%B] > [25%BL = 25%B] > [10%BL = 5%BL]								
DHF								
(+):(-)	0.45±0.124	0.65±0.190	0.60±0.119	0.91±0.201	1.34±0.249	ND	ND	0.60±0.072
5%BL > 25%B > [5%B =10%B = PO = KH]								

3.2.4 Intestinal Microsomal Incubation and Western Blot Analysis

There were no significant differences discernable in the DHF formation in microsomes between fasted rats and those given two doses of peanut oil, with the peanut oil fed:fasted ratio of formation of each enantiomer being 1.1. CYP3A1, CYP2B1 and CYP1A1 were all detected in control and peanut oil treated gut segments (Figure 17). Pre-treatment of small intestine with peanut oil did not significantly affect the expression of CYP3A1 (88.2% of control), CYP2B1 (99.3% of control) or CYP1A1 (97.9% of control) compared with control fasted rats (Figure 17).

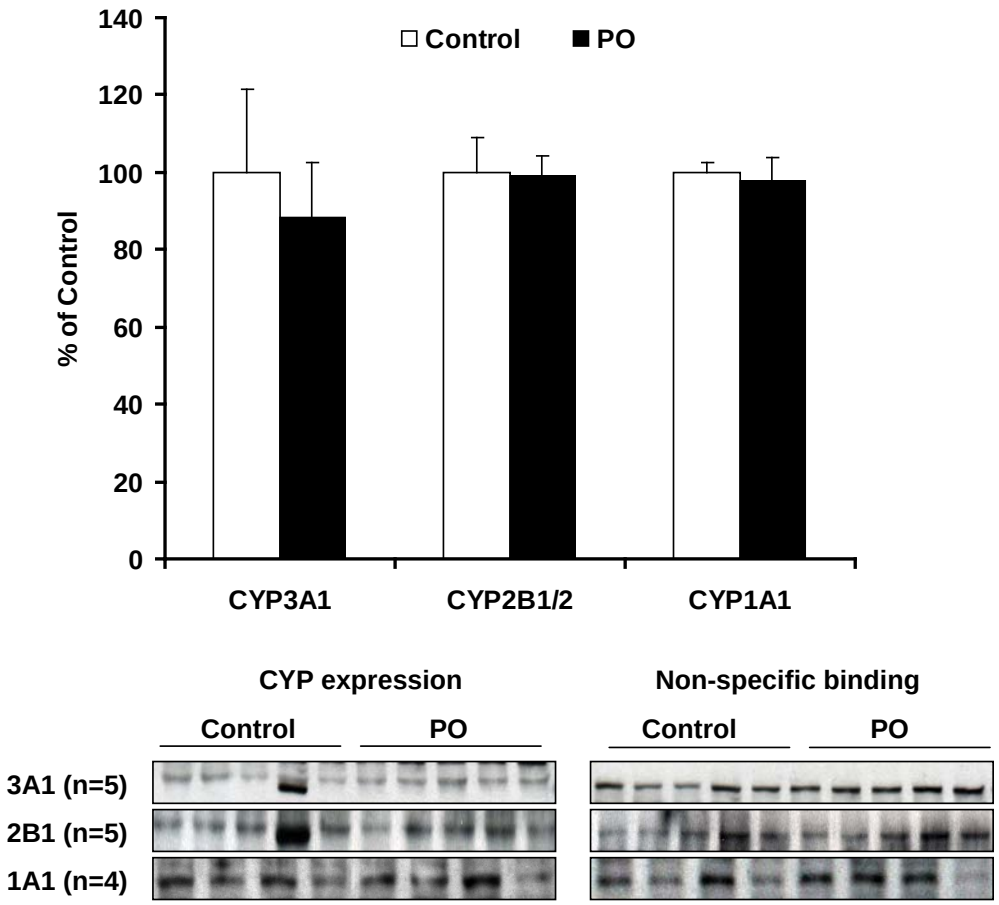

Figure 17: CYP450 protein expression (mean ± SD; n=4 or 5) in the gut microsomes prepared from control or no treatments (white bars) and oral peanut oil pretreatment (PO; black bars). Non-specific binding was used to normalize bands for relative quantitation.

3.3 Effect of hyperlipidemia on the metabolism of HF enantiomers by primary rat hepatocytes *in vitro*

A time dependent increase in the stereoselective metabolism of HF enantiomers was noted upon HF incubation with primary rat hepatocytes. The decrease in the concentration of (-)-HF was higher than (+)-HF in all groups, and was consistent with previous reports involving microsomes and pharmacokinetic evaluations.[8,54,122]

3.3.1 Metabolism of HF after coincubation with media, NL or HL serum

The coincubation of (±)-HF in media with NL and HL rat hepatocytes resulted in stereoselectivity in the apparent metabolism of (±)-HF. The (-)-HF (~80%) was apparently cleared more rapidly than (+)-HF (~30%) by the end of the study (Figure 18). There was no significant difference noted in the metabolism of HF enantiomers between hepatocytes isolated from NL and HL rats (Figure 18).

Coincubation with NL hepatocytes

Compared to media alone, metabolism of HF enantiomers was significantly decreased by the coincubation of drug with HL but not NL serum (Figure 19; Table 9). Compared to NL serum, HL serum coincubations resulted in significant decrease in the metabolism of (-)-HF but not the (+)-HF (Figure 19; Table 9).

Coincubation with HL hepatocytes

Compared to media alone, only HL serum coincubation resulted in a significant decrease in the metabolism of (+)-HF whereas decrease in the metabolism of (-)-HF resulted from both NL and HL serum coincubations (Figure 20; Table 9). Compared to NL serum, metabolism of both HF enantiomers was significantly decreased after coincubation with HL serum (Figure 20; Table 9).

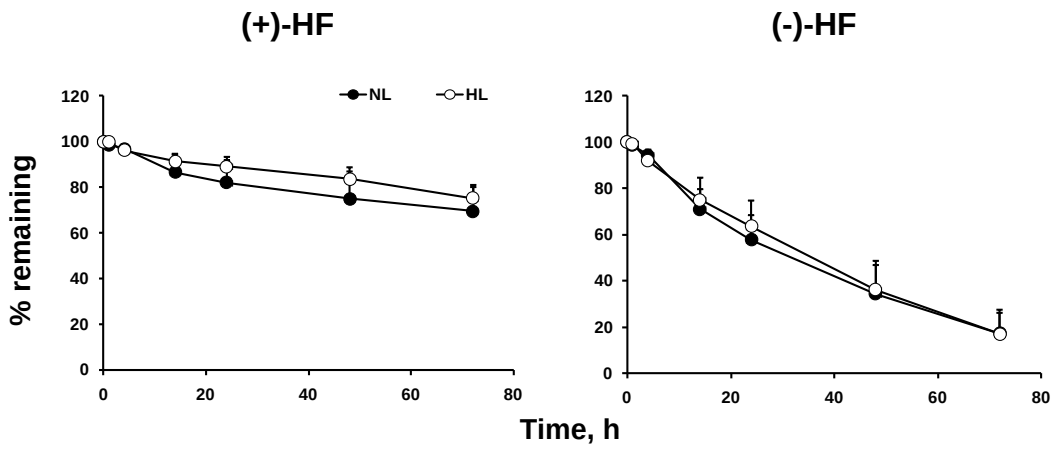

Figure 18: The mean ± SD of % (+)- and (-)-HF remaining to be metabolized by NL and HL hepatocytes (n=6 rats) after coincubation of 500 ng/mL of (±)-HF with media from 0 to 72 hours.

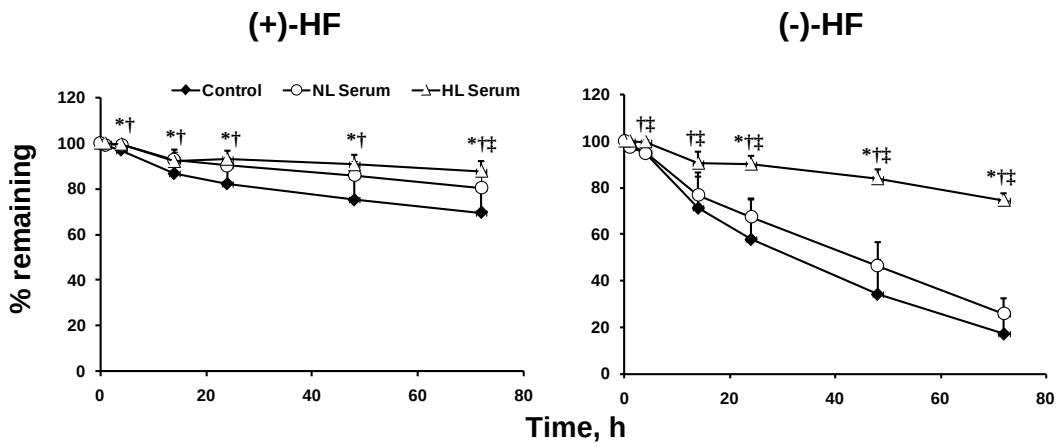

Figure 19: The mean ± SD of % (+)- and (-)-HF remaining to be metabolized by NL hepatocytes after coincubation of (±)-HF (500 ng/mL) with either media alone (control; n=6 rats) or with 5% NL or HL serum in media (n=3 rats) from 0 to 72 hours. *represent significant difference between media and NL serum incubations. †represent significant difference between media and HL serum incubations. ‡represent significant difference between NL and HL serum incubations.

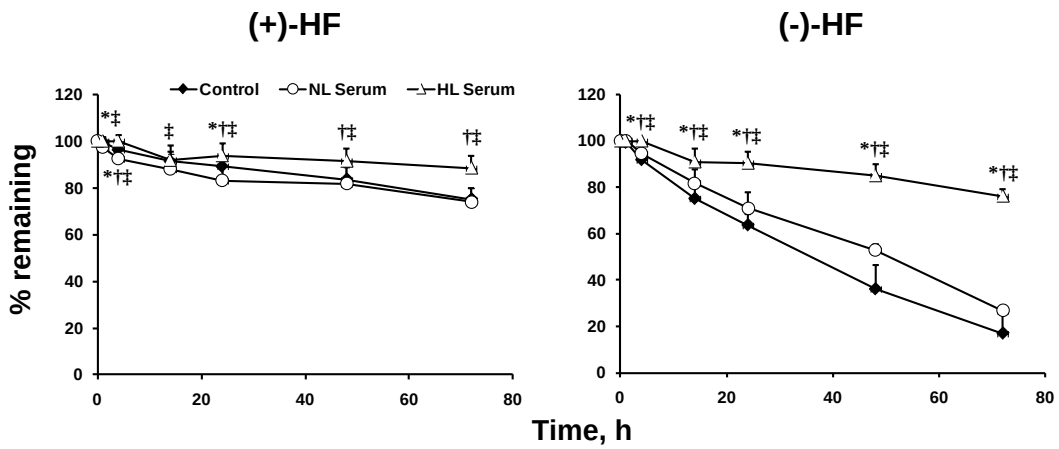

Figure 20: The mean ± SD of % (+)- and (-)-HF remaining to be metabolized by HL hepatocytes after coincubation of (±)-HF (500 ng/mL) with either media alone (control; n=6 rats) or with 5% NL or HL serum in media (n=3 rats) from 0 to 72 hours. *represent significant difference between media and NL serum incubations. †represent significant difference between media and HL serum incubations. ‡represent significant difference between NL and HL serum incubations.

3.3.2 Metabolism of HF after preincubation with media, NL or HL serum

In line with the coincubation group, an increase in stereoselective metabolism of HF enantiomers was noted with time after preincubation of NL hepatocytes with media alone and 5% NL or HL serum in media (Figure 21). It was of note that compared to coincubation, the metabolism of HF enantiomers was significantly higher after preincubation of NL hepatocytes with NL or HL serum (Table 9).

There was no significant difference noted in the metabolism of HF enantiomers between NL hepatocytes preincubated with media and NL serum (Figure 21; Table 9).The preincubation of NL hepatocytes with HL serum resulted in a significant decrease in the metabolism of (-)-HF but not (+)-HF compared to media alone and NL serum (Figure 21; Table 9).

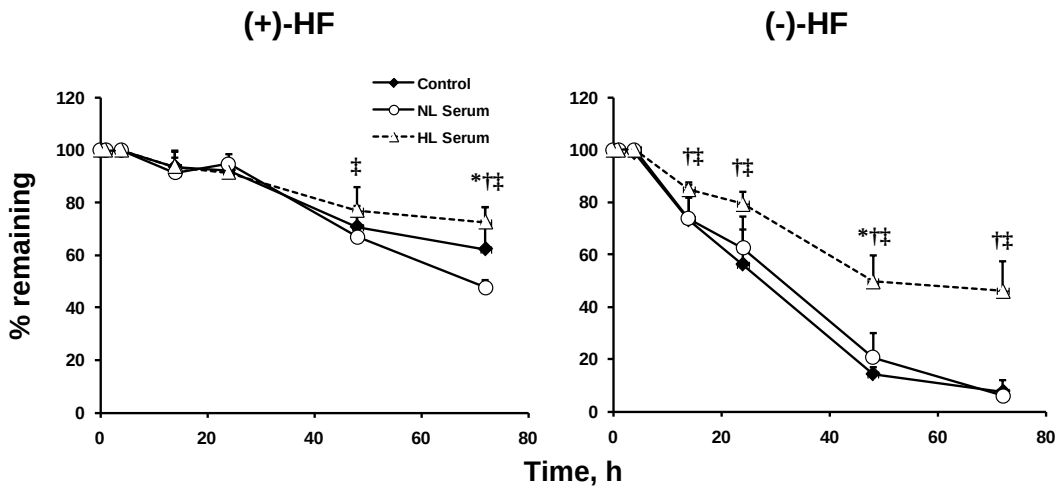

Figure 21: The mean ± SD of % (+)- and (-)-HF remaining to be metabolized by NL hepatocytes after incubation of (±)-HF (500 ng/mL) from 0 to 72 hours. The hepatocytes were preincubated with media alone (control; n=6 rats) or with 5% NL or HL serum in media (n=3 rats) for 24 hours prior to HF incubations. *represent significant difference between media and NL serum incubations. †represent significant difference between media and HL serum incubations. ‡represent significant difference between NL and HL serum incubations.

Table 9: The mean ± SD of area under the curve from 0 to 72 h of percentage HF enantiomers remaining to be metabolized by NL and HL hepatocytes. The (±)-HF (500 ng/mL) was incubated with hepatocytes after coincubation or 24 h preincubation with media alone (control; n=6 rats) or with 5% NL or HL serum in media (n=3 rats).

	Coincubation	
(+)-HF	**NL hepatocytes**	**HL hepatocytes**
Media (Control)	5774 ± 185	6211 ± 97.8
NL Serum	6376 ± 137	5993 ± 168
HL Serum	6621 ± 97.3[*]	6671±122[*†]
(-)-HF		
Media (Control)	3583 ± 219	3747 ± 142
NL Serum	4197 ± 195	4478 ± 87.0[*]
HL Serum	6239 ± 81.5[*†]	6296 ± 100[*†]
	Preincubation	
(+)-HF	**NL hepatocytes**	**HL hepatocytes**
Media (Control)	5851 ± 242	-
NL Serum	5605 ± 176[‡]	-
HL Serum	6108 ± 150[‡]	-
(-)-HF		
Media (Control)	3016 ± 154[§]	-
NL Serum	3280 ± 195[‡]	-
HL Serum	4843 ± 215[*†‡]	-

[*]Significant difference from media (control). [†]Different from NL serum.[§] Different from HL hepatocytes coincubated with media. [‡]Different from NL hepatocytes coincubated with the same type of serum. The Bailer's method of assessing comparisons between AUC was used, with Bonferroni correction being applied to determine $Z_{critical}$ values (p=0.005 and $Z_{critical}$ of 2.81 for effect of lipid on AUC, and p=0.05 and $Z_{critical}$ =2.24 for comparison of enantiomer AUC in the same treatment group).

3.3.3 Effect of NL and HL on stereoselectivity

An increase in the stereoselectivity of HF enantiomers was noted with time after coincubation and preincubation of media and NL serum with NL and HL hepatocytes (Figure 19; Figure 20; Figure 21). A significant difference between enantiomer ratios was noted between all comparison groups (Figure 22). However, it was of note that addition of HL serum in particular led to an apparent decrease in stereoselectivity, mostly due to its apparent inhibitory effect on the metabolism of (-)-HF (Figure 22).

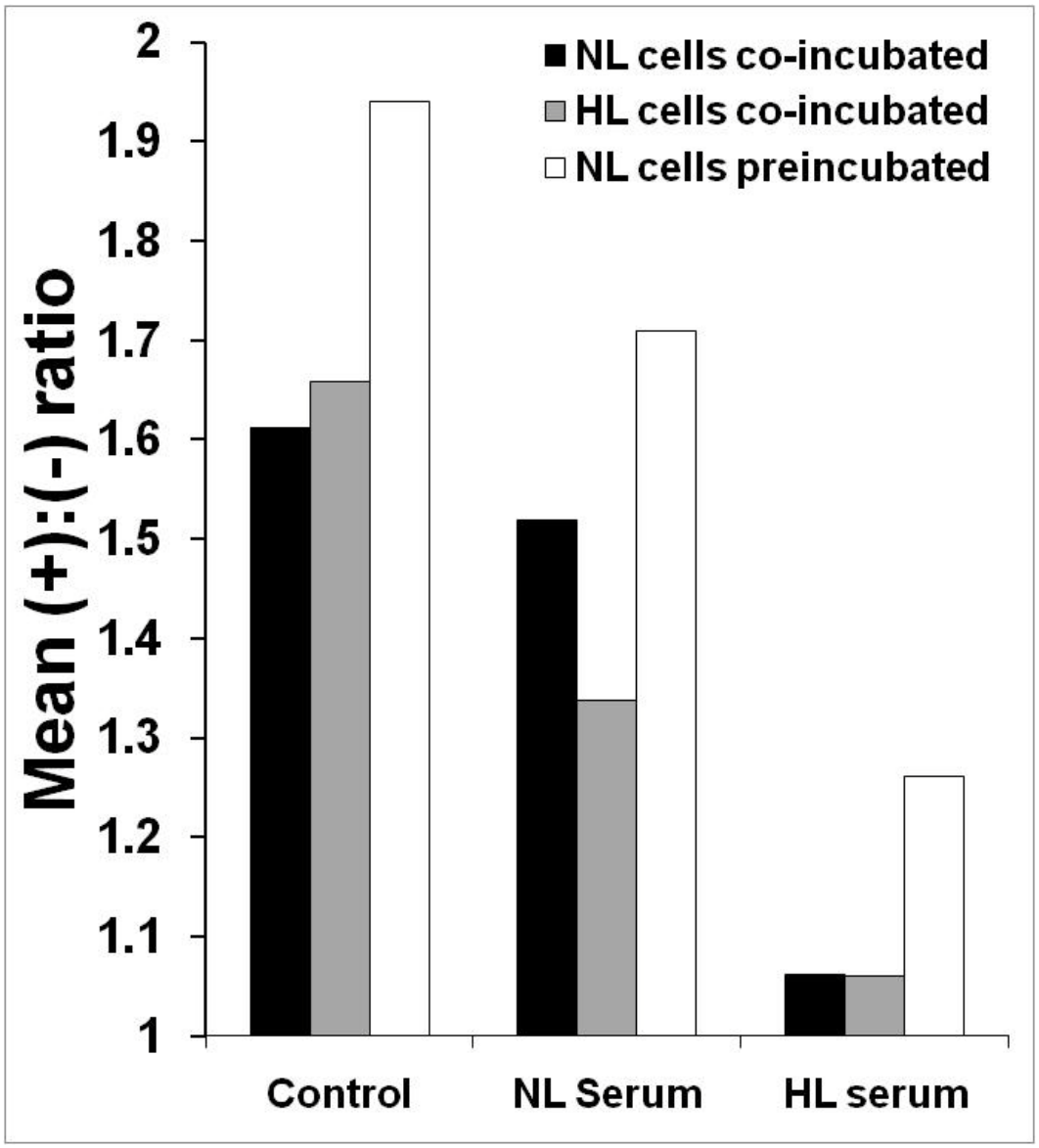

Figure 22: Mean AUC ratios of the (+) to the (-) HF enantiomer in hepatocyte incubations from NL or HL rats, when incubated with media only, or preincubated or coincubated with 5% NL or HL serum.

3.4 Effect of P-glycoprotein on the intracellular accumulation of HF enantiomers and Rh-123 in LLC PK1 and NRK 52E cell lines

3.4.1 Accumulation of HF enantiomers in LLC PK1 cell lines

The uptake of HF enantiomers was linear for 8 h in both uninduced and RF induced LLC PK1 cells (Figure 23). The formation of DHF was not mediated by either uninduced and induced LLC PK1 cells. There was no significant decrease noted in the intracellular accumulation of HF enantiomers in RF induced LLC PK1 cells compared to uninduced LLC PK1 cells (Figure 23). Instead, a significant increase in the accumulation of HF enantiomers was noted for 4 h in RF induced compared to uninduced cells. In the presence of inhibitor, intracellular accumulation of HF enantiomers was higher in RF induced compared to uninduced cells. In uninduced cells, a significantly decreased accumulation of HF enantiomers was noted in the presence of verapamil compared to when verapamil was not present (Figure 24).

3.4.2 Accumulation of rhodamine-123 in LLC PK1 and NRK 52E cell lines

Rh-123 was used as a positive control for P-glycoprotein substrate. As expected, a significant decrease in the accumulation of Rh-123 was noted in RF-induced cells compared to uninduced cells (Figure 25). However, instead of an expected increase in the intracellular accumulation of Rh-123, a significant decrease in the accumulation of Rh-123 was noted in the presence of the P-gp inhibitors AM,

verapamil and CYA in both uninduced and RF-induced LLC PK1 cells (Figure 25). Similar results were also noted upon incubation of Rh-123 with NRK 52E cells in the presence of AM, verapamil and CYA (Figure 26).

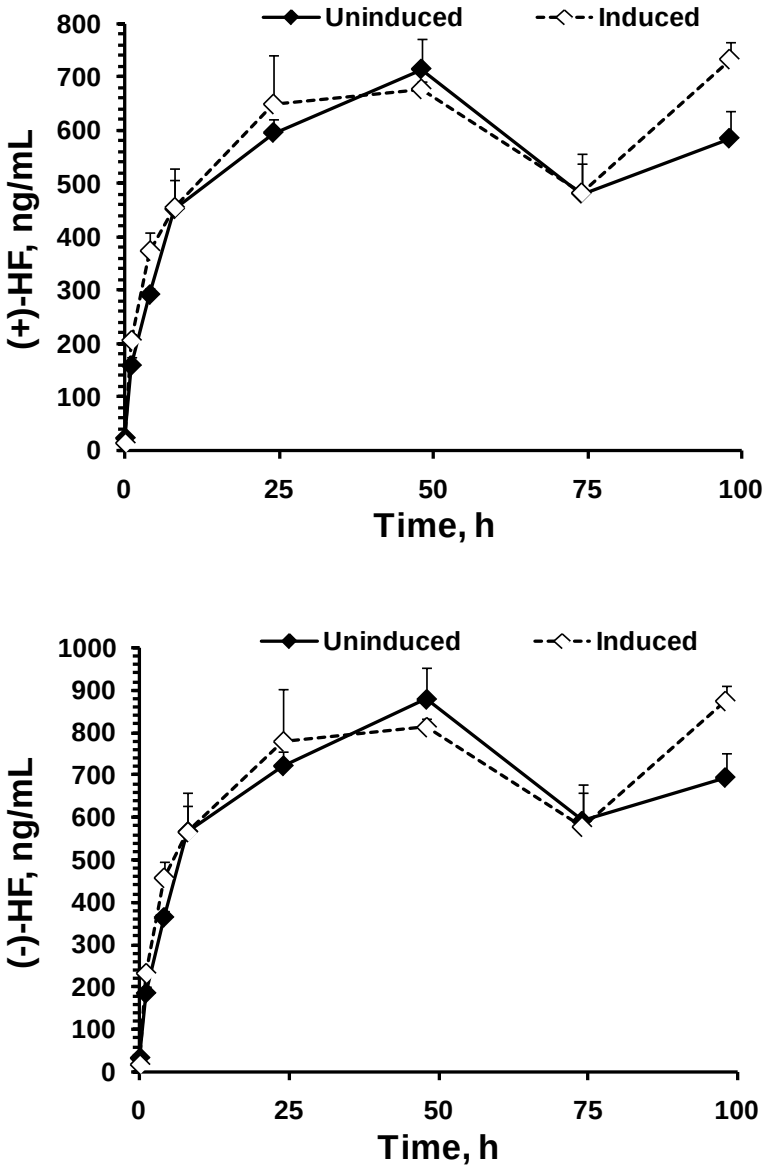

Figure 23: The mean ± SD of intracellular accumulation of (+)- and (-)-HF (ng/mL) upon incubation of (±)-HF (3000 ng/mL) from 0 to 96 h with the uninduced (n=6 wells) and RF induced (n=6 wells) LLC PK1 cell lines.

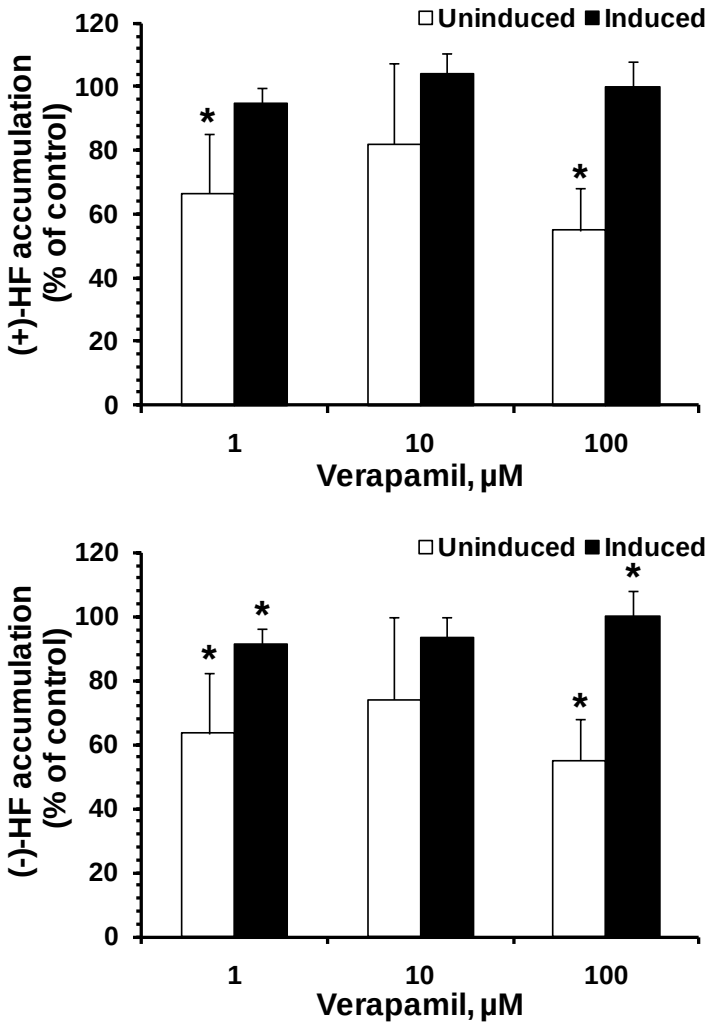

Figure 24: The mean ± SD of intracellular accumulation of (+)- and (-)-HF in the induced (n=6 wells) and uninduced (n=6 wells) LLC PK1 cell lines in the presence of increasing concentrations of verapamil. *represents significant difference from the incubation without inhibitor. Control represents incubation without verapamil incubation.

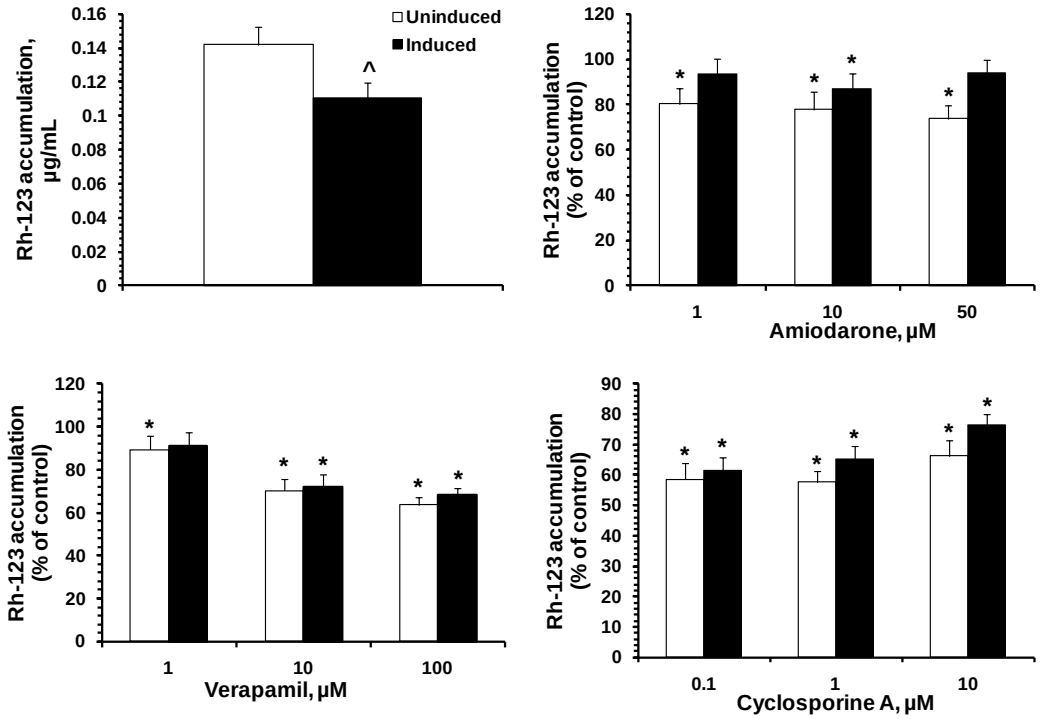

Figure 25: The intracellular accumulation of rhodamine-123 (Rh-123) expressed as mean ± SD in the uninduced (n=6 wells) and RF induced (n=6 wells) LLC PK1 cells in the presence or absence of increasing concentrations of verapamil, AM and CYA. *represents significant difference from the incubation without inhibitor. ^represents significant difference in Rh-123 accumulation between induced and uninduced cells. Control represents incubation without inhibitors.

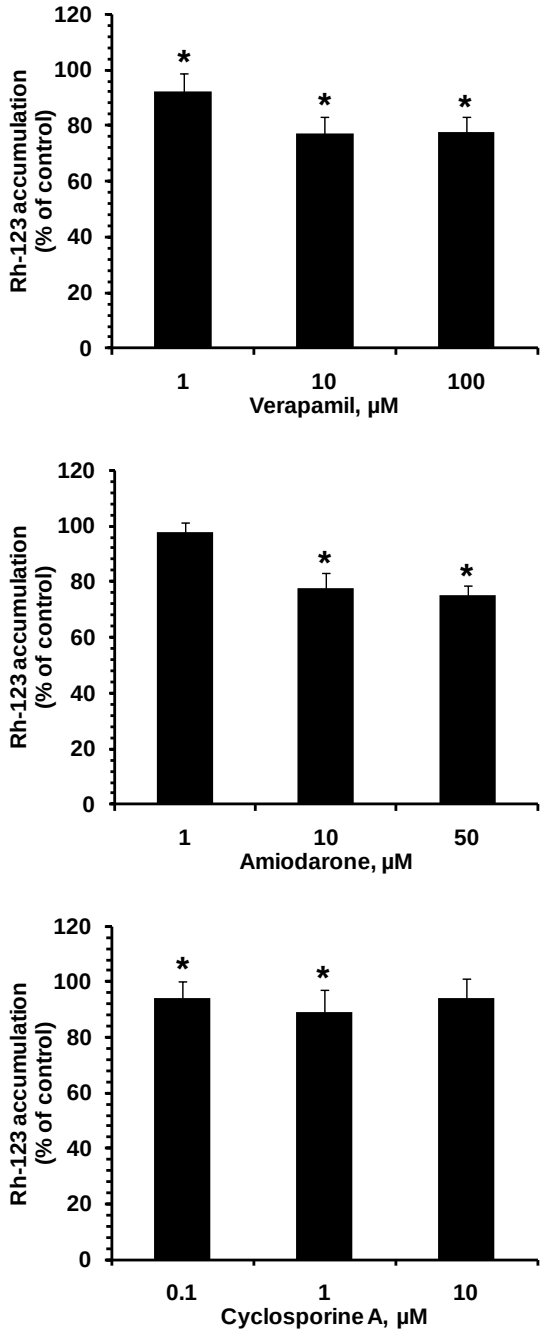

Figure 26: The mean ± SD (n=6 wells) of intracellular accumulation of rhodamine-123 (Rh-123) expressed as a percentage of control in the NRK 52E cell lines in the presence of increasing concentrations of verapamil, AM and CYA. *represents significant difference from the incubation without inhibitor. Control represents incubation without inhibitors.

3.5 The effect of HL on the electrocardiographic (ECG) effects of repeated dose HF

3.5.1 Lipid concentrations

As expected,[104] significant rises in the plasma TC and TG were noted in P407 treated rats. At the end of the study, increases in TC and TG were 12 and 29-fold, respectively, in HL compared to NL rats (Table 10).

3.5.2 Drug and metabolite concentrations

In all animals, the enantiomers of HF and DHF were measurable in plasma and heart at the end of the study (Figure 27). With increases in dose, there were increases in the plasma and heart concentrations although at the 40 $mg \cdot kg^{-1} \cdot d^{-1}$ dose level there seemed to be a plateau in the relationships for HF and DHF enantiomers in plasma. In contrast, in heart no such plateau was noted; rather a greater than proportional increase in heart tissues appeared as the doses increased (Figure 27). Because the numbers of animals in dose groups other than 20 $mg \cdot kg^{-1} \cdot d^{-1}$ were small (n=2-3), however, it is not possible to definitely make conclusions regarding linearity.

In the 20 $mg \cdot kg^{-1} \cdot d^{-1}$ dose group, the accumulation factors for mean plasma concentration of (+)-HF in NL and HL rats, respectively, were 1.74±1.10 and 2.43±0.94 between the first and last doses. In contrast, the corresponding accumulation factors for (-)-HF were apparently less (1.12±0.78 and 1.62±0.69 in NL and HL, respectively). In other dose groups, the accumulation factors for

mean plasma concentration of (+)- HF in NL and HL rats, respectively, were 1.35 and 1.85, 1.94 and 1.77, 1.88 and 2.73 and 2.22 and 2.77 for 4, 10, 30 and 40 $mg \cdot kg^{-1} \cdot d^{-1}$. The corresponding accumulation factors for mean plasma concentrations of (-)-HF were 1.45 and 1.27, 1.60 and 1.33, 1.72 and 1.91 and 1.56 and 1.98. Hence, there seemed to be a trend towards increasing accumulation as the dose increased.

The (+) and (-)-HF enantiomers were strongly correlated (r^2 ranging from 0.90 to 0.98) for both NL and HL rats within plasma and heart. The plasma and heart enantiomer concentrations of DHF were consistently lower than HF in NL and HL rats (Figure 27). Significant positive correlations were seen between DHF and HF enantiomer concentrations in both NL and HL rats (Figure 28). In NL rats these correlations were stronger for (-)-HF than (+)-HF in both plasma (r^2 = 0.75 vs. 0.32) and heart (r^2 = 0.91 vs. 0.70), possibly due to the more rapid metabolism of the (-) enantiomer of HF to DHF.[54,122] However, in HL rats there were no such differences noted between enantiomers either in plasma or heart (Figure 28). The slopes in the DHF vs. HF relationships for both the (+) and (-) enantiomers in plasma were much higher in the NL than the HL rats (Figure 28, upper panels). In comparison, heart uptake of DHF enantiomers increased in a similar manner to increases in HF in both the HL and NL rats (Figure 28, lower panels).

Both HF enantiomers were significantly higher in plasma of HL than NL rats after 20 $mg \cdot kg^{-1} \cdot d^{-1}$ doses (Figure 27 and Table 10). For metabolite, (-)- but not (+)-

DHF was significantly higher in HL compared to NL plasma (Figure 27 and Table 10). In heart, however, concentrations of HF and DHF enantiomers were similar in both NL and HL rats (Figure 27 and Table 10).

Strong positive linear relationships were noted upon comparing heart to total and unbound plasma concentration of HF enantiomers in NL and HL rats (Figure 29). Ten-fold higher slopes were noted between total plasma vs. heart concentration of HF enantiomers in NL compared to HL rats (Figure 29, upper panels). In viewing the estimated unbound plasma vs. heart concentrations, however, this difference in slopes was essentially obliterated (Figure 29, lower panels).

3.5.3 Effects of vehicle and hyperlipidemia on rat electrocardiogram

The HF dosing vehicle had no clear effect on the ECG (Table 10). There were some changes detected in the measures of QT and PR after the first dose of vehicle given to NL rats, although they were numerically very small ($\leq$ 5%). By the end of the study no significant differences were detected in any of the vehicle treated groups from baseline conditions. Hyperlipidemia by itself had virtually no effect on the rat ECG (Table 10).

3.5.4 Electrocardiographic effects of halofantrine

In the 20 $mg \cdot kg^{-1} \cdot d^{-1}$ dose group, the RR and PR intervals were negligibly affected by repeated doses of HF (Table 10). The QT intervals, however, were significantly longer than baseline in both NL (+9.8±2.3%) and HL rats

(+21.4±11.2%) at the end of the study. The QTc values were similarly affected. Furthermore, HF caused the QT and QTc intervals in HL rats to be significantly longer than the NL rats (Table 10). By taking the sum of the enantiomers and relating those concentrations to QT interval, the strength of the correlations seemed to be more in line with those of the (+) enantiomer than the (-) enantiomer in both plasma and heart (Table 11).

As plasma concentrations of HF enantiomers rose, there was a general linear increase in the QT interval in both NL and HL rats (Figure 30, upper panels). The addition of DHF and HF plasma molar concentrations yielded essentially the same strength of relationship with QT interval as noted with HF alone in both NL and HL rats (Table 11). In general, the relationships between heart HF enantiomer concentrations and QT interval mirrored those seen in plasma (Figure 30, lower panels). It was observed that the slopes of heart enantiomer concentration vs. QT interval were very similar between NL and HL rats. This was unlike the situation for plasma, where an 11-fold difference was noted for the (-) enantiomer (Figure 30, upper panels). The sum of DHF and HF heart molar concentrations vs. QT interval resulted in numerically lower r^2 values in NL and HL rats for each enantiomer compared to the respective HF enantiomer vs. QT interval relationships (Table 11).

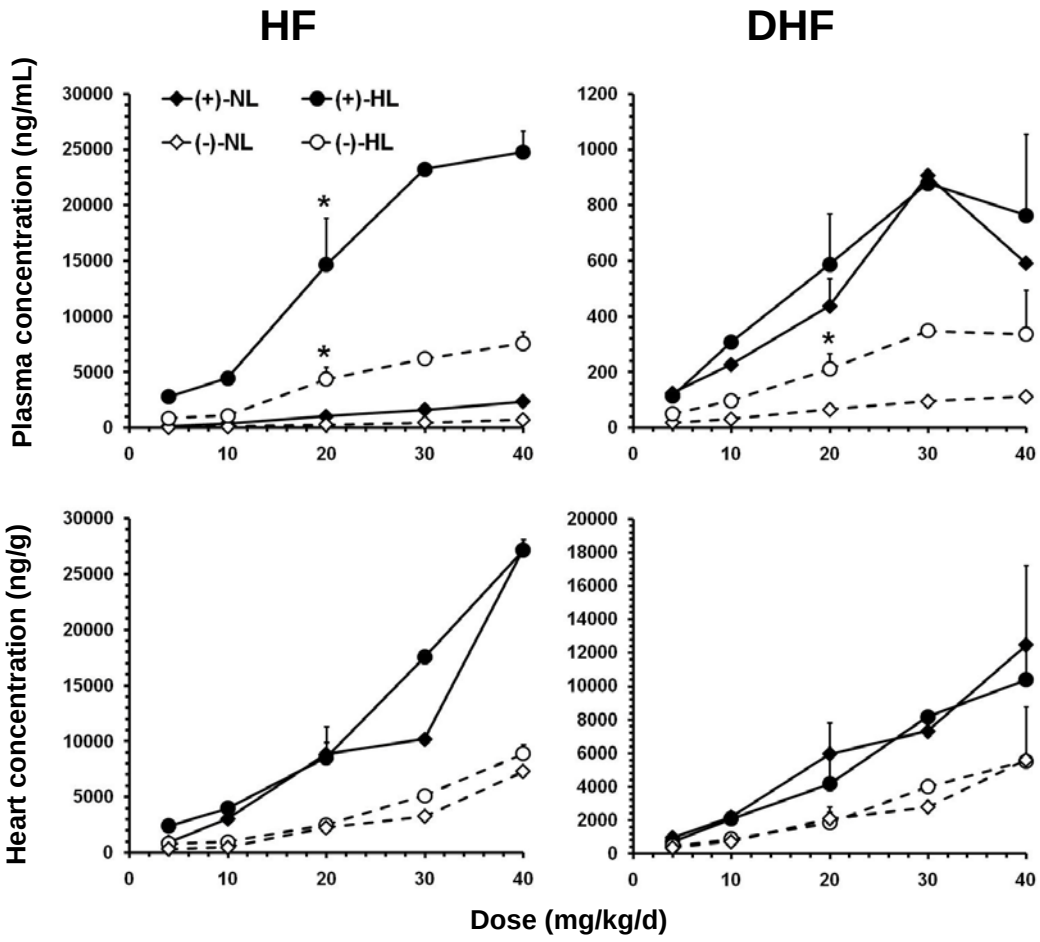

Figure 27: The mean±SD concentrations of HF and DHF enantiomers in NL and HL rats 12 h after the last (±)-HF doses of 4, 10, 20, 30 and 40 mg/kg/d. 20 mg/kg/d group had 7-8 rats whereas all other dose groups had 2-3 rats.

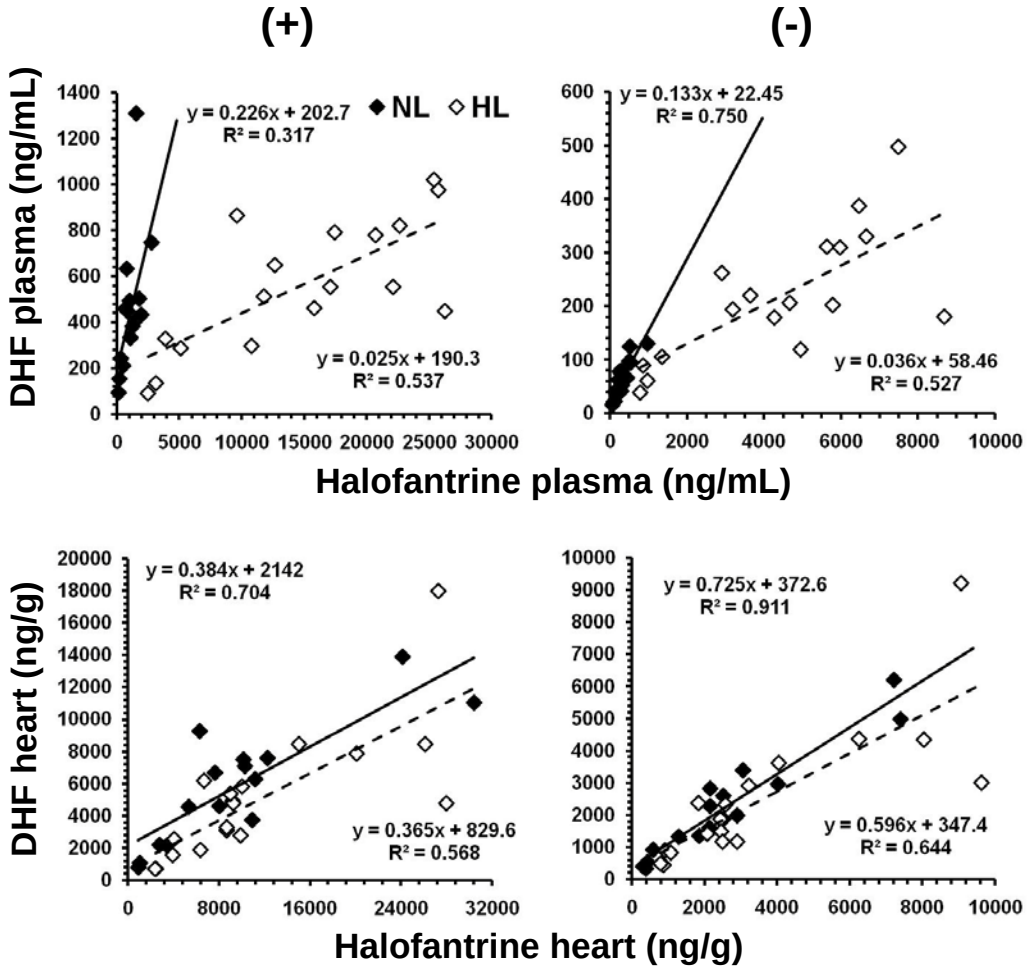

Figure 28: The correlation between plasma and heart concentration of HF enantiomers with corresponding DHF enantiomers in NL and HL rats following their treatment with all dose groups of (±)-HF.

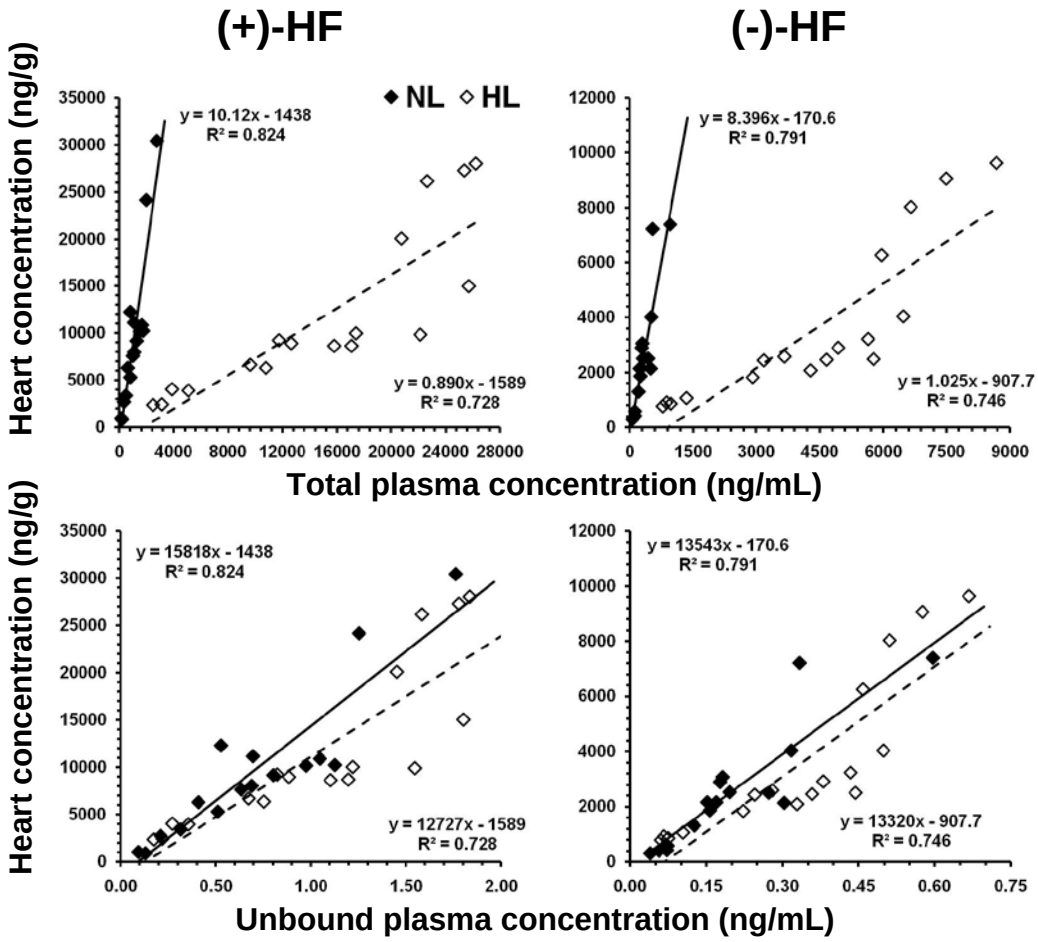

Figure 29: The correlation between heart and total or unbound plasma concentration of HF enantiomers in NL and HL rats following their treatment with all dose groups of (±)-HF.

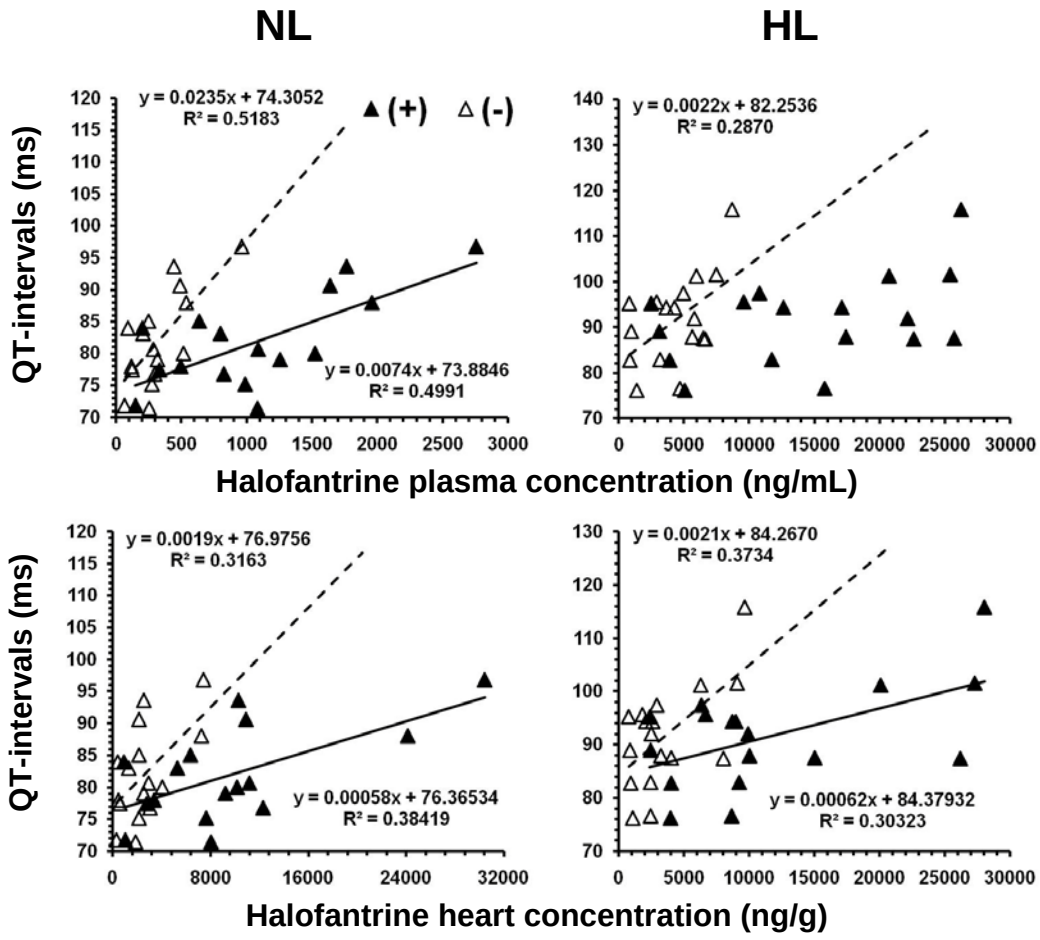

Figure 30: The correlation between QT intervals and plasma or heart concentration of HF enantiomers in NL and HL rats treated with all dose groups of (±)-HF.

Table 10: The mean ± SD of ECG parameters, plasma and heart concentrations of HF and DHF enantiomers and plasma TC and TG in vehicle control and HF treated NL and HL rats 12 h after first and last dose. Blank cells represent values either not measured or calculated. †Significantly different from NL group. *****Significantly different from baseline. ‡Significantly different from vehicle control.

			Vehicle Control		20 mg/kg/d	
			NL	**HL**	**NL**	**HL**
ECG parameters (ms)						
QT	**Baseline**		70.5 ± 1.81	72.9 ± 2.06	73.1 ± 4.94	74.6 ± 7.68
	First dose		74.2 ± 2.23*****	75.9 ± 2.60	72.4 ± 4.86	79.3 ± 8.24
	Last dose		68.9 ± 2.66	71.9 ± 4.83	80.2 ± 6.05*****‡	90.2 ± 7.19*****‡†
QTc	**Baseline**		132 ± 5.26	137 ± 5.06	137 ± 8.99	140 ± 12.9
	First dose		142 ± 5.77	144 ± 3.23	137 ± 10.3	149 ± 13.9
	Last dose		130 ± 3.29	134 ± 10.9	148 ± 10.6*****‡	165 ± 13.6*****‡†
RR	**Baseline**		152 ± 6.57	150 ± 5.55	153 ± 8.04	151 ± 6.25
	First dose		144 ± 8.87	146 ± 5.21	149 ± 14.4	151 ± 11.2
	Last dose		150 ± 8.79	155 ± 8.04	161 ± 7.56	163 ± 8.62*****
PR	**Baseline**		49.6 ± 1.01	49.2 ± 2.79	51.0 ± 2.83	47.9 ± 2.41†
	First dose		47.5 ± 1.00*****	50.1 ± 2.20	52.1 ± 3.65‡	50.0 ± 3.42
	Last dose		48.9 ± 0.89	49.5 ± 2.78	52.6 ± 4.86	50.6 ± 2.53
Halofantrine (µg/[mL or g])						
(+)	**Plasma**	**First dose**	-	-	0.83 ± 0.52	6.24 ± 0.93†
		Last dose	-	-	1.04 ± 0.31	14.7 ± 4.21†
	Heart	**Last dose**	-	-	8.85 ± 2.47	8.56 ± 1.37
(-)	**Plasma**	**First dose**	-	-	0.53 ± 0.49	2.91 ± 0.75†
		Last dose	-	-	0.30 ± 0.086	4.38 ± 1.07†
	Heart	**Last dose**	-	-	2.27 ± 0.56	2.51 ± 0.43
Desbutylhalofantrine (µg/[mL or g])						
(+)	**Plasma**	**Last dose**	-	-	0.44 ± 0.10	0.59 ± 0.18
	Heart	**Last dose**	-	-	5.95 ± 1.85	4.17 ± 1.60
(-)	**Plasma**	**Last dose**	-	-	0.067 ± 0.020	0.21 ± 0.056†
	Heart	**Last dose**	-	-	2.09 ± 0.73	1.85 ± 0.64
Lipid levels (mmol/L)						
TC			-	-	2.83 ± 0.35	35.8 ± 8.69†
TG			-	-	1.70 ± 0.40	58.7 ± 15.4†

Table 11: The regression coefficients (r^2) for the relationships between QT interval and corresponding plasma and heart concentrations. The regressions were performed for both HF alone, and the sum of the molar concentrations of HF plus DHF enantiomers.

	Normolipidemia					
	Plasma			**Heart**		
	(+)	**(-)**	**(+) + (-)**	**(+)**	**(-)**	**(+) + (-)**
HF	0.499	0.518	0.510	0.384	0.316	0.373
HF+DHF	0.413	0.504	0.438	0.359	0.305	0.347
	Hyperlipidemia					
HF	0.187	0.287	0.210	0.303	0.373	0.322
HF+DHF	0.182	0.276	0.204	0.243	0.287	0.256

Table 12: Comparison of ratios of drug residence within rat plasma lipoprotein fractions and change in plasma unbound fraction (fu) in HL rats given P407 relative to NL plasma based on previous reports (Patel et al., 2008; Shayeganpour et al., 2007).

Lipoprotein fraction	Amiodarone	Halofantrine	
		(+)	(-)
Lipoprotein deficient	0.035	0.393	0.298
HDL	0.021	↓	↓
LDL	0.750	0.966	1.07
Triglyceride rich	10.9	3.04	4.09
Change in fu	-96%	-89%	-88%

HDL and LDL denote high and low density lipoprotein fractions; ↓ denotes decrease, drug detected in NL but not HL (ratio not calculable); Triglyceride-rich refers mostly to VLDL in the P407 model.

4 DISCUSSION

4.1 The effect of hyperlipidemia on the stereoselective tissue distribution, lipoprotein association and microsomal metabolism of (±)-halofantrine

When HF was injected intravenously to P407 treated rats, approximately 11-15-fold increases in the plasma concentrations of the enantiomers were observed, which were secondary to approximately 10-fold decreases in unbound fraction and clearance[8] (Figure 8 and Figure 9; Table 4). The data shown here represent the first information describing DHF concentrations after in vivo administration of HF in HL. Although the concentrations of DHF were below the lower limit of quantitation in most of the plasma samples with doses of 2 mg/kg HF HCl, several highly perfused tissues contained large quantities of not only parent drug but also DHF enantiomers (Figure 11 and Figure 12; Table 4 and Table 5). It was known that after a 50% higher dose of HF, concentrations of DHF enantiomers in NL rat plasma were quantifiable but low.[124]

For many protein-bound drugs, normally it is only the unbound drug that is capable of passing through the cell membranes for access to the cells. Given the reduction in unbound fraction and volume of distribution in HL, we would normally expect to see no change in the tissue uptake of a low extraction ratio drug such as HF. However, in this study it was observed that in some tissues, including liver and spleen, concentrations of one or both enantiomers of HF and

DHF were increased. In contrast, decreases were seen in some other tissues (lung, kidney, heart, brain and fat) (Table 4 and Table **5**). Because it is the unbound fraction that is expected to normally control drug penetration into tissues, we also estimated the post-distributive Kpu using unbound fractions determined previously.[8] The use of those estimates is valid assuming that there is no saturation of plasma protein binding. If unbound concentrations are the limiting factor for drug access to tissues, then it would be expected that Kpu would be the same in both NL and HL. What we observed, however, was that there were significant increases in this parameter for lung, kidney, liver and spleen for both HF enantiomers in the post-distributive period. However, for heart decreases of (+)-HF and increases for (-)-HF were noted for Kpu (Figure 10). This suggests that there were factors present, likely to increase drug load in the lipoprotein rich fractions in HL which promoted drug uptake in some tissues beyond that expected based on the decrease in unbound fraction. We have recently reported this observation in a similar study design involving AM.[132]

It was recently reported that in P407-treated HL plasma, marked shifts of AM and its dealkylated metabolite occurred from the LPDP fraction to the TRL-rich fraction.[160] Here a very similar finding was observed for HF and DHF (Table 6) although the increase in HF and DHF in the TRL fraction in HL plasma was not quite as marked as it was for AM and its metabolite. In the NL plasma studied here (Table 6), more drug was present in the TRL and HDL fractions of NL rats than was previously reported.[92] This was an interesting difference; previously the

plasma was obtained from animals that had been fasted overnight. Here, the animals were allowed free access to a maintenance diet of rat chow up to the time of plasma collection. In the normal fed situation, HF enantiomers were bound to a higher degree to lipoproteins than was previously thought. The data illustrates the importance of taking into account the type of NL plasma that is used.

The biodistribution findings (Table 4 and Table **5**) are mostly descriptive, but some attempt to explain them is warranted. The biodistribution of lipoproteins and lipoprotein-associated drugs is affected by the lipoprotein receptor density present in various tissues. In rat, rabbit and human, 60-75%, 67% and 56-80% of LDL-turnover, respectively, is mediated through the actions of LDL receptors.[161-163] The liver is known to be well endowed with LDL and LDL-receptor related protein (LRP) receptors.[95,164] This and the drug being associated with LDL (Table 6) is in line with the observation of increases in the liver AUC of (+)-HF enantiomer and higher Kpu of both enantiomers (Table 4, Figure 10).

Although neither drug nor metabolite was observed to increase in the LDL fraction, it can be assumed that the TRL fraction where the drug had shifted contains mostly VLDL particles. Furthermore, the fate of VLDL particles is to lose TG through the actions of lipoprotein lipase, eventually leading them to evolve into VLDL remnant and LDL particles[34], each of which can be taken up by cells through the action of LRP and/or LDL receptors.[95,164] Hence, even though in our *in vitro* lipoprotein association study the drug was preferentially present in the

VLDL, *in vivo* the actions of LDL receptors can still explain the apparent increase of HF in the liver. In HL, to maintain cholesterol homeostasis, hepatocytes can transiently increase the turnover of LDL receptors on the cell surface which might increase LDL-bound drug uptake. In addition, VLDL is known to act as a ligand for LDL receptor uptake in the liver[95]. Both of these mechanisms could be involved in the increase in amount of LP bound drug in the liver, despite a down-regulation of hepatic LRP in HL.[164] Similar to AM [132], the increase in Kpu of both HF and DHF enantiomers in HL spleen tissues might be explained by the high clearance capacity of chylomicron particles by spleen macrophages from rat blood.[165] This is in line with significant association of HF and DHF enantiomers with the TRL fraction in HL. It was previously suggested that (+)-HF is mainly responsible for concentration dependent QT prolongation observed after HF administration.[123] The current findings of a significant decrease in (+)-HF enantiomer uptake in HL heart tissues is consistent with the decreases noted in HF induced QTc prolongation in a HL rabbit model.[130] The cause of the decrease in heart concentrations is not clear at this time.

Liver microsomal preparations were used to check the possibility of HL induced alterations in the hepatic metabolism of HF (Figure 13). Recently it was found that the expressions of some, but not all, CYP isoenzymes (CYP3A1/2 and 2C11) were lower in P407 treated HL animals. Because CYP3A1 and 2C11 were implicated in HF metabolism in rat[54], a change in metabolism to DHF was

feasible. Although the mean values of Vmax and $CL_{int,1}$ were lower in HL rats, the differences were not statistically significant (Table 7).

4.2 The effect of bile and lipids on the stereoselective metabolism of halofantrine by rat everted-intestinal sacs

The bioavailability of HF is known to increase in humans[55] and beagle dogs[56] when given with a high fat meal, but there was a discrepancy between the amount of increase in parent drug and DHF. The major intent of this study was to seek a mechanism behind the lower metabolite to drug ratio in the fatty meal state. After the ingestion of high fat in most mammalian species, including rat, there is a net increase in the rate of bile salt presentation to the duodenum. These bile salts can help to disperse the TG and the absorption of di- and monoglycerides formed as a consequence of the actions of pancreatic lipoprotein lipase. Based on compositions of rat bile available in the literature[150], the simulated bile salt solutions were prepared in the presence and absence of added TG or CHOL to see the possible impact on HF metabolism by everted intestinal sacs. Besides the everted intestinal sac technique, an Ussing chamber and cultured cell lines can be used for screening the absorption and presystemic metabolism of potential new drug candidates. The rat everted intestinal sac technique[166] is an accepted model for study of the intestinal metabolism of a number of drugs.[40,167-169] It is a relatively simple and inexpensive in vitro model, and has an advantage over some other methods in that it retains the tight epithelial junctions of the intestinal tissues.

The large volume of distribution of HF was in line with the large concentrations of the drug present in the intestinal tissues. Indeed the affinity to the intestinal tissues was so high that the drug was not able to traverse the tissue to gain access to the inner serosal aspect. Another contributing factor to lack of drug on the serosal side of the sac was the lack of a sink effect, normally imparted by blood flow, in the closed system used here. As expected based on the known ability of intestinal microsomes to metabolize HF to DHF[54], DHF was found in most segments incubated with HF.

The HF enantiomer concentration in intestinal segments was not significantly different between KH alone and SBS incubations (Figure 15A). In contrast, the DHF:HF enantiomer ratio in the everted intestinal sacs was decreased significantly with increase in SBS concentration (Figure 15D). The crude bile extract used in the present study to prepare SBS consists of a mixture of hydrophilic and hydrophobic bile acids. Bile acids, such as cholic acid, chenodeoxycholic acid, lithocholic acid, deoxycholic acid and ursodeoxycholic acid are known to be substrates for CYP3A but not other CYPs normally found in the rat small intestine, such as CYP1A1.[170,171] For HF CYP3A is also an important enzyme, although in rat the involvement of CYP2C11, CYP1A1, CYP2C6 and CYP2D1/2 is also documented in vitro.[54] Of these isoenzymes, CYP3A1 has the most activity, and is by far most highly expressed in the rat small intestine.[172] A competition between bile acids and HF enantiomers for intestinal CYP3A metabolism therefore provides a feasible explanation for the observed reduction in

DHF formation in the presence of increasing bile salt concentrations. A cytotoxic influence of bile salts/acids was not apparent, as there was no increase in extracellular LDH activity in incubations involving SBS. Interestingly, unlike the observations for HF, in a recent study using everted rat intestinal sacs there was no apparent effect of increasing bile salts on AM metabolism to desethylamiodarone.[40] Amiodarone differs from HF because its dealkylation is facilitated by CYP1A1 to a much greater extent, with a relative rate of formation being ~2:1 for CYP3A1 compared to CYP1A1[61] (ratio for HF is ~ 24[54]). As such it may not be surprising to see such a difference between the two drugs.

The addition of soybean oil emulsion caused significant decreases in the amount of HF present in the intestinal tissues (Figure 15B and Figure 15C). To compensate for this in assessing the amount of metabolism to DHF enantiomers, the concentration of DHF present in the tissues was normalized to the amount of HF sequestered. When this correction was applied, it was apparent that added lipids within the incubation media caused significant decreases in the DHF:HF ratios of (-), but not (+), enantiomers (Figure 15E and Figure 15F). Soybean oil is mostly composed of polyunsaturated fatty acids, including linoleic acid and linolenic acid (~60%).[83] Both of these fatty acids are also known to competitively inhibit human CYP isoenzymes such as CYP2C9 and CYP2C19 and also to a lesser extent, CYP1A2, CYP2E1 and CYP3A4.[79] Due to the homology between CYP3A4 and rat CYP3A1, it is conceivable that decrease in intestinal transformation of HF to DHF was due to this inhibitory mechanism for (-)-HF.

The (-) enantiomer may have been preferentially inhibited by the fatty acids due to its greater metabolizing efficiency by CYP3A1 than is the case for its antipode.[54]

In contrast to co-incubation of lipids, preexposure of the intestinal tissues to 1% CHOL in peanut oil did not cause a decrease in formation of DHF enantiomers (Figure 15E). Similar to soybean oil, peanut oil also contains a varying composition of saturated, monounsaturated and polyunsaturated fatty acids. Unlike soybean oil, peanut oil is mostly composed of monounsaturated fatty acids (81%)[82], which do not have as much inhibitory activity on CYP3A isoenzymes as do the polyunsaturates, which are more prevalent in soybean oil. Unlike HF, metabolism of AM was significantly lower in 1% CHOL in peanut oil pretreated rats.[40] Although it was not significant, the mean DHF:HF ratio for both enantiomers was lower compared to preparations where lipid was not added either as pretreatment or co-treatment (Figure 15E).

The lack of changes in HF metabolism observed in everted intestinal sacs (Figure 15E) and in intestinal microsomes after oral peanut oil treatment were in line with unchanged expressions of CYP3A1, CYP1A1 and CYP2B1. In the microsomal experiments, any competitive inhibitory effects that might be imparted by fatty acids on CYP activity would be removed from these subcellular enzyme incubations during their preparation.

There were interesting findings in HF enantiomer uptake by everted intestinal sac tissues when the concentrations of CHOL in the incubation media were increased (Figure 16A and Figure 16B). For both enantiomers in the presence of SBS, an increased concentration of CHOL led to significant increases in enantiomer uptake compared to lower CHOL concentrations. It has been shown that CHOL is a substrate for P-glycoprotein (ABCB1) and other members of the ABC family.[33] HF is known to be a substrate for this type of transporter in malarial parasites.[173] It is conceivable therefore that the intestinal tissue concentrations of the drug were increased due to inhibition of efflux transport imparted by the competitive influence of CHOL. Addition of TG and phospholipids by introduction of soybean oil emulsion into the incubations caused lower concentrations of both HF enantiomers in the intestinal tissues, in both the low and high CHOL containing media. In addition, this obliterated the increases observed in the high CHOL containing incubates (Figure 16A and Figure 16B). The decreased concentrations of HF enantiomers in the intestinal tissues after addition of lipids could be explained by formation of large mixed micelles[174], which are not able to traverse the mucosal membrane in this closed in vitro model, which is also devoid of propulsive and mixing movements.

Increased concentrations of CHOL in the presence of SBS led to significant increases in the ratios of DHF:HF enantiomers in the intestinal tissues (Figure 16C and Figure 16D) suggesting the possible influence of CHOL on HF metabolism. The exact mechanism cannot be elucidated from the current study.

However, it is known that following uptake into enterocytes, intracellular CHOL is transported from the plasma membrane to the smooth endoplasmic reticulum, where CYP isoenzymes are also located.[33] It is feasible that CHOL either enhanced the ability of drug present in the enterocytes to access the CYP isoenzymes or increased the functional ability of those enzymes, resulting in an increased DHF:HF ratio.

There were significant differences noted between the uptake and metabolism of the two HF enantiomers by intestinal segments (Table 8). Stereoselectivity has been noted in the metabolism of HF enantiomers with CYP3A1, CYP1A1 and CYP2D1 contributing more towards metabolism of (-)-HF compared to its antipode whereas (+)-HF has more affinity for CYP 2C11 than does (-)-HF.[54] The differences in stereoselectivity between the uptake and metabolism of HF enantiomers can therefore be attributed to the effect of treatments on the metabolism of HF enantiomers rather than enantioselective uptake of HF enantiomers.

4.3 Effect of hyperlipidemia on the metabolism of HF enantiomers by primary rat hepatocytes *in vitro*

Isolated primary hepatocytes are widely used as an *in vitro* tool to study the qualitative and quantitative aspects of hepatic drug metabolism. They provide a full complement of drug metabolizing enzymes (Phase I and II) and transporters

compared to other *in vitro* systems such as liver microsomes and heterologously expressed recombinant human CYP isoenzymes.

The metabolism of HF has been studied in the past utilizing rat and human liver microsomes.[54,175-177] However, the present work is the first attempt to study the metabolism of HF enantiomers in isolated primary rat hepatocytes. Previous studies with NL liver microsomal incubations of HF have reported higher formation rates of (-)-DHF compared to its antipode.[54] Similar results were noted in the present study after HF incubation with isolated rat hepatocytes; the rate of (-)-HF disappearance was faster than that of (+)-HF (Figure 18 and Table 9). The observed stereoselectivity in the disappearance of HF enantiomers can be attributed to the metabolism of HF enantiomers as non-stereoselective plasma protein binding of HF enantiomers has been observed in the past.[8] These findings are also in line with in vivo estimates of clearance[8,122] and in vitro enzyme kinetics parameters (Table 7) except for CYP2C11 which is implicated in the metabolism of (+) more than (-)-HF.[54]

There were three major aims of these studies: 1) To assess the influence of NL and HL in vivo on the metabolic efficiency of hepatocytes for HF, 2) To examine the effect of preincubation of hepatocytes with lipoproteins on hepatocellular metabolism of HF, and 3) To examine the effect of coincubation of lipoproteins on the metabolism of HF.

The major CYP isoenzymes involved in the metabolism of HF enantiomers are CYP3A1/2, 2C11, 1A1, 2C6 and 2D1/2.[54,175] The expression of CYP3A1/2 and CYP2C11 enzymes is significantly decreased in the liver microsomes prepared from HL rats.[74] Despite the observed decrease in the expression of the major CYP enzymes involved in the metabolism of HF enantiomers, V_{max} and $Clint,_1$ values of DHF formation were not significantly different between NL and HL liver microsomes.[128] In line with previous microsomal studies, no significant differences were noted in the metabolism of HF enantiomers between NL and HL hepatocytes in the present study (Figure 18 and Table 9). However, it should be noted that hepatocytes from HL rats were no longer exposed to the HL environment after their isolation and during the time period (up to 72 h) of HF incubation. Therefore, the possibility of suppressed CYP3A1/2 and CYP2C11 proteins in HL hepatocytes regaining their expression with time cannot be ruled out.

The tissue uptake of most protein bound drugs is dictated by their unbound concentrations in the plasma. In addition to this, the lipoprotein receptors present in various tissues including liver[95,164] can also play a major role in the tissue uptake of lipophilic drugs such as HF which are extensively bound to the plasma lipoproteins in the HL state, thus resulting in an unexpected increase in the tissue concentrations. For instance, in HL rats, although the unbound concentration of HF enantiomers was significantly lower, higher Kpu (tissue to plasma unbound concentration) values of HF enantiomers were noted compared to NL livers

(Figure 10).[128] In HL plasma, HF enantiomers are mostly associated with LDL and VLDL particles (Table 6).[128] Owing to their Apo E, both LDL and VLDL are ligands for the LDL receptors which are abundantly expressed in the liver.[95] Therefore, higher than expected uptake of HF enantiomers due to the action of LDL receptors might have been expected to result in faster metabolism of HF enantiomers in the HL state. However, in contrast to the above proposed hypothesis, HF coincubation with HL serum resulted in a significant decrease in the metabolism of (-)-HF but not (+)-HF (Figure 19, 20 and Table 9) in both NL and HL hepatocytes. It is possible that the observed decrease in (-)-HF metabolism in coincubation groups could be due to a decrease in uptake or decreased metabolism. A decrease in the uptake of HF enantiomers could either result from decreased unbound fraction in the presence of HL serum or from down regulation of LDL receptors, or both.

In the P407 mouse model of HL, downregulation of LDL receptors has been reported.[178] Moreover, LDL receptors are membrane proteins which could conceivably be affected during the perfusion step in the isolation of hepatocytes. Both of these could have potentially decreased the metabolism of HF enantiomers in the HL state. The study design used, in which total well content was assayed, could not discern the effect of downregulation of LDL receptors on the uptake of the drug.

To assess the possibility of decreased metabolism as a result of decreased unbound fraction in HL conditions, preincubation of hepatocytes with 5% NL or HL serum was carried out 24 h prior to HF incubations (Figure 21). Preincubation of cells with HL, but not NL, serum had the effect of stereospecifically decreasing the apparent clearance of the (-) enantiomer of HF (Figure 21 and Table 9). The inhibitory impact, however, was not as great as was observed when the HL serum was coincubated with hepatocytes, where the measured AUC was significantly higher than that of preincubated cells (Table 9). Clearly preincubation caused a decrease in metabolism, presumably by decreasing the expression of CYP3A.[77,132] The additional inhibitory effect of coincubation would appear to lie with a decrease in unbound fraction of the HF enantiomers. This is supported by the fact that NL serum also could decrease apparent clearance in the coincubation group (although HF is very highly protein bound in HL plasma, it is also highly bound by NL plasma).

4.4 Effect of P-glycoprotein on the intracellular accumulation of HF enantiomers and Rh-123 in LLC PK1 and NRK 52E cell lines

P-glycoprotein (P-gp) is a plasma membrane-bound glycoprotein which serves as an efflux pump for many xenobiotics, thus decreasing their accumulation into the tissues. P-gp is encoded by the ABCB1 (MDR1) gene and belongs to the superfamily of ATP binding cassette (ABC) transporters.[179] The P-gp homologue Pgh-1 (encoded by *pfmdr*1 gene) in *P.falciparum* malarial parasites has been

associated with the treatment resistance towards many antimalarial drugs including halofantrine.[133]

In the tissue distribution study, it was noted that the brain uptake of (+)-HF was higher in HL compared to NL; the opposite was observed for (-)-HF (Table 4). It was noted that the HL:NL ratio calculated based on brain AUC values was 2.31 for (+)-HF in comparison to 0.31 for (-)-HF. This reversal in the HF enantiomer HL:NL ratio was not noted for plasma or other tissues. This finding was of interest because it has been shown that ingestion of high fat has resulted into decreased function of intestinal transporters.[44] Assuming that HL causes a decrease in P-gp activity, this may suggest that the (+) enantiomer is a substrate for P-gp. However, there are no reports of HF enantiomers being substrates for P-gp. Therefore LLC PK1 (pig kidney cell lines) and NRK 52E (rat kidney cell lines) which are widely used for P-gp efflux assay were used in the present study to elucidate the role of P-gp transport in the accumulation of HF enantiomers in the brain tissue.[153,180]

If HF is a substrate of P-gp, we expect to see less accumulation in the RF induced compared to uninduced LLC PK1 cells. However, no significant decrease was noted in the intracellular concentration of HF enantiomers in RF induced compared to uninduced LLC PK1 cells (Figure 23) suggesting that HF enantiomers are not a substrate of P-gp. To confirm these results, verapamil, a non-specific inhibitor of P-gp was coincubated with HF. However, instead of

expected no change in the accumulation of HF, it was noted that accumulation of HF was decreased in the presence of verapamil in the uninduced LLC PK1 cells (Figure 24) which suggest that HF might be the substrate for uptake transporters. This is in line with decreased accumulation of chloroquine (a P-gp substrate) in the presence of HF, which should have expectedly increased if HF competed with chloroquine for substrate binding site on P-gp.[181] In a recently published report, it has been shown that transport of HF varies markedly between wild type Pgh-1 and polymorphic variants of Pgh-1. It has been shown in this study that HF is a substrate of polymorphic variants of Pgh-1 but not for wild type Pgh-1.[182]

In order to confirm the induction of P-gp in LLC PK1 by RF, Rh-123, a substrate of P-gp was used as a positive control for the P-gp efflux assay.[149] As expected, intracellular accumulation of Rh-123 was decreased in the RF induced compared to uninduced LLC PK1 cells (Figure 25). However, when Rh-123 was coincubated with non-specific inhibitors of P-gp (verapamil, AM and CYA) to confirm the HF accumulation results in the presence of verapamil, the opposite occurred. Similar to HF (Figure 24), a significant decrease in the Rh-123 accumulation in the cells was noted (Figure 25). Similar results were also noted for NRK 52E cells with similar incubation of Rh-123 and verapamil, AM and CYA (Figure 26). These observations are in line with the previous reports which showed the similar decrease in the accumulation of Rh-123 in LLC PK1 cell lines in the presence of verapamil, quinidine and inhibitors of organic cationic transporters cimetidine and TEA.[183] This may suggest that there is a carrier-

mediated uptake mechanism for influx of Rh-123 in uninduced LLC PK1 cells and NRK 52E cells.

4.5 Effect of hyperlipidemia on the electrocardiographic effects of repeated dose halofantrine

The HL state can alter the pharmacokinetic properties of several lipoprotein-bound drugs including HF, CYA, AM, and amphotericin B.[7-9,12,13,184,185] The HL induced changes in the pharmacokinetics of these drugs can also potentially alter their pharmacodynamic properties. For instance, repeated dosing of AM to HL rats has resulted into higher heart uptake of AM which in turn was significantly related to QT interval prolongation.[10] In another study, accentuated nifedipine related reduction in mean arterial pressure was noted despite a decrease in unbound plasma concentrations in HL.[11] Similarly, both CYA[6] and amphotericin B,[127] each of which is bound to lipoproteins, demonstrated enhanced nephrotoxicities in HL. An increase in the IC_{50} of HF was observed when post-prandial serum was incubated with *P. falciparum* culture in vitro.[129]

In HL, increased lipoprotein concentrations cause higher plasma concentrations of (±)-HF and its enantiomers compared to NL (Figure 27).[8,55,73] In HL plasma, elevated levels of HF enantiomers can be attributed to their increased lipoprotein binding, thereby reducing the unbound fraction,[8] and decreased metabolism by reduction in hepatic CYP3A and 2C11.[128,142,147,148] Theoretically, for a low hepatic extraction ratio drug such as HF, a decrease in its plasma unbound

concentration should result in a proportional decrease in its clearance and high volume of distribution. The net effect would be no change in the unbound plasma or total tissue concentration. This expectation is in line with the similarity in total heart uptake of HF enantiomers between NL and HL rats after repeated dosing (Figure 27 and Table 10). In addition, when the measured total (bound +unbound) plasma concentrations of HF enantiomers were converted to estimated unbound concentrations based on the unbound concentrations observed previously[8] in NL and HL rats, the slopes of the lines for NL and HL rats became virtually parallel. This suggests that for HF, it is the unbound concentration in plasma which determines the drug's ability to leave plasma and enter cells, including not only cardiomyocytes, but also the malaria parasite.[129]

This is unlike the case of the antiarrhythmic drug AM in the same animal model of HL.[10] Like HF, large differences were noted in the regression slopes of the drug in plasma vs. heart uptake in the NL and HL animals (similar to Figure 29, upper panel). When the same procedure (Figure 29) was applied to ascertain the unbound concentrations of AM vs. heart uptake, however, the slopes remained very different between the unbound plasma vs. heart concentrations in the NL and HL rats. It was suggested that AM heart uptake was enhanced by its ability to enter into VLDL particles. Heart possesses a high density of VLDL receptors, which is able to efficiently sequester intermediate density lipoproteins derived from VLDL.[36,141] Therefore it was proposed that VLDL receptor mediated uptake

of VLDL-associated AM might have caused an increased cardiac accumulation in the HL state.[10]

Even though AM and HF share an increased uptake in VLDL-containing fractions in HL plasma, their relative uptakes into the various lipoprotein-containing fractions of separated plasma differ (Table 12).[128,147,148,160] In reviewing prior studies, it was noted that the HL:NL ratio of AM in TG-rich fractions was ~10.6-fold, compared to only 3.0 and 4.1-fold for (+)- and (-)-HF, respectively (Table 12). Furthermore, HF enantiomers tended to be more associated with lipoprotein-deficient and LDL fractions in HL plasma. The HL state also has a greater impact on unbound fraction of AM compared to HF enantiomers (Table 12). Therefore it is clear that AM has a higher affinity for VLDL than does halofantrine in this HL rat model. Therefore, in contrast to AM, the lack of change in heart uptake of HF enantiomers could be due to their lower affinity towards VLDL particles in HL plasma.

The prolongation of the QT interval by HF is a major concern in the clinical use of the drug.[137,186] In line with human data,[135] both HF enantiomers showed concentration-dependent increases in the prolongation of QT intervals in NL rats. Similar relationships were noted with HL rats for (-)-HF but not (+)-HF (Figure 30, upper panels). Previously, Wesche et al. showed that (+)- was more potent compared to (-)-HF in its prolongation of QT intervals in isolated feline cardiomyocytes.[123] It was of interest though that in taking the sum of the two

enantiomers and correlating the values to QT interval that the regression coefficients generally were more in line with the (+) than the (-) enantiomer (Table 11). We cannot conclusively differentiate between the relative potency of HF enantiomers on QT interval prolongation, however, because racemic HF was administered rather than individual enantiomers, and because concentrations of the enantiomers were strongly correlated in plasma and heart.

There have been some conflicting views expressed regarding the effect of DHF on QT interval prolongation.[123,126,187] Wesche et al. (2000) had reported that HF was considerably more potent at extending the QT interval in isolated perfused cat hearts than DHF.[123] McIntosh et al. later examined the effect of DHF on the rabbit QT interval.[126] They found that not only could DHF significantly prolong the QT interval, but also that it was probably equipotent to HF. Although HF was not given to animals directly, the claim was based on the findings of Lightbown et al. who gave HF but not metabolite, using essentially the same study design.[188] Based on a smaller cumulative dose, the QT prolonging activity of DHF indeed seemed to be similar to HF. Some reflection is needed here, however. For example, in rats the areas under the curve of DHF enantiomers in heart are considerably higher after administration of preformed metabolite, compared to that of equivalent doses of HF.[125] Furthermore, prolonged anesthesia with mechanical ventilation could have modified the intrinsic QT prolonging effects of drug or metabolite.[143,144] The ECG measures were also recorded shortly after dosing of the rabbits with either HF or DHF, which could have potentially

obscured some differences between the drug and metabolite if the pseudoequilibrium between plasma and tissues had not been fully realized. The possibility of interspecies differences between rabbit and cat in response to HF and DHF was offered as an explanation for the apparent high effect of DHF in the rabbit.

Although we did not administer preformed DHF to our rats, we did measure considerable DHF concentrations in the rat heart after repeated doses of HF. When the molar cardiac HF and DHF enantiomer concentrations in rats were added together and correlated with QT interval, there was no appreciable strengthening (indeed weakening of the relationship in HL rats) of the relationships (Table 11) which is not as expected if DHF was equipotent to HF.

In patients free of drugs known to extend the QT interval, it was noticed that elevated concentrations of LDL CHOL are associated with an increase in the QT interval.[189] Similarly, in rabbits given extended diets of CHOL for up to 16 weeks, a prolongation was observed in the QT interval.[190] In our rats HL did not result in longer QT by itself, but it did accentuate the QT prolonging effect of HF (Table 10). This is an interesting finding. In combination with the elevated levels of TG and CHOL noted in the malarial patients[145,146] as discussed in the rationale, our findings in rat raises the possibility of HL contributing to the QT interval prolongation caused by HF in patients; more studies are needed to examine this possibility.

Our findings are not in agreement with those of McIntosh et al. (2004), who reported a lack of QTc interval prolongation after HF administration to anesthetized rabbits administered a single short-term (30 min) intravenous soybean emulsion.[130] The reason for this disparity is not clear, although the study designs were very different. Although soybean emulsion does increase serum lipid concentrations, it more likely mimics a post-prandial condition rather than HL. Furthermore, because the lipid micelles are introduced with an absence of embedded apoproteins, their handling by the body probably differs from chylomicron particles or other lipoprotein classes. Because a chronic high fat diet can lead to hypercholesterolemia and a prolonged QT interval in rabbits,[190] the lack of prolongation in QT observed by McIntosh et al. in response to HF seems not due to the species used, but perhaps other attributes (study duration, dosing, anesthesia, ventilation, etc.) of their experimental design, as outlined above and in the rationale.

5 CONCLUSION

The HL state caused a shift in association of HF and DHF enantiomers from LPDP to higher TG-containing plasma fractions. Metabolism of HF to DHF enantiomers by microsomal protein was not affected by HL. Biodistribution in HL was modified in a complex manner, with increases and decreases in HF enantiomers being noted in selected tissues. For a highly lipoprotein-bound drug such as HF, distribution to tissues appears to be not only influenced by plasma unbound fraction, but also perhaps to a directed uptake of lipoprotein-bound drug by lipoprotein receptors present in various tissues.

The less than proportional increases observed in the AUC of DHF compared with HF in humans and beagle dogs following the administration of HF post-prandial may be at least in part explained by an oral lipids mediated inhibition of the intestinal metabolism of HF enantiomers. Competitive influences seem to be the underlying basis for the inhibition, because there was no evidence of changes in CYP expression or in intestinal microsomal formation of the DHF enantiomers from HF. Further mechanistic studies are needed to definitively explain the cause of the decrease in metabolism within the intestinal tract.

The QT interval prolongation of HF enantiomers was enhanced in the P407 rodent model of HL, despite similar heart concentrations in NL and HL rats. In HL, the plasma concentrations of HF enantiomers were substantially higher after repeated doses. Uptake of the drug by heart tissues was apparently controlled by the

unbound fraction of the drug in plasma. Based on the regression analysis, there was no evidence that DHF caused any significant prolongation of the QT interval in the rat.

Both decreased uptake and direct inhibition of CYP enzymes by HL serum resulted into decrease in the metabolism of HF enantiomers by primary rat hepatocytes. Further studies are needed to explain the lack of increased metabolism expected based on the in vivo observations from our previous studies.

From our results, HF does not appear to be a substrate of mammalian P-glycoprotein efflux transporters. Therefore, it can be concluded that the observed differences between the brain uptake of HF enantiomers in HL state cannot be due to the inhibition of P-gp efflux transport. This study has shown some indirect evidence of HF being a substrate for influx transporters. The brain endothelial cells have shown to express two members of the LDL receptor gene family namely LDL receptor and LDL receptor related protein-1 (LRP-1)[191] and some of the influx transporters, namely Oatp1a4, Oat1, Oat3, Oct1 and Oct3.[192] Both of these could contribute towards the difference noted in the accumulation of HF enantiomers in HL brain tissue. Further studies are needed to elucidate the underlying mechanism for these observations.

Unfortunately, lipoprotein binding of drugs has been poorly studied, and when reported is underappreciated in terms of the influence that it can potentially impart

on the disposition of drugs. In terms of its influence on pharmacokinetic properties, lipoprotein binding is normally viewed in the same light as other types of binding events to plasma proteins such as albumin or α1-acid glycoprotein. Binding to lipoproteins, however, can potentially result in sometimes unexpected consequences on the properties of drug disposition, largely secondary to the influence of lipoprotein receptors, which can modify the tissue uptake of drug. This includes access to drug to their eliminating organs and pharmacological site of action, which can have consequences on the metabolism and pharmacodynamic properties of the drug. Further studies are warranted to provide more complete insight into the effects of lipid constituents on pharmacokinetics and pharmacodynamics of drugs.

6 FUTURE DIRECTIONS

Both everted gut and hepatocyte studies have shown that oral lipids and circulating lipoproteins caused a preferential decrease in the metabolism of (-)-halofantrine. Specific studies are required to elucidate the exact mechanism through which oral lipids and serum lipoproteins affect the metabolism of HF enantiomers. Based on the results from hepatocyte study, it would be worthwhile to examine the effect of serum lipoproteins on the expression and activity of efflux and uptake transporters responsible for HF transport across the cell membrane.

Studies involving LLC-PK1 and NRK 52E cell lines have shown some indirect evidence of HF enantiomers being substrates for uptake transporters. Further studies can be explored to determine the role of specific uptake transporters in the transport of HF enantiomers.

In the HL state, prolongation of QT interval was noted despite of no changes in the HF heart concentrations. Further studies are required to clarify the exact mechanisms through which HL state increase the susceptibility of heart towards QT prolonging effects of HF.

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