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***PHARMACOKINETIC INTERACTIONS
AFFECTING THE ANTIDIABETIC REPAGLINIDE***

by Lauri Kajosaari

ACADEMIC DISSERTATION

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To my family

To Elina

“There’s nothing more exciting than science. You get all the fun of sitting still, being quiet, writing down numbers, paying attention...

–Science has it all!”

- Principal Seymour Skinner -

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ABBREVIATIONS AND DEFINITIONS

Ahr	Arylhydrocarbon receptor
ANOVA	Analysis of variance
ARNT	Ahr nuclear translocator
ATP	Adenosine triphosphate
AUC _{0-t}	Area under the concentration-time curve from 0 to t hours
BCRP	Breast cancer resistance protein
CAR	Constitutive androstane receptor
CI	Confidence interval
CL	Clearance
CL _{int}	Intrinsic clearance
C _{max}	Peak concentration
COMT	Catechol O-methyltransferase
CV	Coefficient of variation
CYP	Cytochrome P450
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid; cDNA = complementary DNA
EMEA	The European Agency for the Evaluation of Medicinal Products
ER	Endoplasmic reticulum
FAD	Flavin adenine dinucleotide
FMO	Flavin mono-oxygenase
f _u	Unbound fraction of drug
GST	Glutathione transferase
HbA _{1c}	Glycosylated haemoglobin
HDL	High-density lipoprotein
HLM	Human liver microsomes
HNMT	Histamine N-methyltransferase
HPLC	High-performance liquid chromatography
IC ₅₀	Inhibitor concentration causing 50% inhibition of the activity
ISEF	Intersystem extrapolating factor
k _{el}	Elimination-rate constant
K _i	Inhibition constant for competitive inhibition
K _I	Inhibitor concentration where inactivation rate of metabolism-dependent inhibition is 50% of maximum
K _{inact}	Rate of enzyme inactivation for metabolism-dependent inhibition
K _m	Michaelis-Menten kinetic constant
LC-MS-MS	Liquid chromatography-tandem mass spectrometry
LDL	Low-density lipoprotein
MAO	Monoamine oxidase
MRP	Multidrug-resistance-related protein
m/z	Mass-to-charge ratio
NADPH	Nicotinamide adenine dinucleotide phosphate
NAT	Arylamine N-acetyltransferase

OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
PAH	Polycyclic hydrocarbon
PEPT	Peptide transporter
PON	Paraoxonase
PPAR	Peroxisome proliferator-activated receptor
PXR	Pregnane X receptor
RAF	Relative activity factor
rhCYP	Recombinantly expressed human CYP enzyme
RNA	Ribonucleic acid; mRNA = messenger RNA
RXR	Retinoid X receptor
SD	Standard deviation
SNP	Single nucleotide polymorphism
SULT	Sulfotransferase
$t_{1/2}$	Elimination half-life
t_{max}	Time to peak concentration
TPMT	Thiopurine methyltransferase
UGT	UDP-glucuronosyltransferase
V_d	Volume of distribution
V_{max}	Maximum reaction velocity
XRE	Xenobiotic response element

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text by the Roman numerals I to VI.

- I** Kajosaari LI, Laitila J, Neuvonen PJ, Backman JT
Metabolism of repaglinide by CYP2C8 and CYP3A4 *in vitro*: effect of fibrates and rifampicin
Basic & Clinical Pharmacology & Toxicology 2005; 97: 249-256.
- II** Kajosaari LI, Backman JT, Neuvonen M, Laitila J, Neuvonen PJ
Lack of effect of bezafibrate and fenofibrate on the pharmacokinetics and pharmacodynamics of repaglinide
British Journal of Clinical Pharmacology 2004; 58: 390-396.
- III** Niemi M, Kajosaari LI, Neuvonen M, Backman JT, Neuvonen PJ
The CYP2C8 inhibitor trimethoprim increases the plasma concentrations of repaglinide in healthy subjects
British Journal of Clinical Pharmacology 2004; 57: 441-447.
- IV** Kajosaari LI, Niemi M, Neuvonen M, Laitila J, Neuvonen PJ, Backman JT
Cyclosporine markedly raises the plasma concentrations of repaglinide
Clinical Pharmacology & Therapeutics 2005; 78: 388-399.
- V** Kajosaari LI, Niemi M, Backman JT, Neuvonen PJ
Telithromycin but not montelukast increases the plasma concentrations and effects of the CYP3A4 and CYP2C8 substrate repaglinide
Clinical Pharmacology & Therapeutics 2006; 79: 231-242.
- VI** Kajosaari LI, Jaakkola T, Backman JT, Neuvonen PJ
Pioglitazone, an *in vitro* inhibitor of CYP2C8 and CYP3A4, does not increase the plasma concentrations of the CYP2C8 and CYP3A4 substrate repaglinide
European Journal of Clinical Pharmacology 2006; 62: 217-223.

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ABSTRACT

INTRODUCTION Repaglinide is a short-acting oral insulin secretagogue, used to reduce postprandial hyperglycaemia in type 2 diabetic patients. Repaglinide is extensively metabolised, and due to considerable first-pass metabolism its oral bioavailability is about 60%. Repaglinide is mainly eliminated by excretion into bile as inactive metabolites. In previous studies, the cytochrome P450 (CYP) 3A4 inhibitors itraconazole and clarithromycin have moderately increased the area under the concentration-time curve (AUC) of repaglinide. Gemfibrozil, a CYP2C8 inhibitor, has greatly increased repaglinide AUC, enhancing and prolonging its blood glucose-lowering effect. Rifampicin has decreased the AUC and effects of repaglinide.

AIMS The aims of this work were to investigate the contribution of CYP2C8 and CYP3A4 to the metabolism of repaglinide, and to study other potential drug interactions affecting the pharmacokinetics of repaglinide, and the mechanisms of observed interactions.

METHODS The metabolism of repaglinide was studied *in vitro* using recombinant human CYP enzymes and pooled human liver microsomes (HLM). The effect of trimethoprim, cyclosporine, bezafibrate, fenofibrate, gemfibrozil, and rifampicin on the metabolism of repaglinide, and the effect of fibrates and rifampicin on the activity of CYP2C8 and CYP3A4 were investigated *in vitro*.

Randomised, placebo-controlled cross-over studies were carried out in healthy human volunteers to investigate the effect of bezafibrate, fenofibrate, trimethoprim, cyclosporine, telithromycin, montelukast and pioglitazone on the pharmacokinetics and pharmacodynamics of repaglinide. Pretreatment with clinically relevant doses of the study drug or placebo was followed by a single dose of repaglinide, after which blood and urine samples were collected to determine pharmacokinetic and pharmacodynamic parameters.

RESULTS *In vitro*, the contribution of CYP2C8 was similar to that of CYP3A4 in the metabolism of repaglinide ($< 2 \mu\text{M}$). Bezafibrate, fenofibrate, gemfibrozil, and rifampicin moderately inhibited CYP2C8 (K_i 9.7 μM , 30.4 μM , 92.6 μM , and 30.2 μM , respectively) and repaglinide metabolism (IC_{50} 37.7 μM , 111 μM , 164 μM , and 13.7 μM , respectively), but only rifampicin inhibited CYP3A4 (K_i 18.5 μM) *in vitro*.

Bezafibrate, fenofibrate, montelukast, and pioglitazone had no effect on the pharmacokinetics and pharmacodynamics of repaglinide *in vivo*. The CYP2C8 inhibitor trimethoprim inhibited repaglinide metabolism by HLM *in vitro* (IC_{50} 129 μM) and increased repaglinide AUC by 61% *in vivo* ($P < .001$). The CYP3A4 inhibitor telithromycin increased repaglinide AUC 1.8-fold ($P < .001$) and enhanced its blood glucose-lowering effect *in vivo*. Cyclosporine inhibited the CYP3A4-mediated (but not CYP2C8-mediated) metabolism of repaglinide *in vitro* and increased repaglinide AUC 2.4-fold *in vivo* ($P < .001$). The effect of cyclosporine on repaglinide AUC *in vivo* correlated with the *SLCO1B1* (encoding organic anion transporting polypeptide 1, OATP1B1) genotype.

CONCLUSIONS The relative contributions of CYP2C8 and CYP3A4 to the metabolism of repaglinide are similar *in vitro*, when therapeutic repaglinide concentrations are used. *In vivo*, repaglinide AUC was considerably increased by inhibition of both CYP2C8 (by trimethoprim) and CYP3A4 (by telithromycin). Cyclosporine raised repaglinide AUC even higher, probably by inhibiting the CYP3A4-mediated biotransformation and OATP1B1-mediated hepatic uptake of repaglinide. Bezafibrate, fenofibrate, montelukast, and pioglitazone had no effect on the pharmacokinetics of repaglinide, suggesting that they do not significantly inhibit CYP2C8 or CYP3A4 *in vivo*.

Coadministration of drugs that inhibit CYP2C8, CYP3A4 or OATP1B1 may increase the plasma concentrations and blood glucose-lowering effect of repaglinide, requiring closer monitoring of blood glucose concentrations to avoid hypoglycaemia, and adjustment of repaglinide dosage as necessary.

INTRODUCTION

Repaglinide is a short-acting blood glucose-lowering drug aimed to be taken with meals in the treatment of type 2 diabetic patients.¹ Repaglinide stimulates insulin release from the pancreas,² and it can be used as monotherapy, or in combination with e.g. metformin or a glitazone.²⁻⁴ Repaglinide is extensively metabolised by the cytochrome P450 (CYP) system to inactive metabolites, and the main route of excretion of repaglinide and its metabolites is via bile into faeces.⁵

CYP enzymes play a central role in the metabolism of drugs, and inhibition or induction of these enzymes can alter the plasma concentrations and effects of their substrate drugs.⁶ CYP2C8 and CYP3A4 participate in the biotransformation of repaglinide, but their contributions at clinically relevant repaglinide concentrations *in vitro* have been unclear.⁷ Clarithromycin⁸ and itraconazole⁹ (inhibitors of CYP3A4) have increased repaglinide area under the concentration-time curve (AUC) by about 40%. Gemfibrozil has caused the largest increases in the plasma concentrations of repaglinide, an approximately 8-fold increase in the AUC, and has strongly enhanced its blood glucose-lowering effects.⁹ Rifampicin (inducer of CYP enzymes and transporters) has decreased the plasma concentrations and effects of repaglinide.¹⁰⁻¹²

When this work was initiated, the mechanism of the drastic interaction between gemfibrozil and repaglinide was unclear. Gemfibrozil had been shown to increase the plasma concentrations of the CYP2C8 substrate cerivastatin,¹³ but the inhibitory effect of gemfibrozil on CYP2C8 *in vitro* had been only moderate.¹⁴ Furthermore, gemfibrozil does not inhibit CYP3A4,¹⁵ and it seemed unlikely that moderate inhibition of only one of the two CYPs participating in the metabolism of repaglinide would explain the observed interaction.⁹ In addition, the cause of the variation in the observed effects of rifampicin on repaglinide pharmacokinetics¹⁰⁻¹² was not known, and the effect of transporter inhibition on repaglinide pharmacokinetics had not been studied.

The aim of this work was to investigate repaglinide metabolism *in vitro* using therapeutic repaglinide concentrations, particularly the relative contributions of CYP2C8 and CYP3A4. The susceptibility of repaglinide to other pharmacokinetic interactions was studied in humans. The drugs studied with repaglinide *in vivo* included fibrates (bezafibrate and fenofibrate), selective *in vitro* inhibitors of CYP2C8 (trimethoprim and montelukast),^{16,17} and the CYP3A4 inhibitor telithromycin.¹⁸ The effect of pioglitazone (inhibitor of CYP2C8 and CYP3A4 *in vitro*)¹⁹ on repaglinide was also studied to determine whether their synergistic effect, observed in diabetic patients,²⁰ was caused by inhibition of repaglinide metabolism. The effect of cyclosporine, an inhibitor of CYP3A4, organic anion transporting polypeptide 1 (OATP1B1), and P-glycoprotein,²¹⁻²⁴ on repaglinide was also investigated.

REVIEW OF THE LITERATURE

1 Drug interactions

The pharmacologic effects of drugs are based on interaction of the unbound drug with their targets (enzymes, receptors) in their target tissues.²⁵ Route of administration, dose, drug pharmacokinetics, the distribution to the site of action, the concentration-response relationship, and drug removal (redistribution, elimination) determine the rapidity and duration of pharmacological effect.^{25,26} Drug exposure is usually described by the peak drug concentration (C_{max}) and AUC, and drug elimination by half-life ($t_{1/2}$) and clearance (CL).²⁵

With the increasing availability of new drugs, the concurrent use of multiple medications is very common, particularly in elderly patients. Drug interactions are an important aspect of clinical drug application in patients receiving multiple drug regimens. In drug interactions, the combined effects of interacting drugs are greater (or less) than the arithmetic sum of their individual actions, usually due to pharmacokinetic or pharmacodynamic interference. The underlying mechanism of pharmacokinetic drug interactions is usually a change in the systemic clearance or distribution of one drug by another drug. In pharmacodynamic drug interactions the coadministered drugs have overlapping pharmacologic mechanisms or similar target systems. Drug interactions can lead to therapeutic failure (due to lack of effect) or drug overdose (exaggerated pharmacological response and/or drug toxicity), but drug interactions may also be modest and lack clinical significance.^{27,28}

When any two drugs are administered together, the risk for interaction has been reported to be 6%,^{29,30} increasing exponentially with the number of coadministered drugs.³¹⁻³³ Up to 10% of all hospital admissions in the elderly have been reported to be drug-related,²⁹ with fatal adverse drug effects being the 6th leading cause of death in the USA.³⁴ Of all adverse drug reactions, up to 20 to 30% are assumed to be caused by drug interactions.^{29,35,36} In the pharmacological therapy of type 2 diabetes, drugs with additive glucose-lowering effects are used to gain better glycemic control, but such combinations carry a higher risk of hypoglycaemic episodes.³⁷

1.1 Pharmacokinetic drug interactions

In pharmacokinetic drug interactions, e.g. the absorption, distribution, protein binding, metabolism or excretion of a drug is altered by a coadministered drug. The concentrations of the victim drug are altered, and if the change is great enough, the clinical effect of the drug may also be changed.³⁸

Reversible inhibition of cytochrome P450 (CYP) enzymes is probably the most common cause of pharmacokinetic interactions, because CYPs have a pivotal role in the metabolism majority of drugs. Whether a reversible inhibitor will cause a clinically significant impairment of drug metabolism, will depend on the affinities of the substrate (K_m) and inhibitor (K_i) to the drug-metabolising enzyme, and the concentration of each drug at the relevant site of the biological process (affected by dose, distribution, protein binding, etc.).^{38,39}

A pharmacokinetic interaction caused by direct competitive inhibition of drug metabolism (or transport) starts as the target protein is exposed to the inhibitor, and ends when the inhibitor is no longer present. The onset and offset of effect are rapid, and no prior exposure to the inhibitor is needed. Inhibitor concentration at the target site and its inhibitory potency determine the magnitude of interaction. The effect of induction of drug metabolism (or transport) is seen more slowly, and depends also on the duration of exposure to the inducer. Onset and offset of the inducing effect are slow, and pharmacokinetics of the victim drug can remain altered even if the inducer is no longer present.^{27,38,39}

Orally administered drugs that have a low bioavailability due to presystemic metabolism are particularly susceptible to inhibitory interactions. Potent inhibition of their metabolism can cause drastic increases in their bioavailability and peak plasma concentrations, greatly affecting their clinical effects and adverse reactions. For drugs with high bioavailability that are also eliminated by metabolism, potent inhibition of their metabolism probably leads to prolonged elimination half-life and effects, but after a single dose of the victim drug, the peak concentrations are unlikely to be significantly affected.^{27,38,39}

1.2 Pharmacodynamic drug interactions

In pharmacodynamic interactions the effects of one drug are changed by the presence of another drug at its site of action. Though the interaction can be direct (e.g. two drugs share the same target receptor), pharmacodynamic interactions are often indirect, and result from interference with physiological mechanisms.⁴⁰

If two drugs that have the same pharmacological effect are given together, their effects can be additive. For example, even moderate amounts of alcohol can cause excessive drowsiness, if therapeutic doses of sedative drugs are taken simultaneously. Additive effects can occur both with the main effect of drugs, as well as with their adverse effects. For example, increased nephrotoxicity can occur with coadministration of otherwise unrelated nephrotoxic drugs.⁴⁰

In contrast to these additive pharmacodynamic effects, some drug combinations can oppose the activities of each other. For example, increased vitamin K intake can cancel the therapeutic effects of oral anticoagulants that inhibit vitamin K activation, and glucocorticoids can oppose the hypoglycaemic effect of oral antidiabetic drugs.⁴⁰

2 Drug metabolism and transport

Drugs are eliminated from the body by excretion and metabolism. Most drugs are lipid-soluble compounds, which require biotransformation into more hydrophilic form before they can be excreted from the body. A drug may be excreted by the kidneys into urine, by the liver cells into the bile and pass into the intestine, or end up in saliva (or milk). Drugs are most often metabolised in the liver by enzymes localised in the endoplasmic reticulum of hepatocytes, but significant drug metabolism occurs also in other tissues, e.g. gut wall during drug absorption.^{41,42}

The metabolism of drugs has been traditionally divided into "phase I" reactions (e.g. oxidation or hydrolysis) and "phase II" reactions (e.g. conjugation).⁴¹ Phase I reactions usually produce functionalised and pharmacologically less active metabolites, whereas phase II reactions increase the water-solubility of the compounds and enhance their excretion.⁴³ Drug transporters are involved in the cellular uptake of many drugs, and in the export of their metabolites.⁴⁴

Oxidation is a common pathway of xenobiotic metabolism, taking most often place in an intracellular organelle called the endoplasmic reticulum by the CYP system. Reduction is a relatively uncommon pathway of drug metabolism, but hydrolysis of drugs by CYP enzymes and non-specific esterases is common.^{41,45,46}

Typically, the pharmacologically less active drug metabolite produced by oxidative (or other) reactions is conjugated to a water-soluble compound to produce an end product that can be more readily excreted. The biotransformation of a drug may produce several, even dozens of different metabolites.⁴⁵

The CYP system is often a crucial step in the overall elimination of drugs, and therefore a change in the activity (e.g. by inhibition) or amount (e.g. by induction) of CYP enzymes often results in alteration of the pharmacokinetics of drugs.^{6,47-50} Inhibition or induction of drug transporters may also alter the absorption, distribution or elimination of drugs.⁴⁴

2.1 CYP enzyme system

2.1.1 Overview

The cytochromes P450, CYP enzymes, are a superfamily of heme-containing enzymes, of which over 2700 individual members are currently known to exist in nature.⁵¹ They have been named after their characteristic absorption wavelength maximum (450 nm), seen when the reduced form of the enzyme is bound to carbon monoxide.⁵² The CYPs are able to metabolise a diverse group of substrates, both endogenous and xenobiotic, most often by catalysing oxidative reactions.

The CYP system is arguably our most important xenobiotic metabolizing system, and approximately 80-90% of human drug metabolism is CYP-mediated.⁵³⁻⁵⁵ Most of the CYP-catalysed reactions lead to detoxification of xenobiotics, involving the formation of more polar metabolites that are more readily excretable. However, the CYPs can also activate pro-drugs, or produce

more toxic and reactive metabolites. To date, a total of 57 CYP enzymes have been identified in humans.⁴⁶ The families CYP1, CYP2, and CYP3 are primarily associated with the metabolism of exogenous compounds, whereas the other CYPs have mainly endogenous roles.⁴⁶ Degree of similarity in the amino acid sequence divides the CYPs into families (>40% identical, e.g. CYP1 family), subfamilies (>55% identical, e.g. CYP1A subfamily), and individual enzymes with unique sequence (e.g. CYP1A1 enzyme).⁵⁶

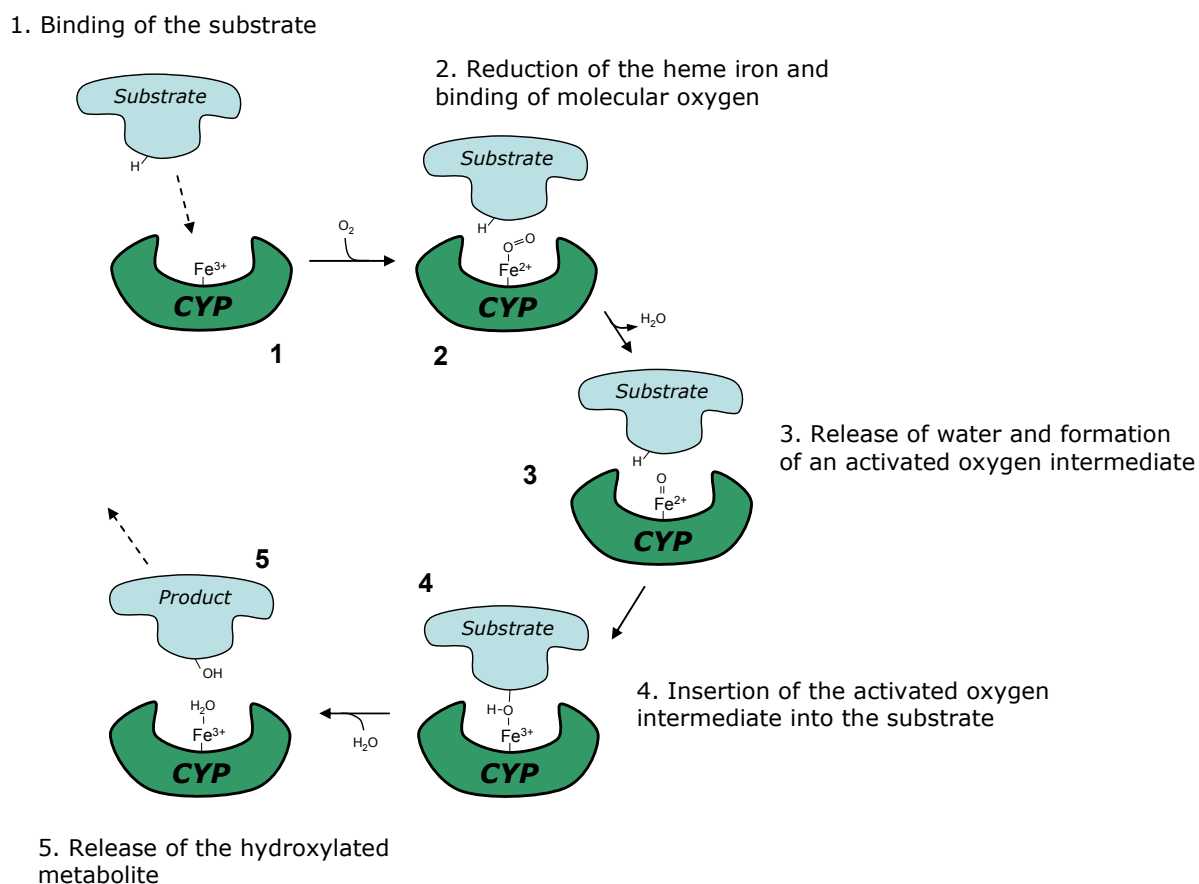
STRUCTURE AND TISSUE DISTRIBUTION The function of CYP enzymes is based on the catalytically active centre of the protein, formed around a heme.⁵⁷ The overall sequence variability among CYP proteins is great, but they have a high conservation of their central structure and general topography.⁵⁸ The most variable regions are associated with anchoring to membrane, and substrate binding and recognition. The substrate-recognition sites are flexible and move upon substrate binding to facilitate the catalytic process.⁵⁹ CYPs are anchored on the outer face of the endoplasmic reticulum (ER) by hydrophobic anchors, with the active site exposed at the cytosolic face of the membrane.⁴⁵

The functions of CYPs are extremely diverse –from biosynthesis and catabolism of signalling molecules and steroid hormones to detoxification or activation of xenobiotics- and they can be found in all types of tissues, with developmentally regulated patterns of expression.⁶⁰ The highest levels of CYPs are found in the liver, where they were first described.⁶¹ The small intestine has the second highest CYP content,⁶² and CYPs are present in lower quantities in many other tissues, such as the kidney, brain, and the respiratory tract.⁶³

In the liver, CYP3A4 is quantitatively the most important, with CYP2C8, CYP2C9, CYP2A6, CYP2E1 and CYP1A2 present in somewhat lower quantities; CYP2C19 and CYP2D6 are of relatively minor quantitative importance, but their clinical importance is high.⁶⁴ CYP3A4 is the major form of CYP expressed in enterocytes, and members of CYP2C subfamily are also significantly expressed.⁶² Microsomal protein content decreases along the small intestine from the duodenum to ileum.⁶⁵

CATALYTIC MECHANISM CYPs most often catalyse the insertion of one of the atoms of molecular oxygen into the substrate being biotransformed, while the second atom of oxygen is reduced to water. The most frequently catalysed reaction is hydroxylation, but the result of CYP-mediated catalysis can be e.g. a dealkylation, dehydrogenation, isomerisation or carbon-bond cleavage.⁶⁰ The variety of CYP enzyme structures and the intrinsic reactivity of all their substrates explains the diversity of reactions catalysed by CYPs.⁴⁶

The details of the mechanism by which CYPs carry out all types of reactions are not fully understood. The best-documented aspect is the hydroxylation reaction that is common to most CYPs (Figure 1).

Figure 1. A simplified scheme of a hydroxylation reaction, common to most CYPs.⁴⁶

SUBSTRATE SELECTIVITY The CYPs have broad and often overlapping substrate specificities. It is possible that two or more CYPs contribute to the metabolism of a single compound, or that a single CYP can catalyse two or more metabolic reactions for the same substrate. Although more than one CYP can catalyse the biotransformation of a drug, they may do so with markedly different affinities. The selectivity of a substrate towards a particular CYP enzyme is based on differences in the number and spatial disposition of the relevant complementary structures on both substrate and enzyme molecules. The lipophilic character and molecular mass of the substrate also play a role in substrate recognition. Drug biotransformation *in vivo* is often determined by the CYP with the highest affinity for the drug. Thus, the rate of elimination of drugs can be largely determined by a single CYP enzyme or a combination of specific CYPs.^{6,51}

VARIATION IN CYP ACTIVITY The levels and activity of each CYP have been shown to vary from one individual to the next due to genetic, environmental, and host factors. Environmental and host factors include medications (e.g. anticonvulsants, rifampicin, antifungals, macrolides), foods (e.g. cruciferous vegetables, grapefruit), habits (alcohol consumption, smoking), age, hormonal status and disease status (infections, hyper- or hypothyroidism, hepatic failure).^{39,66}

Genetic variation observed in genes encoding CYP enzymes, are most commonly single nucleotide polymorphisms (SNP), but complete deletions and

insertions of another gene copy also occur. Only a fraction of genetic variations have any functional consequence, but some of them lead to altered gene product function.⁶⁷ In addition to interindividual differences, there are differences between ethnic groups in the frequencies of polymorphic variations of CYPs.⁶⁸ A genetic polymorphism is defined as an inherited genetic difference that occurs with >1% population frequency. The gene encoding the enzyme protein (e.g. CYP1A1 enzyme) is presented in italics (e.g. *CYP1A1*), and specific alleles are presented by * and allele mark (e.g. *CYP1A1*3* allele). The allele considered prevalent is called the "wild-type" allele.⁶⁹

2.1.2 Mechanisms of CYP induction

CYP induction increases the capacity for metabolic detoxification. This is usually advantageous, protecting the individual against exposure to xenobiotics, but can also be harmful, e.g. if the induced enzyme catalyses the activation of a procarcinogen. Induction can alter drug efficacy or lead to drug-drug interactions. The predominant mechanism of human enzyme induction is increased gene transcription, leading to an increase in the amount of induced enzyme. Enhanced enzyme activity can also result from reduced degradation of the enzyme or stabilisation of the messenger RNA (mRNA). Although a given inducer can be quite selective for a single enzyme, there may be significant inductive effects on one or more additional CYPs and other enzymes.^{70,71}

Induction usually involves a transient increase in the rate of transcription that begins soon after exposure to the inducer, and returns to basal level when the inducer is eliminated from the body. Transcriptional activation of CYP gene expression occurs via binding of an inducing agent to an intracellular receptor, binding of the inducer-receptor complex to DNA response elements, followed by enhanced gene transcription. The overall inductive effect is a combination of several nuclear receptor-mediated processes. For example, the glucocorticoid receptor contributes indirectly to CYP induction by upregulating the nuclear receptors which themselves control the expression of CYPs.⁷²

The induction of the genes in the CYP1 family is under the control of the arylhydrocarbon receptor (Ahr), a transcription factor located in the cytosol and activated by binding of the appropriate inducing chemical (e.g. polycyclic hydrocarbons, PAHs).^{70,71} Three "orphan" nuclear receptors, constitutive androstane receptor (CAR), pregnane X receptor (PXR), and peroxisome proliferator activated receptor (PPAR), are key elements in the induction of the hepatic CYP2, CYP3, and CYP4 families. Their endogenous ligands were previously unknown (hence the name "orphan"), but recently several ligand candidates have been identified (e.g. androstenol, corticosterone and arachidonic acid).⁷¹ Following activation by binding of appropriate ligand (e.g. phenobarbital for CAR, rifampicin for PXR, and clofibric acid for PPAR), they heterodimerise with retinoid X receptor (RXR) and bind to corresponding response element on the DNA, followed by subsequent increase in gene transcription. In addition to CYP enzymes, other drug-metabolising enzymes (e.g. UGTs)⁷³ and P-glycoprotein⁷⁴ have been shown to be regulated by these nuclear receptors. CAR and PXR bind to the same DNA response elements, suggesting that their regulatory functions are overlapping.⁷⁵⁻⁷⁷

2.1.3 Mechanisms of CYP inhibition

A drug can inhibit the activity of the CYP that metabolises the drug itself, but

inhibition of other CYPs that play no role in the metabolism of the inhibitor itself also occur. The mechanism of CYP inhibition divides the inhibitors into reversible and irreversible (and quasi-irreversible) categories.³⁹

REVERSIBLE INHIBITION Reversible CYP inhibition is thought to be the most common mechanism causing pharmacokinetic drug–drug interactions. This type of inhibition is dose-dependent, and when the inhibitor is eliminated from the body, the normal metabolic function of the inhibited enzyme continues. Reversible inhibition can be further divided into competitive (prevalent), noncompetitive and uncompetitive types (Table I). The affinity with which the inhibitor binds to the enzyme is described by the inhibition constant (K_i), used to describe the inhibitory potency of a competitive inhibitor. The subtypes of reversible inhibition can be recognised according to the changes they produce in the observed kinetic constants of affected substrates *in vitro*, and K_i can be determined with graphical plotting methods or nonlinear regression models.⁷¹

In competitive inhibition, the substrate and inhibitor compete with each other for the active site of the enzyme. The inhibitor can share structural similarity with the substrate(s) of the inhibited CYP, but this may not be apparent. Binding of the inhibitor to the active site of the enzyme prevents the binding and biotransformation of the substrate.⁷¹ In non-competitive inhibition, the inhibitor binds to the enzyme at a site distinct from the active site. The binding of the substrate to the enzyme active site is not affected, but the catalytic function of the enzyme-inhibitor-complex is not normal.⁷¹ In uncompetitive inhibition, the inhibitor binds only to the enzyme-substrate complex, that is, binding of the substrate to the enzyme must precede binding of the inhibitor.⁷¹

IRREVERSIBLE INHIBITION Irreversible and quasi-irreversible inhibition require metabolic activation of the inhibitor by the affected CYP enzyme before forming an enzyme-inhibitor complex (Table I).⁷⁸ Irreversible inhibitors are commonly called “catalysis-dependent”, “mechanism-based” or “suicide” inhibitors. Covalent binding of the activated inhibitor to the enzyme leads to irreversible inactivation of the enzyme.⁷⁹ The formed covalent bonds between an irreversible inhibitor and enzyme cannot be broken to regenerate the enzymatic activity. *In vivo*, this sort of inhibition may start more slowly than reversible inhibition, but the final effect is usually more profound and is reversed only by synthesis of new CYPs.⁷⁹

Table I. The typical characteristics of different types of CYP inhibitors.

Type of inhibition		Onset of inhibition	In vitro characteristics
Reversible	Competitive	Inhibitor binds to the enzyme active site	V_{\max} remains constant, but K_m decreases with increasing inhibitor concentration
	Noncompetitive	Inhibitor binds to a site distinct from the enzyme active site	K_m remains constant, but V_{\max} decreases with increasing inhibitor concentration
	Uncompetitive	Inhibitor binds to the enzyme-substrate complex	V_{\max} and K_m decrease with increasing inhibitor concentration, but their ratio remains constant
Quasi-irreversible		Metabolically activated inhibitor forms a quasi-irreversible complex with the enzyme	Enzyme activity can be restored under experimental conditions
Irreversible		Metabolically activated inhibitor binds covalently to the enzyme	Enzyme activity cannot be restored; K_{inact} describes the rate of enzyme inactivation and K_I is the inhibitor concentration where this rate is 50% of maximal

2.1.4 CYP1 family

The members of the CYP1 family (CYP1A1, CYP1A2, and CYP1B1) are involved in the metabolism of many aromatic compounds and in the generation of carcinogenic metabolites from PAHs.⁸⁰⁻⁸²

CYP1A2 CYP1A2 is the most important isoform of the family, accounting for approximately 10% of the total liver CYP content (Table II).^{64,83} CYP1A2 is mainly expressed constitutively in the liver,⁶⁴ and it has about 10-20 different substrate drugs (e.g. melatonin, caffeine, clozapine, and theophylline).^{6,81,84} CYP1A2 inhibitors include furafylline and fluvoxamine.^{85,86} The plasma concentrations and effects of the CYP1A2 substrate tizanidine are drastically increased by fluvoxamine.^{87,88}

2.1.5 CYP2 family

This is the largest and most diverse of the CYP families. Its members are primarily expressed in the liver, but CYP2C enzymes are found in significant quantities also in the intestine.⁸⁹

CYP2A6 CYP2A6 is predominantly a hepatic enzyme, representing about 10% of hepatic CYP proteins, and its substrates include nicotine and coumarine (Table II).⁶ CYP2A6 is inhibited by e.g. methoxalen, and it is induced by phenobarbital.⁶ CYP2A6 is a highly polymorphic enzyme, with at least 16 distinct alleles identified, associated with absent or decreased enzymatic activity. CYP2A6 polymorphism may have a role determining smoking behaviour as CYP2A6 is the major nicotine oxidase and defective CYP2A6 variants have decreased nicotine clearance.⁹⁰⁻⁹²

CYP2B6 Hepatic expression of CYP2B6 has been reported to vary from 0.2 to 7 % of total hepatic CYP, and it metabolises e.g. bupropion and propofol (Table II).^{93,94} CYP2B6 activity is inhibited by e.g. clopidogrel and ticlopidine,⁹³ and it is inducible by phenobarbital and rifampicin. In addition to the wild type allele, seven variant forms of *CYP2B6* have been identified, but their functional significance is not completely understood.^{50,67}

CYP2C SUBFAMILY This family comprises CYP2C8, CYP2C9, CYP2C18, and CYP2C19, which collectively are responsible for the metabolism of about 20% of clinically prescribed drugs.⁸¹ CYP2C enzymes are expressed in the liver and intestine, where they are the most expressed CYP subfamily after CYP3A.^{65,89}

CYP2C8 CYP2C8 constitutes about 12% of total microsomal CYP content in the liver (Table II), and the enzyme is characterised by a relatively large active site.⁸³ Though CYP2C8 is found also in the intestine, intestinal CYP2C8 content is small and unlikely to play an important role in drug metabolism.⁸⁹

CYP2C8 metabolises arachidonic acid and retinoid acid,^{95,96} and it also plays an important role in the metabolism of drugs such as amiodarone,⁹⁷ cerivastatin,⁹⁸ paclitaxel,⁹⁹ and repaglinide.⁷ Extrahepatically, CYP2C8 appears to be important in the activation of arachidonic acid in the kidney.¹⁰⁰ CYP2C8 inhibitors include trimethoprim, montelukast, quercetin, and gemfibrozil.^{101,102} CYP2C8 expression is induced by e.g. rifampicin, phenobarbital, and dexamethasone.⁶

In addition to the wild-type allele, at least four variant *CYP2C8* alleles are known. *CYP2C8*2* is very rare, *CYP2C8*3* has a frequency of 13% and *CYP2C8*4* a frequency of 7.5% in Europeans; in Africans the frequencies are different. Variant *CYP2C8* forms are associated with a decreased activity compared to the wild type enzyme when using paclitaxel as substrate.¹⁰³

CYP2C9 CYP2C9 is expressed both in the liver and in the intestine, and the CYP2C9 content of the liver is approximately 10-fold higher than in the intestine (Table II). CYP2C9 is the predominant CYP2C isoform both in the intestine and liver.⁸⁹ Substrates of CYP2C9 include S-warfarin, S-ibuprofen, losartan, and phenytoin.¹⁰⁴ CYP2C9 is inhibited by e.g. sulfaphenazole, voriconazole, and fluconazole,¹⁰⁴ and it is induced by rifampicin, dexamethasone, and phenobarbital.⁶

Several variant *CYP2C9* alleles have been described, characterised by impaired enzymatic activity. The variants forms alter the pharmacokinetics of CYP2C9 substrate drugs, and about 1% of Caucasians have a low CYP2C9 activity.^{50,67} For example, a relationship between warfarin dose requirement and CYP2C9 genotype has been found in several studies.¹⁰⁵⁻¹⁰⁷

CYP2C19 CYP2C19 is functionally expressed in the liver and intestine,⁸⁹ and its substrates include diazepam, protein pump inhibitors (e.g. omeprazole), and citalopram (Table II).⁵³ CYP2C19 is inhibited by fluconazole and fluvoxamine, and induced by phenobarbital and rifampicin.⁶ Some variant *CYP2C19* alleles (*CYP2C19*2* and **3*) do not produce functional protein, leading to impaired metabolism. For example, the absence of functional CYP2C19 has been associated to better response to peptic ulcer treatment with omeprazole and impaired diazepam elimination.^{50,67} Recently identified *CYP2C19*17* allele has been reported to cause ultrarapid metabolism by enhancing CYP2C19

expression.¹⁰⁸

CYP2D6 CYP2D6 is expressed in the gut wall and the liver, and it represents about 2% of total hepatic CYP content (Table II).⁵¹ CYP2D6 substrates include amitriptyline, codeine, and metoprolol.⁵³ CYP2D6 is inhibited by e.g. quinidine, and it has not been shown to be inducible by drugs. There is considerable genetic variation in CYP2D6 activity, and people can be divided into poor, extensive, and ultrarapid metabolisers based on the CYP2D6 genotype.^{50,67}

CYP2E1 CYP2E1 is mainly found in the liver, and it accounts for about 7-9% of hepatic CYP content (Table II). CYP2E1 substrates include acetone, ethanol, benzene, halothane, isoflurane, and paracetamol,⁵³ and CYP2E1 is inhibited by e.g. disulfiram. Increased metabolic function of CYP2E1 in response to inducing agents (e.g. ethanol, acetone) is mediated through multiple induction mechanisms. CYP2E1 is the major enzyme converting paracetamol to its reactive intermediate, and CYP2E1 induction (by e.g. excess ethanol consumption) predisposes to liver damage from therapeutic paracetamol doses.^{50,67}

2.1.6 CYP3 family

The CYP3A subfamily accounts for approximately 30-40% of the total CYP content in the human liver,⁵³ and CYP3A4 is involved in the metabolism of about 50% of drugs.⁸¹ CYP3A genes are highly inducible, by e.g. rifampicin, barbiturates and dexamethasone.¹⁰⁹

CYP3A4 CYP3A4 is the most abundant CYP isoform in the liver and intestine (Table II).⁶⁴ Observed CYP3A4 activity varies considerably between individuals, at least partly due to substrate overlap with variably expressed CYP3A5 and CYP3A7 enzymes.⁸¹

CYP3A4 substrates include, for example, midazolam, simvastatin, cyclosporine, and triazolam.⁵³ CYP3A4 inhibitors include e.g. ketoconazole, itraconazole, erythromycin, and grapefruit juice.¹¹⁰ CYP3A4 inducers include rifampicin, phenytoin, phenobarbital, and carbamazepine.^{6,111} Induction of CYP3A4 has been shown to occur both in the liver and in the intestine.¹¹²

A substantial number of variant CYP3A4 alleles have been described, but their population frequencies are very low, and they are unlikely to explain the variability of CYP3A4 activities. The observed activity differences may be explained by variation in the functions of transcriptional regulators of CYP3A4 (e.g. PXR).^{50,67}

CYP3A5 CYP3A5 is found mainly in the liver but also in extrahepatic tissues such as the intestine.¹¹³ The substrate specificity of CYP3A5 is similar (but not identical) to that of CYP3A4, but the catalytic efficiency of CYP3A5 is usually smaller than that of CYP3A4. Erythromycin and midazolam are metabolised efficiently by CYP3A4 and CYP3A5, whereas CYP3A4 is a more efficient metaboliser of quinidine, testosterone, and irinotecan than CYP3A5.⁶ CYP3A5 shows a polymorphic expression pattern, and significant amounts of the enzyme (product of *CYP3A5*1*) are expressed in only 10-20% of caucasians. *CYP3A5*3*, producing a nonfunctional protein, is very common in all ethnic groups.^{50,67}

CYP3A7 CYP3A7 is normally expressed only in the fetal liver, but some adults continue to express this enzyme. CYP3A7 expression may be a

contributory factor to the high overall variability of CYP3A activity in adults.^{50,67}

2.1.7 Other drug-metabolising enzymes

In addition to CYP enzymes, there are also many other enzymes localised in the endoplasmic reticulum or cytosol that contribute to drug metabolism, and many of these enzymes catalyse the same reactions as CYPs. Flavin mono-oxygenases catalyse the oxidation of e.g. nortriptyline, verapamil, and cimetidine.¹¹⁴ Monoamine oxidases (MAOs) metabolise the brain transmitters dopamine and serotonin, and MAO inhibitors are used as antidepressants (e.g. moclobemide) or to treat Parkinson's disease (e.g. selegiline).¹¹⁴⁻¹¹⁶ Alcohol and aldehyde dehydrogenases catalyse the oxidation of alcohols and aldehydes to carboxylic acid metabolites.¹¹⁴ Xanthine oxidase metabolises purine compounds to uric acid, and its inhibitor allopurinol is used in the treatment of gout.¹¹⁴ Members of the paraoxonase enzyme family hydrolyse several insecticides, and appear to be protective against the development of atherosclerosis by hydrolysing derivatives of oxidised cholesterol.^{114,117}

Table II. Examples of substrates, inhibitors, and inducers of CYP enzymes, and their relative abundances in the human liver.

Relative hepatic abundance of CYP isoforms*		Substrates	Inhibitors	Inducers	
~20-29%	CYP3A4 #	Amiodarone Cyclosporine Felodipine Midazolam	Nifedipine Simvastatin Tacrolimus Triazolam	Erythromycin Itraconazole Ketoconazole Ritonavir	Carbamazepine Phenobarbital Phenytoin Rifampicin
~18%	CYP2C9 †	Glimepiride Glipizide Ibuprofen	Phenytoin S-Warfarin Tolbutamide	Amiodarone Fluconazole Sulfamethoxazole	Phenobarbital Rifampicin
~12%	CYP2C8	Amiodarone Cerivastatin Paclitaxel	Repaglinide Rosiglitazone	Gemfibrozil Trimethoprim Montelukast	Phenobarbital Rifampicin
~8-13%	CYP1A2	Caffeine Clozapine Olanzapine	Ropivacaine Theophylline Tizadinide	Cimetidine Fluvoxamine Furafylline	Omeprazole Tobacco smoke
~4-13%	CYP2A6	Coumarin Nicotine		Methoxalen	Phenobarbital
~7-9%	CYP2E1	Ethanol Halothane	Paracetamol	Disulfiram Methoxypsoralen	Ethanol Isoniazid
~4%	CYP2C19 †	Citalopram Diazepam	Omeprazole S-mephenytoin	Fluconazole Fluvoxamine	Phenobarbital Rifampicin
~0.2-7%	CYP2B6	Bupropion Halothane	Nevirapine Propofol	Clopidogrel Ticlopidine	Phenobarbital
~2%	CYP2D6 †	Nortriptyline Metoprolol	Dextromethorphan Tramadol	Quinidine Fluoxetine	Not known

* Individual CYP contents vary considerably, data from Rodrigues 1999 (n=12) and Shimada *et al.* 1994 (n=60).

Polymorphically expressed CYP3A5 shares several substrates with CYP3A4.

† These enzymes exhibit significant polymorphism.

Table adapted from Pelkonen *et al.* 1998 and Ingelman-Sundberg 2003.

2.2 Conjugating enzymes

The conjugating enzymes use the functional groups originally present in their substrates, or introduced by drug-metabolising enzymes, to attach them to additional molecules. The addition of a conjugate, such as glucuronic acid, usually results in pharmacological inactivation of the drug or metabolite, but in some cases this can be an activating step. The product conjugates are more water-soluble and more readily eliminated from the body.⁴¹

UDP-GLUCURONOSYLTRANSFERASES (UGT) The UGTs are a superfamily of membrane-bound enzymes that catalyse the conjugation of glucuronic acid to their substrates. The tissue distribution and intracellular location of UGTs is similar to that of CYPs, but their active site is inside the endoplasmic reticulum. UGTs have broad and overlapping substrates specificities and can conjugate different types of functional groups. Their substrates include endogenous compounds such as steroids, bilirubin and retinoid acid in addition to xenobiotics.^{41,118}

SULFOTRANSFERASES (SULT) Sulfonation modulates the biological activity of xenobiotics, hormones and neurotransmitters, but also the receptor activity of e.g. estrogens and androgens. SULTs are enzymes found primarily in the cytosol, and they catalyse the transfer of a sulfonate group to their substrates. SULTs form a large superfamily of genes; SULT1 and SULT2 families are responsible for the sulfonation of the greatest number of compounds.^{41,119}

GLUTATHIONE TRANSFERASES (GST) The GSTs are mainly cytosolic enzymes that catalyse the conjugation of glutathione group to suitable substrates, but act also to remove reactive oxygen species. The conjugates produced by GSTs are water-soluble, and require active transport out of the cell by e.g. multispecific organic anion transporter (MOAT) or P-glycoprotein.^{41,120}

ARYLAMINE N-ACETYLTRANSFERASES (NAT) The function of NATs is to conjugate an acetyl group to appropriate substrates. Humans express two distinct isoenzymes, NAT1 and NAT2. Clinically relevant NAT substrates include the antiarrhythmic procainamide, several antibacterial sulphonamides, caffeine, and many industrial and environmental carcinogens.^{41,121}

METHYLTRANSFERASES Methyltransferases catalyse the methylation of many drugs, neurotransmitters and hormones, and even macromolecules such as proteins and DNA.⁴¹ Catechol O-methyltransferase (COMT) is found in a cytosolic and a membrane-bound form, and its substrates include neurotransmitters dopamine, noradrenalin and adrenaline and drugs L-dopa and methyldopa. Thiopurine methyltransferase (TPMT) is a cytosolic enzyme, which catalyses the S-methylation of e.g. 6-mercaptopurine. Methylation mediated by the cytosolic histamine N-methyltransferase (HNMT) has been shown to be a major pathway of histamine metabolism.^{41,122}

2.3 Drug transporters

The physicochemical properties of a drug (pK_a , size, lipophilicity) affect drug movement through biological barriers, but the action of drug transporters also has a significant impact on drug disposition and elimination. The substrate

selectivity, direction of action (moving substrates into or out of the cell), intracellular location (in the basolateral or apical cell membrane), and tissue distribution (in e.g. the intestine, kidney, liver, or brain) of transporters determine how they affect drug movement between biological compartments. In addition to drugs, these transporters also transport endogenous substances (sugars, lipids, amino acids, bile acids and hormones).^{44,123-125}

In the small intestine, enterocytes contain several transporters that are important in the absorption of dietary constituents and drugs, but also transporters that limit drug absorption. P-glycoprotein is a particularly important efflux transporter that limits the extent of drug absorption by pumping its substrate drugs back into the gut lumen.⁴³

In the liver, uptake transporters (e.g. OATPs) expressed on the basolateral membrane of hepatocytes extract their substrate drugs from the portal blood. Once inside, drugs often undergo metabolic transformation and/or conjugation, or they may be excreted unchanged into bile. The final step of transport of drugs from portal blood into bile can be mediated by efflux transporters (e.g. P-glycoprotein) localised on the canalicular membrane of the hepatocyte.^{44,123-125}

In the kidney, combined function of uptake and efflux transporters localised to the basolateral and apical membranes of tubular cells can affect the direction and efficacy of drug passage. Other factors, such as urine pH and glomerular permeability of the drug are often more important in determining the amount of drug excreted into urine.⁴³

In the blood-brain barrier, formed mainly by capillary endothelial cells, drug transporters are an important component limiting the access of drugs into brain tissue. While uptake transporters act to import glucose and amino acids to brain, efflux transporters such as P-glycoprotein prevent entry of their substrate drugs.⁴³

The placenta is the structural barrier between the mother and the developing fetus, and there are various transporters both in the maternal-facing and fetal-facing placenta membranes. For example, P-glycoprotein has a major functional role in the maternal blood-placenta barrier,¹²⁶ while OAT4 is localised in the fetal-facing surface, and thought to have a role in the placental uptake of fetal-derived compounds.^{44,123-125}

Compared to the CYP enzymes, relatively little is known about the functions of drug transporters. Differentiation of the role of a particular transporter in drug pharmacokinetics is difficult, because the substrate specificities of different (efflux and influx) transporters, expressed variably in organs relevant to pharmacokinetics (e.g. intestine, liver, and kidney) may overlap considerably. A complicating factor is that in studies conducted in humans, drug concentrations are usually measured only from blood (and urine), and knowledge of tissue concentrations is lacking. Furthermore, the study of drug transporters *in vitro* is more complicated than that of drug-metabolising enzymes, because the action of transporters is directional and requires a cell membrane.^{44,123-125}

The induction and inhibition of drug transporters follow the same principles described for drug-metabolising enzymes. Induction of several drug transporters

is mediated by the same nuclear receptors that regulate CYP gene expression. PXR activation has been shown to induce P-glycoprotein, MRP2 (also induced by CAR activation), and OATP1B1. Inhibition of drug transporters important to drug elimination (e.g. hepatic uptake or biliary/renal excretion) or limiting absorption (in the intestine) can lead to drug-drug interactions. For example, inhibition of P-glycoprotein by clarithromycin has been shown to increase the AUC of digoxin (a high-affinity P-glycoprotein substrate).^{44,127,128}

2.3.1 Uptake transporters

Uptake (influx) transporters facilitate the entry of drugs into cells. Uptake transporters include the organic anion transporting polypeptide (OATP), the organic anion transporter (OAT), organic cation transporter (OCT), and peptide transporter (PEPT) families (Table III).^{44,123,129}

Three organic anion transporting polypeptides (OATPs) have been identified in the human liver, located at the sinusoidal membrane of hepatocytes and extract compounds from portal venous blood. Efflux of intracellular glutathione has been suggested to be a driving force for OATP-mediated transport.^{44,124,129}

Organic anion transporters (OATs) are multispecific transporters expressed in a variety of tissues, such as kidney, liver, brain and placenta. The transport mechanism of OAT1 and OAT3 utilises the Na⁺ gradient of the plasma membrane, but the mechanism of OAT2- or OAT4-mediated transport is not well characterised. In the kidney, OAT1 and OAT3 are localised on the basolateral membrane of the proximal tubule cells and take up drugs from blood.^{44,124,129}

The members of the organic cation transporter (OCT) family act to transport various small organic cations, but interact also with larger cations.^{44,124,129} Peptide transporters (PEPTs) are located in the membrane of intestinal and renal epithelial cells, where they mediate the uptake of peptides produced by protein digestion, as well as structurally related compounds (e.g. β -lactam antibiotics).^{44,130}

2.3.2 Efflux transporters

Efflux transporters act to limit the entry of drugs or enhance their removal from the cell, and are present in many tissues (e.g. intestine, blood-brain barrier, hepatocytes, and placenta). P-glycoprotein (MDR1), multidrug resistance-associated protein family (MRP), and the breast cancer resistance protein (BCRP) are the efflux proteins most relevant to drug disposition, but their substrates also include sugars, amino acids, cholesterol and proteins.^{123,128,131}

P-GLYCOPROTEIN (MDR1) P-glycoprotein is found at the hepatocyte canalicular membrane, on the apical side of gastrointestinal epithelial cells, on the surface of endothelial cells of brain capillaries, in the placenta, and in the testis. P-glycoprotein reduces the absorption in the intestine and enhances the excretion of drugs into the bile or into urine. P-glycoprotein plays also an important role in protecting the brain and testis by limiting their exposure to drugs.^{127,131-133}

Table III. The tissue distribution of selected uptake transporters, and examples of their substrates.

<i>Transporter family</i>	<i>Location</i>	<i>Examples of substrates</i>
Organic anion transporting polypeptides (OATPs)	OATP1B1, OATP1B3, and OATP2B1 in the liver; OATP2B1 also in the lung, brain, kidneys	OATP1B1: methotrexate, rifampicin, atorvastatin, pravastatin, rosuvastatin, fexofenadine
Organic anion transporters (OATs)	Kidney, liver, brain, and placenta	OAT1: NSAIDs, β -lactam antibiotics, diuretics and ACE-inhibitors; OAT2: NSAIDs, antibiotics; OAT3: steroid conjugates, NSAIDs, and diuretics; OAT4: steroid conjugates, antibiotics.
Organic cation transporters (OCTs)	OCT1: liver, intestine; OCT2: kidney	OCT1: dopamine, cimetidine, metformin
Peptide transporters (PEPTs)	PEPT1: small intestine, kidney, liver; PEPT2: kidney, brain, lung, mammary gland	PEPT1, PEPT2: Dipeptides, tripeptides, β -lactam antibiotics, ACE inhibitors

References in text.

The substrates, inhibitors, and inducers of P-glycoprotein are structurally diverse, but overlap with those of CYP3A4. It has been suggested that P-glycoprotein and CYP3A4 function together to limit oral drug bioavailability, but evidence in humans is limited.¹³⁴ P-glycoprotein substrates include digoxin, doxorubicin and vinblastine, and its activity is inhibited by e.g. quinidine, verapamil or cyclosporine. Inducers of P-glycoprotein include e.g. rifampicin and St. Johns wort.^{127,131-133}

MDR-RELATED PROTEINS (MRP) MRPs are organic anion efflux pumps that transport glutathione, glucuronide or sulphate conjugates of drugs, and also unconjugated drugs (e.g. macrolide antibiotics). They are found in several tissues, e.g. in the liver, gut, kidney, brain and placenta. Due to their cellular locations, MRP1 and MRP3 secrete drugs into, while MRP2 moves drugs out of the body.^{128,131,135}

MRP1 is located in various tissues, but has low expression in the liver. MRP2 is located in the canalicular membrane of the hepatocyte, on the apical membrane of intestinal enterocytes, and in the proximal tubule in kidney. MRP2 substrates include pravastatin, ceftriaxone, and ciprofloxacin, and its activity is inhibited by e.g. probenecide. MRP3 is expressed in the basolateral membrane of the hepatocyte and basolateral membrane in kidney tubules.^{128,131,135}

BREAST CANCER RESISTANCE PROTEIN (BCRP) BCRP is present in the placenta, intestine, liver, and capillaries, and its substrates include mitoxantrone, anthracyclines, and topotecan. The role of BCRP in the hepatic distribution of drugs remains to be determined, but it appears to have an active role limiting drug entry into CNS.^{128,131}

3 *In vitro* studies on drug metabolism

In vitro drug metabolism studies are used to estimate risks of drug-drug interactions, and also to predict human pharmacokinetics in the development of drugs. The properties of drugs characterised using *in vitro* methods can be used to guide the planning of studies conducted in humans. For example, when the principal enzymes responsible for the biotransformation of a drug have been identified *in vitro*, estimates can be made of its susceptibility to drug-drug interactions caused by inhibition of its metabolism. Conversely, the inhibitory properties of a drug can be used to estimate its effect on the metabolism of other drugs.^{83,136,137}

Compared to clinical studies, the *in vitro* drug metabolism studies are faster and cheaper to conduct, and do not require human exposure. However, they have numerous intrinsic problems (e.g. non-specific binding of drugs to microsomes, the need for organic solvents) and cannot incorporate many determinants of human drug disposition (e.g. plasma protein binding, hepatic blood flow). Though *in vitro* data can be used to predict the likelihood of clinical drug interactions, studies conducted in humans are needed to draw reliable conclusions. Human liver microsomes (HLM) are the most widely used source of human enzymes used in *in vitro* drug metabolism studies, while other alternatives include cDNA-expressed CYP enzymes (rhCYPs), isolated/cultured hepatocytes, liver slices, and purified CYP enzymes.¹³⁸⁻¹⁴⁰

3.1 *In vitro* systems

HUMAN LIVER MICROSOMES (HLM) HLM are prepared from homogenised liver tissue by differential centrifugation. HLM contain all the CYP enzymes expressed in the human liver in the same proportions that they occur *in vivo*. By pooling several liver tissue samples, the individual variation in enzyme content can be minimised, but each HLM batch is still slightly different. Pooled HLM contain the "average" levels of all CYPs expressed in the livers taken into the pool. The ratio of NADPH-reductase to CYP, the amount of cytochrome b₅, and the type of lipids are same as those in the intact liver. The same sample of pooled HLM can be used to study all CYPs of interest, and they contain the enzymes required to study metabolism-dependent inhibition. HLM contain, however, large amounts of lipids and proteins that can significantly bind the studied drugs, though this problem is common to all *in vitro* systems. In HLM, enzyme-selective inhibitors must be used when assessing the role of a particular CYP in the metabolism of a substrate.^{83,141-143}

cDNA-EXPRESSED HUMAN CYP ENZYMES (rhCYP) rhCYPs can be produced e.g. in insect cells and human lymphoblast-derived cells, and in this system all the activity comes from the particular expressed CYP enzyme. In contrast to HLM, the metabolism of the substrate by a specific CYP can be directly evaluated. The variable expression of cytochromes b₅ and/or NADPH-reductase can affect the V_{max} determined for a given enzyme, although the K_m values towards marker substrates are generally comparable between rhCYPs and HLM. When a substrate is metabolised by several CYPs, the K_m observed in HLM differs from that obtained using a single rhCYP. rhCYPs are useful when e.g. trying to differentiate relative importance of different CYPs that metabolise the same compound. Generally, rhCYPs are not suitable for metabolism-dependent

inhibition experiments, if the target of the inhibitory metabolite is other than the enzyme responsible for its formation. Studies with rhCYPs give information only about one CYP at a time, and the properties of e.g. different polymorphic CYPs can be compared.^{83,137,141-143}

PURIFIED RECONSTITUTED CYP ENZYMES A functional CYP can be reconstituted from homogenous purified enzymes by mixing CYP, NADPH-reductase and phospholipids. However, it is often difficult to reconstitute them reproducibly, and the amount of NADPH-reductase is often higher than that present in HLM. Furthermore, not all CYPs are available in purified form.^{83,137,141-143}

HEPATOCTES AND LIVER SLICES Hepatocytes offer some advantages over HLM system and are useful for the study of induction or integrated metabolism, but have several additional problems. For example, the interaction between two specific drugs might be studied successfully in hepatocytes if the mechanism of inhibition is complex. Michaelis-Menten enzyme kinetic equations do not usually apply to hepatocytes, and it is extremely difficult to interpret the obtained data mechanistically. The inhibitor may compete for cellular uptake in addition to metabolic biotransformation, and conjugation of the substrate may complicate the determination of metabolite formation. Isolated hepatocytes are hard to obtain, and repetition of experiments is difficult as CYP expression of hepatocytes is markedly decreased in culture. Liver slices, in addition to having the problems mentioned above, have a barrier to the diffusion of drugs to the cells inside the slice.^{83,137,141-143}

3.2 Enzyme kinetics

Most CYP-mediated reactions follow simple Michaelis-Menten (MM) kinetics. The MM model assumes that the active site of the enzyme contains one binding site, where the catalytic process occurs, and that the velocity of the reaction can be characterised as a hyperbolic saturating profile. The classic enzyme kinetic concept is:¹⁴¹



where E=enzyme, S=substrate, ES=enzyme-substrate complex, and P=product.

The reaction rate (v) depends on the concentration of both unbound enzyme and substrate. When the enzymes are saturated by substrates, the enzyme catalytic rate reaches a maximum (V_{max}). K_m is the substrate concentration where the reaction velocity is 50% of V_{max} .¹⁴¹

$$\text{MM equation: } v = \frac{V_{max} \cdot [S]}{K_m + [S]}$$

Some kinetic observations *in vitro* are less straightforward, with some CYPs (e.g. CYP3A4) exhibiting unusual properties such as substrate activation, substrate inhibition, partial inhibition and differential kinetics. Multiple binding site-models have been proposed to describe these "atypical" enzyme kinetics. For example, the two-site model hypothesises that two (similar or different) substrate molecules can bind simultaneously to the enzyme active site. Due to steric,

allosteric or electronic effects they may interact in a way that leads to altered kinetic properties.¹⁴⁴

3.3 Identification of CYP enzymes responsible for drug metabolism

“Reaction phenotyping” or “enzyme mapping” is the process of identifying CYPs responsible for the metabolism of a given compound. This helps to predict the effect of polymorphisms, environmental factors, and other drugs on the overall metabolism and elimination of the drug of interest.^{83,136,137,142}

There are several available approaches for reaction phenotyping, and a combination of methods is usually needed to identify which human CYPs are responsible for the metabolism of a given drug. The rate of metabolism of the drug can be measured in several HLM samples, and reaction rates correlated with the variation in the CYP levels or activities of the individual samples. The importance of individual CYPs in the metabolism of the drug can be studied using pooled HLM and known chemical inhibitors or inhibitory antibodies of selected CYPs. Marked inhibition of the total metabolism of a drug in the presence of a selective CYP isoform inhibitor implies that this CYP significantly metabolises the drug. Using rhCYPs one can determine whether a particular CYP is able to metabolise the drug, but the importance of this enzyme in the overall metabolism of the drug is difficult to reliably predict from rhCYP data alone due to scaling problems.^{83,136,137,142}

The relative importance of CYPs can vary at different substrate concentrations, as the substrate selectivity of CYPs diminishes with increasing substrate concentration. For example, if *in vitro* metabolism studies are performed at substrate concentrations greatly exceeding the *in vivo* concentrations, the enzymes that appear to be most important in the biotransformation of a drug may not be the same as those responsible for its biotransformation *in vivo*.^{83,136,137,142}

3.4 Characterising the inhibitory properties of drugs

CYP inhibition studies enable the prediction of the effect of the drug of interest on the metabolism and elimination of other drugs. The inhibitory efficiency of a drug can be described by determining IC_{50} to a reaction or K_i value for a specific CYP. IC_{50} value, the inhibitor concentration that causes 50% inhibition of measured activity, is dependent of the experimental conditions (type and concentration of the substrate, protein concentration, incubation time etc.). The K_i value is more laborious to determine, but is more reliable. The K_i is a measure of the affinity of the inhibitor for the enzyme and can be reproduced from one laboratory to another regardless of the precise experimental conditions.^{136,137,140,142,145,146}

A designed pool of HLM is more suitable than random HLM samples to overcome individual variability in the CYP content, and experiments are ideally performed under initial-rate reaction conditions, where the metabolic consumption of the substrate does not exceed 20%. For each CYP model reaction studied, variable amounts of microsomes and incubation times are required to allow sufficient and reliable generation of metabolites. Both excessive binding and metabolism of the

inhibitor may cause limitations to the amount of drug available to inhibit the CYP.^{136,137,140,142}

When determining the IC_{50} values for direct CYP inhibition, various (e.g. four to six) concentrations of the inhibitor are incubated with HLM in the presence of the same concentration of the marker substrate. The data obtained from these samples is used to calculate the IC_{50} value by nonlinear regression. More data is needed for determining the K_i value; this experiment can be thought of as four or five separate sets of IC_{50} assays with a different substrate concentration in each. The type of inhibition can be determined visually from the plotted data, and nonlinear regression can be used to determine the K_i value.^{137,140,143,145,146}

The possibility of metabolism-dependent inhibition (e.g. mechanism-based) must also be studied. For detection of metabolism-dependent inhibition, the inhibitor is preincubated with NADPH in HLM to allow for the generation of possible inhibitory metabolites. After the preincubation, the marker substrate is added, and the incubation continued to measure residual CYP activity. If the preincubation substantially increases the observed inhibition, the inhibitor is at least partially metabolism-dependent.^{137,140,143}

There are several problems when determining the inhibitory characteristics of drugs. Many drugs have poor aqueous solubility at physiological pH, limiting the highest concentration of the drug that can be achieved *in vitro*. Dissolving the drug in an organic solvent (such as methanol, acetonitrile, etc.) or weakly acidic solutions, delivers the drug to the incubation mixture. Organic solvents, however, tend to be also substrates for CYPs, and can potently and selectively inhibit their activities. No organic solvent is optimal for all CYPs and the final concentration should be kept as low as possible.^{138,140,145,146}

The CYP marker reaction studies should be studied under initial-rate conditions, i.e. where the formation of the metabolite is directly proportional to incubation time and protein concentration, and the percentage of substrate metabolism does not exceed 20% (preferably 10%). The effect of substrate overmetabolism is seen as an apparent decrease in the inhibitory capacity of the drug (and higher K_i).¹⁴¹

3.5 *In vitro-in vivo* extrapolation

The use of *in vitro* inhibition results to make *in vivo* interaction predictions is difficult due to the intrinsic differences between the simplified non-physiological *in vitro* models and the dynamic (and only partially understood) drug-eliminating systems of human bodies. The ratio of the plasma concentration of an inhibitor $[I]$ *in vivo* (e.g. C_{max}) to its inhibitory constant (K_i) can be used to predict the probability with which the inhibitor would cause a clinically significant interaction with a substrate for the affected isoenzyme. Consideration should not include only the estimated AUC ratio alone, but include also the margin of safety of possible victim drugs, and their pharmacokinetic profiles. For substrates metabolised via multiple pathways, the inhibition of a minor metabolic pathway (e.g. <30% of total clearance) is not likely to cause a pharmacokinetically significant effect.^{145 136,137,140}

The extent of interaction can be predicted according to the following equation:¹⁶

$$\text{AUC ratio 1} = R1 = 1 + [I]/K_i$$

In this equation it is assumed that the substrate is metabolised 100% by the inhibited CYP. The fraction of substrate metabolised via inhibited pathway (f_m) can be incorporated by use of the following equation:¹⁶

$$\text{AUC ratio 2} = R2 = 1 / [(f_m/R1) + (1-f_m)]$$

Intrinsic clearance (CL_{int}) for rhCYP or HLM can be calculated from $CL_{int} = K_m/V_{max}$. To directly compare rhCYPs reaction data with that obtained using HLM, scaling factors are needed because the enzyme content of the systems is different. One approach is to use the relative activity factor (RAF), describing the amount of rhCYP required for equal reaction velocity in HLM.¹⁴⁷

$$\text{RAF}(V_{max}) = \frac{V_{max}(\text{HLM})}{V_{max}(\text{rhCYP})}, \text{ where } V_{max} \text{ refers to metabolism of a probe substrate}$$

by an individual CYP. However, RAFs are specific for the enzyme and the substrate used, and the use of intersystem extrapolating factors (ISEF), based on data from several reactions and CYP abundances in HLM, has been suggested. ISEF scaling factors are specific for one CYP enzyme, and have been determined using differences in either V_{max} or CL_{int} of various reactions.¹⁴⁷

From the CL_{int} determined in HLM, the human liver CL_{int} can be estimated using scaling factors for the microsome content of human liver (48.8 mg microsomal protein/g liver),¹⁴⁸ and the liver weight for a given body weight (25.7 g liver/kg body weight).¹⁴⁹ From this, the hepatic blood clearance (CL_h) can be estimated from human liver CL_{int} using the well-stirred model:¹⁵⁰

$$CL_h = Q \cdot f_u \cdot CL_{int} / (Q + f_u \cdot CL_{int})$$

where Q = hepatic blood flow (20 mL/min/kg)¹⁵¹

f_u = the unbound fraction of drug in blood

SELECTION OF [I] AND K_i The selection of the most appropriate value of inhibitor concentration used in *in vitro-in vivo* interaction predictions can be difficult. The most conservative choice is the peak plasma concentration, but presumably the unbound concentration in plasma is closer to the intracellular drug concentration. Estimates have been made to determine the most accurate way to calculate $[I]/K_i$ ratio, but there is no clearly established method to directly predict clinical drug interactions on the basis of *in vitro* CYP inhibition data.^{136,137}

Though more reliable than the IC_{50} value, the experimental K_i may also vary depending upon the *in vitro* approach used. Microsomal protein concentration, and nonspecific binding of substrate to microsomes are determinants of the apparent inhibitory potency of CYP inhibitors.¹³⁹ Relative activity factors and intersystem extrapolation factors have been recommended for application of K_i values derived from rhCYP systems.¹⁴⁷ Failure to account for atypical kinetics in *in vitro* experiments that are not described by Michaelis-Menten equation can also give rise to errant values of K_i .¹⁴⁴

4 Repaglinide in the treatment of type 2 diabetes

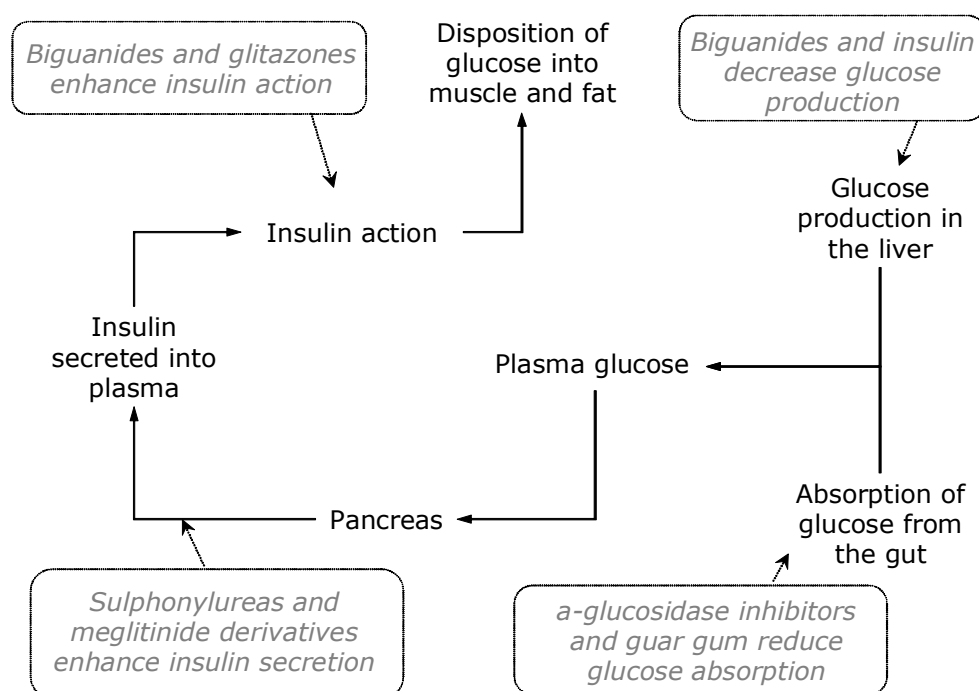
4.1 Treatment principles of type 2 diabetes mellitus

Diabetes mellitus (DM) is a disorder of carbohydrate metabolism characterised by elevated blood glucose, where the action of insulin is reduced because of impaired secretion or decreased insulin sensitivity. Complications such as retinopathy, and neuropathy, cardiovascular disease and foot ulceration follow the progress of the disease. Type 1 DM usually develops before adulthood, and the patients require exogenous insulin therapy to live. Type 2 DM typically develops later in life and in this condition normal (or even excessive) insulin secretion is insufficient to compensate for the developing insulin resistance.¹⁵²⁻¹⁵⁴

Type 2 DM is a progressive disease where the symptomatic phase is preceded by a preliminary phase of impaired glucose tolerance. Type 2 DM is associated with obesity, and treatment of the obese patient should be started with changes in diet, increased exercise and weight reduction. Management of the disease usually requires stepwise adjustment of drug therapy in addition to lifestyle changes. If treatment with one oral antidiabetic fails, then a different type of drug, or a combination of different agents with complementary modes of action may improve the situation, but patients may ultimately need insulin injections as the disease advances. Tight glycemic control in type 2 DM is important in the prevention complications, but the management of dyslipidaemia (with e.g. a statin or fibrate) is also crucial for hyperlipidaemic diabetic patients.¹⁵²⁻¹⁵⁴

The two major classes of oral antidiabetic drugs are sulphonylureas and biguanides. Sulphonylureas act mainly by increasing endogenous insulin secretion, while biguanides act by decreasing hepatic glucose production and increasing peripheral consumption of glucose.^{37,152,153,155} They both require at least some endogenous insulin production for their function, and are often used together. More recently developed classes of oral antidiabetics include the glitazones (thiazolidinediones), which act by increasing insulin sensitivity by altering glucose metabolism,^{152,156} and meglitinide analogs (e.g. repaglinide). Meglitinides have a mechanism of action similar to that of sulphonylureas, but they are more rapidly absorbed and eliminated. They are taken with meals, and due to their shorter duration of action, the risk of hypoglycaemia is smaller compared to sulphonylureas.^{152,157} Other alternatives include alpha-glucosidase inhibitors and guar gum, which delay and decrease glucose absorption from the gut (Figure 2).^{152,153}

Figure 2. The mechanism of action of different oral antidiabetic drugs (adapted from Scheen et al).³⁷



4.2 Repaglinide

4.2.1 Pharmacokinetics of repaglinide

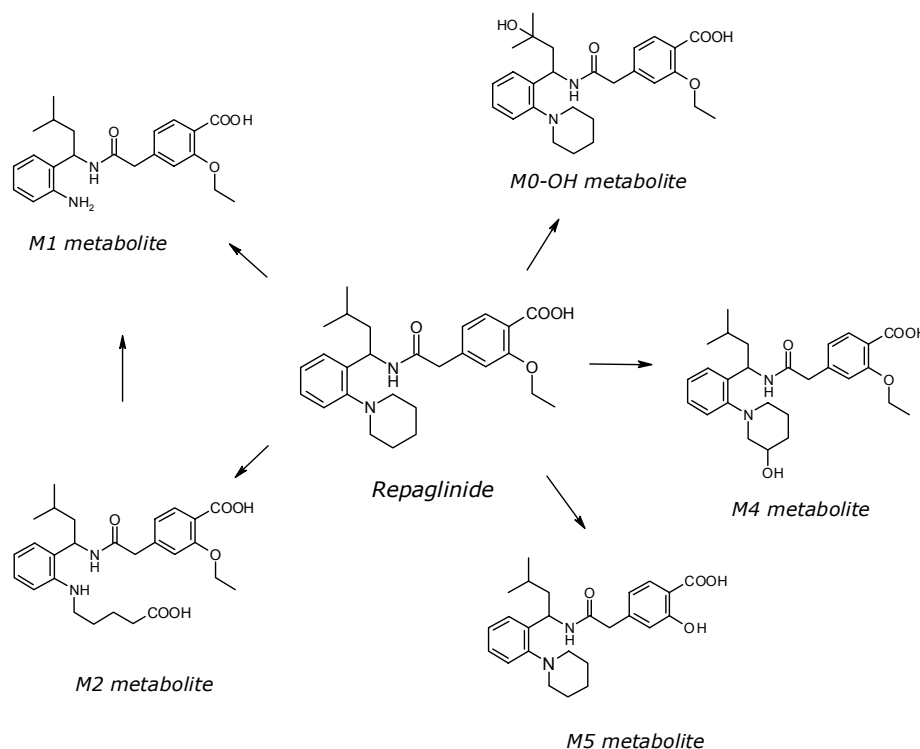
After oral administration, repaglinide is rapidly absorbed, and C_{max} is reached in approximately 1 h.^{158,159} Oral bioavailability of repaglinide is about 60% (range 56 to 63%) due to considerable first-pass metabolism.^{2,160} The elimination of repaglinide is also rapid, its terminal elimination $t_{1/2}$ is about 1 h.^{2,159} C_{max} and AUC of repaglinide increase dose-dependently in patients with type 2 DM receiving doses ranging from 0.125 to 16 mg, but t_{max} remains similar irrespective of the dose.² The interindividual variation in repaglinide AUC is 30 to 70%, whereas intraindividual variation is lower.² Repaglinide is highly bound to plasma proteins (>98%), mainly to albumin.¹⁶¹

Repaglinide is extensively metabolised, and only <2% of the dose is excreted unchanged in humans.⁵ The metabolism of repaglinide produces inactive metabolites, which are excreted mainly into bile (about 90% of the dose) and to a lesser extent into urine (about 8%).⁵ In humans, the main metabolites of repaglinide are M2 (about 68% of the dose), M1 (about 4%) and M4, while other metabolites are produced only in very small amounts.⁵ The most important enzymes participating in the biotransformation of repaglinide *in vitro* are CYP2C8 and CYP3A4. *In vitro*, both CYP2C8 and CYP3A4 have been shown to catalyse the biotransformation of repaglinide to its major *in vivo* metabolites, with M1 and M2 mainly formed by CYP3A4 and M4 mainly by CYP2C8.⁷ Furthermore, repaglinide is not a substrate for P-glycoprotein, according to an unpublished study conducted by Novo Nordisk A/S.¹⁶²

In vivo (in rat, mouse, rabbit, dog, monkey, and humans), numerous repaglinide metabolites have been identified: repaglinide can be metabolised by formation of

an aromatic amine (M1), opening of the piperidine ring to form a dicarboxylic acid (M2), hydroxylation of the piperidine ring (M4), de-ethylation (M5), formation of a tauride (M6), formation of an acylglucuronide (M7), and by N-oxidation (M12).¹⁶² M1 can be formed directly from repaglinide, or by oxidative N-dealkylation from M2.¹⁶² *In vitro*, repaglinide (M0) can also be metabolised to M0-OH (Figure 3).⁷

Figure 3. The main biotransformation pathways of repaglinide (22 μ M) *in vitro*, as described by Bidstrup *et al.*⁷



Age has not significantly influenced repaglinide pharmacokinetics, but compared to healthy elderly volunteers, elderly type 2 diabetics have had higher repaglinide AUC and C_{max} values. Compared to volunteers with normal hepatic function, the AUC, C_{max} and $t_{1/2}$ of repaglinide have been significantly higher in those with liver disease. Mild to moderate renal impairment has not significantly increased repaglinide AUC in diabetic patients or in otherwise healthy volunteers, but severe renal impairment has caused significant increases in repaglinide AUC.²

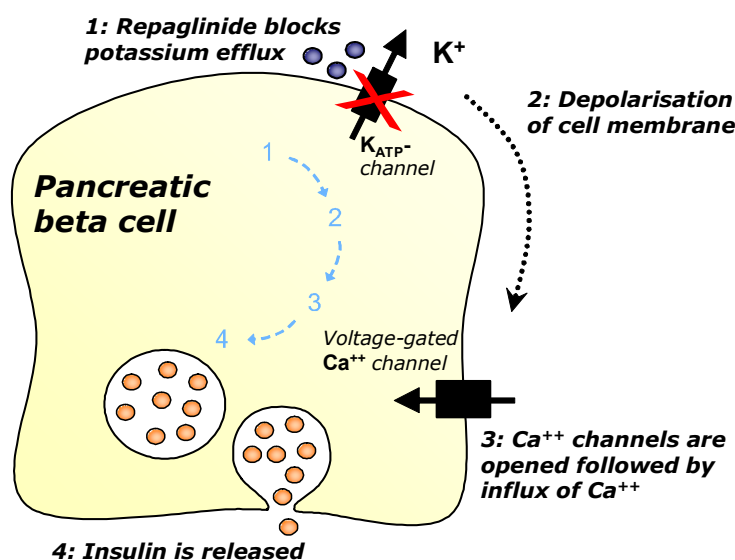
Polymorphism in the gene encoding CYP2C8 has been associated with altered repaglinide pharmacokinetics. Surprisingly, repaglinide (0.25 mg dose) mean AUC was 30-50% lower in subjects with the *CYP2C8**1/*3 genotype (n=10) compared with those with the *CYP2C8**1/*1 genotype (n=41),^{163,164} although in another study where a 2 mg repaglinide dose was used no association was found (*CYP2C8**1/*3 n=11; *1/*1 n=24).¹⁶⁵ Incidentally, there is a strong association between the major variant alleles of CYP2C8 and CYP2C9 (*CYP2C8**3 and *CYP2C9**2).¹⁶⁶

4.2.2 Pharmacodynamics of repaglinide

The pharmacodynamic mechanism of action of repaglinide is to increase insulin secretion from pancreatic β cells. Repaglinide binds to (and closes) the ATP-sensitive K^+ channels on the plasma membrane of β cells, causing depolarisation of the cell without entering it. Depolarisation opens voltage-gated Ca^{++} -channels, leading to influx of Ca^{++} ions and triggering the release of insulin by exocytosis (Figure 4). Sulphonylureas and repaglinide both act by closing the same ion channels, but their binding sites within the protein are different.^{2,167}

Repaglinide does not stimulate insulin release in the absence of glucose, and its effect is dependent on the β cell secretory capacity. The peak serum insulin concentrations are reached in 2 to 2.5 hours and maximum hypoglycaemic activity in 3 to 3.5 hours, following administration of a single oral dose of repaglinide.²

Figure 4. The pharmacodynamic mechanism of repaglinide.



4.2.3 Therapeutic use of repaglinide

Repaglinide is aimed to be used in patients with type 2 DM, whose blood glucose is not adequately controlled by diet and exercise alone. Repaglinide dosage is determined individually, and dose titration requires monitoring of fasting and/or postprandial blood glucose levels. Repaglinide is administered before each main meal, and the recommended starting dose is 0.5 mg per meal. A higher starting dose (1 or 2 mg) can be used in patients with HbA_{1C} levels over 8%. The maximum recommended single dose is 4 mg, and the total daily dose should not exceed 16 mg.^{1,2}

Placebo-controlled studies have demonstrated the antidiabetic efficacy of repaglinide monotherapy, and improvements have been shown in all indicators of blood glucose control.¹⁶⁸⁻¹⁷¹ Compared to other oral antidiabetic drugs, the efficacy of repaglinide has been equal to that of metformin^{170,172} and glibenclamide.¹⁷³ Combination therapy with repaglinide and metformin,¹⁷² pioglitazone,²⁰ or rosiglitazone³ has been shown to be more effective than the respective monotherapies. Results from one cohort study showed that switching

to repaglinide from other oral hypoglycaemic agents improved patient eating patterns, as the diminished fear of hypoglycaemia reduced snacking between meals.¹⁷⁴ However, the frequent dosing regimen of repaglinide can also be viewed as a disadvantage, and its effectiveness in reducing long-term vascular complications of type 2 DM has not been established.

4.2.4 Adverse effects of repaglinide

Adverse events occurring during repaglinide therapy, most commonly hypoglycaemia, are similar to those caused by sulphonylurea treatment, and in general mild to moderate in intensity. The incidence of hypoglycaemia has been significantly higher in those receiving repaglinide than placebo, but similar for repaglinide and sulphonylurea recipients. Serious and nocturnal hypoglycaemias have occurred more often with sulphonylureas than with repaglinide, but when repaglinide is used in combination with metformin, glitazone or bed-time insulin the risk of hypoglycaemia is increased compared to repaglinide monotherapy. Upper respiratory tract infections have been reported to be the second most common adverse effect of repaglinide treatment. Furthermore, headache, gastrointestinal effects, and musculoskeletal pain have been described in repaglinide users, but they have occurred with a similar incidence also in placebo recipients.¹

4.2.5 Drug interactions of repaglinide

The effect of CYP inhibition and induction on the pharmacokinetics of repaglinide has been studied in several cross-over studies in healthy volunteers. The CYP3A4 inhibitors clarithromycin, itraconazole, and ketoconazole have all been shown to moderately increase repaglinide plasma concentrations.^{8,9,12} Clarithromycin and itraconazole increased repaglinide AUC by about 40%,^{8,9} ketoconazole by 15%.¹² Gemfibrozil, a CYP2C8 inhibitor, has increased repaglinide AUC 8-fold, considerably prolonging and enhancing the blood glucose-lowering effect of repaglinide.⁹ Combination of gemfibrozil and itraconazole has caused a nearly 20-fold increase in repaglinide AUC, and further enhanced the blood glucose-lowering effect of repaglinide.⁹ In patients taking both gemfibrozil and repaglinide, events of serious hypoglycaemia have been reported, and their concomitant use has been contraindicated by EMEA.¹⁷⁵

The CYP3A4 inducer rifampicin has decreased repaglinide plasma concentrations, and similar effects can be expected with other inducers of CYP3A4. In one study, where repaglinide was administered 12h after the last rifampicin pre-treatment dose, repaglinide AUC was reduced by 57%,¹⁰ whereas in another study where repaglinide was administered simultaneously with the last rifampicin dose a 31% reduction of repaglinide AUC was seen.¹² Possible drug interactions between repaglinide and CYP inhibitor cimetidine, CYP3A4 substrates ethinylestradiol (also a CYP2C9 and CYP2C19 inhibitor),¹⁷⁶ simvastatin and nifedipine, and drugs with narrow therapeutic margin (digoxin, theophylline or warfarin) have also been investigated, but no significant pharmacokinetic changes were observed.^{12,177}

5 Drugs studied with repaglinide *in vivo*

5.1 Bezafibrate and fenofibrate (Study II)

Fibrates (bezafibrate, fenofibrate and gemfibrozil) are agonists of the nuclear transcription factor PPAR- α , expressed in metabolically active tissues (e.g. liver, kidney, heart). They alter the regulation of lipid metabolism, leading to increased fatty acid intake into cells and their subsequent oxidation. Fibrates reduce plasma triglyceride levels by about 25-50%, LDL-levels only slightly, and usually raise HDL-levels by about 10-15%.¹⁷⁸

Fibrates are used primarily in the treatment of hypertriglyceridemia, and as second-line agents in hypercholesterolic patients intolerant of statins. Bezafibrate is given as a single daily dose of 400 mg, or 200 mg 2-3 times daily, depending on the tablet properties. Fenofibrate is given as a single daily dose, or divided doses, depending on the formulation. Dose is adjusted according to response, usually between 200 and 400 mg daily (Table IV).¹⁷⁸

The most common adverse reactions of bezafibrate and fenofibrate are gastrointestinal disturbances such as anorexia, nausea and gastric discomfort. Other adverse effects reported to occur less frequently include headache, dizziness, vertigo, fatigue, skin rashes, and pruritus. Bezafibrate and fenofibrate may enhance the effects of oral anticoagulants and the dose of anticoagulants should be reduced when treatment with a fibrate is started.¹⁷⁸

5.2 Trimethoprim (Study III)

Trimethoprim is a dihydrofolate reductase inhibitor, it inhibits the synthesis of bacterial DNA, and acts in the same bacterial metabolic pathway as the sulphonamides. Trimethoprim is active against a wide range of Gram-negative and Gram-positive aerobes, as well as some protozoa, but anaerobic species are usually resistant.¹⁷⁹

Trimethoprim is primarily used for the treatment and prevention of urinary-tract infections, but it is used also in gastroenteritis and respiratory tract infections. Trimethoprim can be combined with sulphonamides, most commonly with sulfamethoxazole. The usual adult dose of trimethoprim in acute infection is 100-200 mg twice daily orally, or 200-300mg once daily (Table IV). Trimethoprim is well tolerated in general; the most often reported adverse effects are pruritus, skin rash, and mild gastrointestinal disturbances.¹⁸⁰ Trimethoprim may increase the concentrations and potentiate the effects of several drugs, e.g. phenytoin, digoxin and procainamide.¹⁸¹ Trimethoprim is a selective inhibitor of CYP2C8 *in vitro*,¹⁷ and has been shown to increase rosiglitazone concentrations *in vivo*.¹⁸²

5.3 Cyclosporine (Study IV)

Cyclosporine is a powerful immunosuppressant, which acts mainly on lymphocytes (helper T-cells). By inhibiting the activation of calcineurin, cyclosporine decreases the production of lymphokines and depresses the cell-mediated immune response. Cyclosporine has been the cornerstone of organ and tissue transplantation, where it is used in the prevention and management of graft rejection. Corticosteroids and/or other immunosuppressants are usually used

with cyclosporine. It is also used in severe forms of atopic dermatitis, psoriasis, and rheumatoid arthritis.¹⁸³

In organ transplantation, the usual initial dose is 10 to 15 mg/kg daily, and in psoriasis, atopic dermatitis, or rheumatoid arthritis approximately 2.5 mg/kg daily, reduced gradually to the lowest effective maintenance dose (Table IV). Monitoring of cyclosporine concentrations is usually used to guide its dosing, particularly when higher doses are used, and monitoring of renal and hepatic function, blood pressure and serum electrolytes is also required.¹⁸⁴

Nephrotoxicity is the major adverse effect of cyclosporine treatment, occurring in about 1/3 of the patients during long-term treatment. Other frequent adverse effects include hypertension, gastrointestinal disturbances, hepatotoxicity, headaches, muscle cramps and gingival hyperplasia. There is an increased incidence of malignancies and predisposition to infections in patients receiving long-term cyclosporine therapy.^{185,186} Cyclosporine is a CYP3A4 substrate, and cyclosporine concentrations may be affected by inducers or inhibitors of CYP3A4. *In vitro*, cyclosporine potently inhibits the transporter proteins P-glycoprotein and OATP1B1, and it moderately inhibits CYP3A4.^{22,187} *In vivo*, cyclosporine has raised the plasma concentrations of several statins, at least partially by inhibiting their OATP1B1-mediated hepatic uptake.¹⁸⁸

5.4 Telithromycin (Study V)

Telithromycin is a bactericidal ketolide antimicrobial agent. It is highly active against Gram-positive bacteria such as streptococci, including *S. pneumoniae* and *S. pyogenes*, and also shows good activity against Gram-negative organisms *H. influenzae* and *M. catarrhalis*.¹⁸⁹ Telithromycin is used for the treatment of respiratory tract infections, given orally in a usual dose of 800 mg once daily (Table IV). The most common adverse effects of telithromycin are diarrhoea and other gastrointestinal disturbances. Elevation of liver enzymes and cholestatic jaundice has also occurred during telithromycin treatment, and other effects less commonly reported include dizziness, headache, and drowsiness.^{190,191} Telithromycin inhibits CYP3A4 and CYP2D6 *in vitro*, and it can considerably raise the plasma concentrations of CYP3A4 substrates also *in vivo*. For example, the AUC of simvastatin is raised about 10-fold by telithromycin.¹⁹²

5.5 Montelukast (Study V)

In the pathophysiology of asthma, cysteinyl leukotrienes released from mast cells and eosinophils cause airway oedema, smooth muscle contraction, and alter inflammatory processes. By binding to the cysteinyl leukotriene receptor, montelukast inhibits the actions of cysteinyl leukotrienes, and helps to relieve the symptoms of asthma.¹⁹³ Montelukast is used in the management of chronic asthma, usually in addition to inhaled corticosteroids, and seasonal allergic rhinitis, given in daily doses of 10 mg (Table IV). Montelukast has no use in the treatment of acute asthma attacks. Headaches, increased incidence of respiratory tract infections and GI disturbances have been reported with montelukast. Other adverse effects include myalgia, fever, dizziness, elevations in liver enzymes and hypersensitivity reactions.¹⁹⁴⁻¹⁹⁶ Phenobarbital has decreased the AUC of single-dose montelukast about 40%, but montelukast has not altered the pharmacokinetics of coadministered warfarin or digoxin.^{197,198}

Montelukast is a potent and selective inhibitor of CYP2C8 *in vitro*, and depending on microsomal protein concentration its K_i for CYP2C8 has ranged from 0.0092 to 0.15 μM .¹⁶

5.6 Pioglitazone (Study VI)

The antidiabetic pioglitazone is an insulin-sensitising agent that acts by activating the nuclear receptor α PPAR- γ , and gradually decreases insulin resistance in muscle, adipose tissue and liver by altering glucose metabolism. Pioglitazone has also been found to reduce triglyceride levels and increase HDL concentrations.¹⁹⁹ In patients with type 2 diabetes, pioglitazone is given orally as monotherapy, or combined to metformin, sulphonylurea, or a meglitinide, when monotherapy is inadequate. The usual dose is 15 or 30mg once daily, increased to a maximum of 45mg if necessary (Table IV).²⁰⁰ Pioglitazone may cause hypoglycaemia, headache, weight gain, and anemia; other reported adverse effects include upper respiratory tract infections, gastrointestinal disturbances, dizziness, and pruritus. Pioglitazone can also cause oedema, which can precipitate or worsen cardiac failure.²⁰⁰ *In vivo*, concomitant rifampicin has decreased pioglitazone AUC by 54%,²⁰¹ whereas gemfibrozil has increased pioglitazone AUC 3.2-fold.²⁰² *In vitro*, pioglitazone has been reported to inhibit both CYP2C8 and CYP3A4 enzymes.¹⁹ Based on these *in vitro* findings and usual plasma concentrations of pioglitazone, it has been estimated that pioglitazone would have the potential to inhibit CYP2C8 also *in vivo*.¹⁹

Table IV. The pharmacokinetic characteristics of drugs studied with repaglinide in vivo.

<i>Drug</i>	<i>Oral bioavailability (t_{max})</i>	<i>Plasma protein binding</i>	<i>Plasma elimination $t_{1/2}$</i>	<i>Excretion</i>	<i>Remarks</i>
Bezafibrate ^{178,203}	100% (1 to 2 h)	95%	1.5 to 3 h	Mainly in urine, about 50% unchanged, 50% as metabolites; a fraction in faeces	
Fenofibrate ^{178,204}	60% (-)	>99%	19 to 27 h	Mainly in urine as glucuronide conjugates	Rapidly metabolised to the active compound fenofibric acid
Trimethoprim ¹⁸⁰	>90% (1 to 4 h)	45%	8 to 10 h	Mainly in urine, 40 to 60% unchanged	
Cyclosporine ¹⁸³ (microemulsion)	40% (1½ to 2 h)	90%	5 to 20 h	Mainly via bile into faeces as metabolites, 6% in urine	In blood, about 50% is in erythrocytes, about 15% in leukocytes, remainder in plasma
Telithromycin ¹⁹²	60% (1 to 3 h)	65%	8 to 13 h	Via bile into faeces and in urine, about 2/3 as metabolites and 1/3 unchanged	
Montelukast ²⁰⁵	64% (2 to 4 h)	>99%	5 to 7 h	Via bile into faeces as metabolites	
Pioglitazone ^{200,206}	>80% (1 to 2 h)	>99%	4 to 9 h	Via bile into faeces and in urine as metabolites	The $t_{1/2}$ of pioglitazone metabolites is up to 24 h

t_{max} , time to peak concentration; $t_{1/2}$, half-life.

AIMS OF THE STUDY

The aims of this thesis work were to investigate the relative contributions of CYP2C8 and CYP3A4 in the metabolism of repaglinide, potential drug interactions of repaglinide, and their mechanisms.

Specific aims of the studies were:

- I** To investigate the relative contributions of CYP2C8 and CYP3A4 in the metabolism of repaglinide, and to study the effects of bezafibrate, fenofibrate, gemfibrozil, and rifampicin on the metabolism of repaglinide and CYP2C8 and CYP3A4 activities *in vitro*.
- II** To investigate the effects of bezafibrate and fenofibrate on the pharmacokinetics and pharmacodynamics of repaglinide *in vivo*, in order to compare their interaction potential with repaglinide to that of gemfibrozil.
- III** To investigate the effects of trimethoprim, a selective CYP2C8 inhibitor on the pharmacokinetics and pharmacodynamics of repaglinide *in vivo*, and the effects of trimethoprim on the metabolism of repaglinide by HLM *in vitro*.
- IV** To investigate the effects of cyclosporine, an inhibitor of CYP3A4 and OATP1B1, on the pharmacokinetics and pharmacodynamics of repaglinide *in vivo*, to determine the effect of cyclosporine on the metabolism of repaglinide *in vitro*, and to study the effect of genetic variation (selected CYP enzymes and drug transporters) on the extent of possible interaction.
- V** To investigate the effects of telithromycin (CYP3A4 inhibitor *in vivo* and *in vitro*), montelukast (potent CYP2C8 inhibitor *in vitro*), and their combination on the pharmacokinetics, pharmacodynamics and metabolic fate of repaglinide *in vivo*.
- VI** To investigate the effects of pioglitazone (CYP2C8 and CYP3A4 inhibitor *in vitro*) on the pharmacokinetics and pharmacodynamics of repaglinide *in vivo*, in order to clarify whether their synergistic effect in diabetic patients is caused by inhibition of repaglinide metabolism by pioglitazone.

MATERIALS AND METHODS

1 *In vitro* studies

Using human liver microsomes and recombinant CYP enzymes, the details of repaglinide metabolism, and the effects of potential metabolic inhibitors on repaglinide metabolism and CYP marker reactions were studied *in vitro*.

In study I, the relative contributions of CYP2C8 and CYP3A4 to repaglinide metabolism, and the effect of fibrates and rifampicin on repaglinide metabolism and CYP2C8 and CYP3A4 activities were investigated. The effect of trimethoprim on repaglinide metabolism was determined in study III. In study IV, the effect of cyclosporine on the metabolism of repaglinide to M1, M2, and M4, as well as the contributions of CYP2C8 and CYP3A4 to the formation of these metabolites was investigated.

1.1 Design of *in vitro* studies

The *in vitro* incubations were conducted in duplicate in a shaking water bath at 37 °C, and the incubation times and microsomal protein concentrations (HLM and rhCYP) were within the linear range for reaction velocity. In inhibition studies, preincubation assays were conducted to assess possible metabolism-dependent inhibition. Intra-assay coefficient of variation (CV) was less than 15% for the duplicates, and mean values were used in the calculations. The incubations were carried out in 0.1 M sodium phosphate buffer (pH 7.4), containing 5.0 mM MgCl₂. The stock solutions of all the drugs were prepared in methanol (final concentration 1% v/v in the incubation mixture). The drug(s), buffer, and microsomes were premixed and incubations were commenced by the addition of β-NADPH (final concentration 1.0 mM), and quenched depending on the substrate used. The microsomes were vortexed each time before dilution, and the incubation mixtures were vortexed at the start and end of each incubation. Preliminary assays were conducted to determine appropriate microsomal protein concentrations, and rhCYP concentrations with corresponding reaction velocities.

1.1.1 HLM and rhCYPs

The pooled human liver microsomes (HLM) were purchased from Gentest Corp (Woburn, MA, USA). Human liver tissue had been collected in the USA in accordance with all pertinent regulations, and permissions from the donors' families had been obtained prior to organ collection. The procedures of organ collection had been accepted by the respective institutional Human Subjects Committee, and liver samples were mostly from Caucasian subjects. Recombinant human CYP2C8 and CYP3A4 (Supersomes™) from baculovirus-infected insect cells were purchased from Gentest. HLM and recombinant CYPs were kept at -70°C until use, thawed in a water bath and placed on ice, and diluted as appropriate before addition into the incubation mixture. Incubation mixtures and diluted mixtures were kept on ice until the incubations were started. The HLM system was used in studies I, III, and IV, the rhCYP system was used in studies I and IV.

1.1.2 Metabolism of repaglinide by HLM, CYP2C8 and CYP3A4

REPAGLINIDE DEPLETION (I) To determine the apparent kinetic constants (K_m and V_{max}), the metabolism of repaglinide was studied in HLM and rhCYPs using the substrate depletion method. Varying concentrations of repaglinide were incubated with HLM (0-150 μ M), rhCYP2C8 (0-50 μ M) and rhCYP3A4 (0-80 μ M), and the amount of unchanged repaglinide was measured from samples taken 0, 10, 20 and 30 min from the start of the incubation.

FORMATION OF REPAGLINIDE METABOLITES M1, M2, AND M4 (IV) To determine the contribution of CYP2C8 and CYP3A4 to the formation of repaglinide metabolites M1, M2, and M4, repaglinide (2 μ M) was incubated with rhCYP2C8 and rhCYP3A4. Reaction velocity was determined from the amount of metabolite formed.

1.1.3 Inhibition of repaglinide metabolism, CYP2C8 and CYP3A4

EFFECT OF CYP2C8 AND CYP3A4 INHIBITION ON REPAGLINIDE METABOLISM IN HLM (I) This assay was designed to determine the effect of inhibition of CYP2C8, CYP3A4 and both CYP2C8 and CYP3A4 to the metabolism of repaglinide in HLM. Quercetin (25 μ M, CYP2C8 inhibitor), itraconazole (3 μ M, CYP3A4 inhibitor), their combination and vehicle only were incubated with repaglinide (0.2 and 2 μ M). These inhibitor concentrations were estimated to cause at least 80% inhibition of respective enzyme activities.^{101,207,208} Substrate depletion method was used, as described above, and inhibition described as percentage of control activity.

EFFECT OF FIBRATES AND RIFAMPICIN ON REPAGLINIDE METABOLISM IN HLM (I) Repaglinide (2 μ M) was incubated with bezafibrate (0-80 μ M), fenofibrate (0-200 μ M), gemfibrozil (0-200 μ M) and rifampicin (0-120 μ M) to determine their respective effects on the metabolism of repaglinide in HLM. Substrate depletion method was used, as described above, and inhibition described by IC_{50} values.

EFFECT OF FIBRATES AND RIFAMPICIN ON CYP2C8 AND CYP3A4 IN HLM (I) The effect bezafibrate (0-60 μ M), fenofibrate (0-150 μ M), gemfibrozil (0-160 μ M) and rifampicin (0-260 μ M) on the marker reactions of CYP2C8 (paclitaxel 6 α -hydroxylation) and CYP3A4 (midazolam 1 α -hydroxylation) were investigated in HLM. Reaction velocities were determined from the amount of metabolite formed, and K_i values were determined when inhibition was observed.

EFFECT OF TRIMETHOPRIM ON REPAGLINIDE METABOLISM IN HLM (III) The effect of trimethoprim (0-200 μ M) on the metabolism of repaglinide (0.22 μ M) was studied in HLM, and IC_{50} value was determined. Substrate depletion method was used as described above, but samples were taken at 0, 15, and 30 min from the start of the incubation.

EFFECT OF CYCLOSPORINE ON REPAGLINIDE METABOLISM IN HLM (IV) The effect of cyclosporine (0-30 μ M) on the formation of M1, M2, and M4 metabolites of repaglinide (2 μ M) was studied in HLM. Reaction velocity was determined from the amount of metabolite formed, and IC_{50} values were determined when inhibition was observed.

1.2 Determination of drug concentrations

REPAGLINIDE The concentrations of repaglinide (and its metabolites in IV) were measured using either high-performance liquid chromatography with ultraviolet detection (HPLC-UV), or liquid chromatography-tandem mass spectrometry (LC-MS-MS) (in the low-concentration kinetic studies and the inhibition studies with rifampicin, quercetin, and itraconazole). Repaglinide metabolite concentrations were given in arbitrary units (units per mL) relative to the ratio of the peak height of each metabolite to that of the internal standard in the chromatogram.

In the HPLC analysis, ibuprofen served as internal standard, and quantification was performed with UV-detection at 243 nm. The limit of quantification for repaglinide was 0.1 μM , and the day-to-day CVs were <6% at relevant concentrations. In the LC-MS-MS analysis, PE SCIEX API 3000 liquid chromatography-tandem mass spectrometry system (Sciex Division of MDS Inc, Toronto, Ontario, Canada) was used and nateglinide served as internal standard. The mass spectrometer was operated in the turbo ion spray mode with positive ion detection and the ion transitions monitored were those representing the product ions of the $[\text{M}+\text{H}]^+$ ions. The limit of quantification for repaglinide was 5 nM and the CVs were <10% at relevant concentrations.

1-OH MIDAZOLAM To measure 1-OH midazolam concentrations, HPLC-analysis with UV-detection at 245 nm was used.²⁰⁹ The limit of quantification for 1-OH midazolam was 10 nM, and the CVs were <8% at relevant concentrations.

6-OH PACLITAXEL 6-OH Paclitaxel concentrations were measured with HPLC-UV²¹⁰ or LC-MS-MS²¹¹ (the inhibition study with rifampicin). In the HPLC analysis, UV-detection at 230 nm was used and the limit of quantification for 6-OH paclitaxel was 50 nM; the CVs were <7% at relevant concentrations. In the LC-MS-MS analysis, PE SCIEX API 2000 liquid chromatography tandem mass spectrometry system (Sciex Division of MDS Inc) was used and docetaxel served as internal standard. The limit of quantification for 6-OH paclitaxel was 25 nM, and the CVs were <8% at relevant concentrations.

1.3 Data analysis

SUBSTRATE DEPLETION METHOD For each substrate concentration (and inhibitor concentration, when applicable), pseudo-first order rate constants (k) for repaglinide metabolism were determined from the concentration-time points. k was calculated by linear regression from the natural logarithm of repaglinide concentration versus incubation time using the program MK-model (version 5.0, Biosoft, Cambridge, UK). The initial reaction velocity (v_0) was calculated by the equation $v_0 = C_0 \cdot k$, where C_0 is the initial concentration of repaglinide.

Reaction velocities were expressed as pmol/min/mg protein or pmol/min/pmol CYP. IC_{50} , K_m and V_{max} values were determined from reaction velocity data by nonlinear regression analysis using the program Fig.P (version 6.0, Biosoft, Cambridge, UK); simple Michaelis-Menten kinetic model was the best to fit the data.

METABOLITE FORMATION METHOD Reaction velocities were calculated for the CYP2C8 and CYP3A4 marker reactions (I) and formation of repaglinide metabolites (IV). The K_i values were determined by nonlinear regression analysis

using the program SigmaPlot (version 8.0, SPSS Inc., Chicago, IL, USA). Lineweaver-Burk and Dixon plots of the data were used to determine the type of inhibition; the model for competitive inhibition was the best to fit the data. IC₅₀ values were determined by nonlinear regression analysis using the program Fig.P.

CALCULATIONS Intrinsic clearance (CL_{int}) values for the metabolism of repaglinide by rhCYPs and HLM were calculated by the equation $CL_{int} = K_m / V_{max}$. The relative activity factor (RAF, describing the amount of rhCYP required for equal reaction velocity per mg of HLM) was calculated for CYP2C8 and CYP3A4 using activity data provided by Gentest.

The relative contributions of CYP2C8 and CYP3A4 in the *in vivo* hepatic metabolism of repaglinide were estimated by multiplying recombinant enzyme CL_{int} values with the respective RAF. The relative contributions of CYP2C8 and CYP3A4 in the hepatic metabolism of repaglinide were also estimated from the CL_{int} values using CYP enzyme abundances in HLM and intersystem extrapolating factors (ISEF) for rhCYPs and HLM.¹⁴⁷ The CL_{int} determined in HLM was expressed per kilogram body weight using standard scaling factors.^{148,149} Hepatic blood clearance (CL_h) value was estimated using the well stirred model: $CL_h = Q \cdot f_u \cdot CL_{int} / (Q \cdot f_u \cdot CL_{int})^{150}$ where Q is the hepatic blood flow and f_u is the unbound fraction (0.015) of repaglinide in blood.¹⁶¹

2 *In vivo* studies

A total of five *in vivo* studies were conducted in healthy human volunteers to investigate the effects of bezafibrate, fenofibrate, trimethoprim, cyclosporine, telithromycin, montelukast, and pioglitazone on the pharmacokinetics and pharmacodynamics of repaglinide.

The studies were carried out in the facilities of the Department of Clinical Pharmacology, University of Helsinki. The study protocols were approved by the Ethics Committee for Studies in Healthy Subjects and Primary Care (Hospital District of Helsinki and Uusimaa) and by the Finnish National Agency for Medicines. Before entering the studies the volunteers had given their written informed consent, and were ascertained to be healthy by medical history, physical examination, and routine laboratory tests.

2.1 Design of *in vivo* studies

The studies were randomised, placebo-controlled, cross-over studies, which consisted of 2 to 4 phases (Figure 5). In each phase, a pre-treatment period with the study drug or placebo was followed by the study day, when repaglinide was ingested. Following an overnight fast, 0.25 mg repaglinide was ingested with 150 mL water at about 9.00, and the volunteers spent the next 3 h seated. During the study days, timed blood samples were taken for monitoring of blood glucose levels and for determination of drug concentrations. In studies IV and V, urine was collected cumulatively for 12 h after repaglinide administration for the quantification of repaglinide and metabolite excretion. During the study days, the volunteers were under direct medical supervision, and food intake was strictly controlled. Between the phases was a 2 or 4 week wash-out period.

The pre-treatment medications (Table V) and matched placebos were supplied, packed, and labelled according to a randomisation list for each subject by the Pharmacy of the Helsinki University Central Hospital, which supplied also repaglinide used in studies.

2.2 Subjects

57 subjects (50 male, 7 female) healthy volunteers participated in the studies (Table VI). None of the subjects used continuous medication (not even oral contraceptives), and use of grapefruit juice or any pharmaceuticals was not allowed for 2 weeks before the study. Participation in other studies and donation of blood were not allowed for one month before and after the study. The main exclusion criteria for the studies were: a history of intolerance to the study drugs, concomitant drug therapy of any kind, an existing disease of possible significance, age <18 or >45 years, a history of haematological, endocrine, metabolic, or gastrointestinal disease, pregnancy or nursing.

2.3 Blood sampling

On the days of repaglinide administration, timed blood samples (10 mL each) were drawn from a cannulated forearm vein and collected into tubes containing ethylenediaminetetra-acetic acid (EDTA) (Table VII). Blood glucose concentrations were measured immediately after each blood sampling by the glucose oxidase method (Precision G Blood Glucose Testing System, Medisense,

Bedford, MA, USA). Plasma was separated within 30 min after blood sampling, and samples were stored at -70 °C until analysis.

Figure 5. The overall design of the *in vivo* studies.

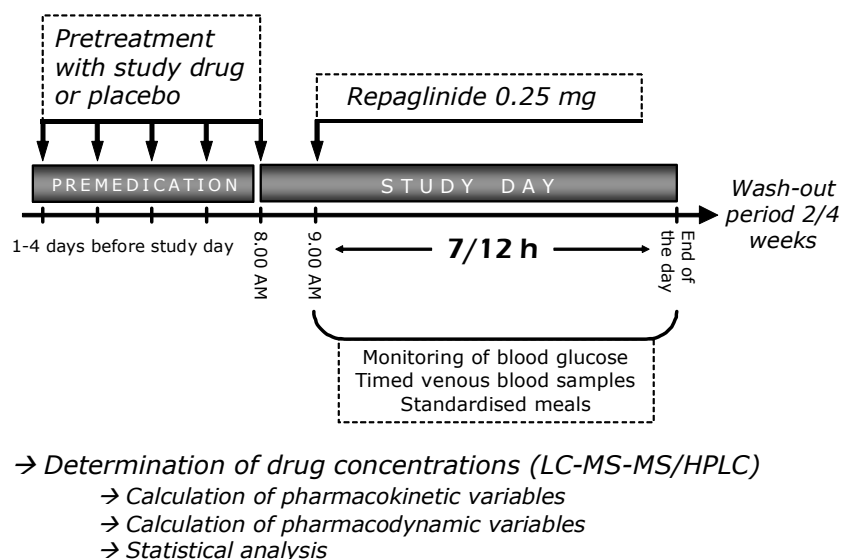


Table V. The formulations and manufacturers of drugs used in studies II-VI.

Drug	Medicinal product	Manufacturer
Repaglinide	NovoNorm 0.5 mg tablet (halved)	Novo Nordisk, Bagsvaerd, Denmark
Bezafibrate	Bezalip 400 mg tablet	Roche, Mannheim, Germany
Fenofibrate	Lipanthyl 200 mg capsule	Fournier S.A., Fontaine Les Dijon, France
Trimethoprim	Trimetin 160 mg tablet	Vitabalans, Hämeenlinna, Finland
Cyclosporine	Sandimmun Neoral 100 mg capsule	Novartis Pharma SA, Huningue, France
Telithromycin	Ketek 400 mg tablet	Aventis Pharma, Scoppito, Italy
Montelukast	Singulair 10 mg tablet	Merck, Sharp & Dohme, Haarlem, Holland
Pioglitazone	Actos 30 mg capsule	Lilly, Madrid, Spain

Table VI. The characteristics of subjects, pre-treatment medication, and wash-out period in studies II-VI.

Study	Subjects			Pretreatment medication	Wash-out period (weeks)
	Number (male/female)	Age range (y)	Weight range (kg)		
II	12 (12/0)	21-26	58-100	Placebo, bezafibrate (400 mg) or fenofibrate (200 mg) at 08 for 5 days	2
III	9 (8/1)*	19-23	62-97	Placebo or trimethoprim (160 mg) at 08 and 20 for 3 days	2
IV	12 (12/0)	19-25	56-100	Placebo or cyclosporine (100 mg) for 2 days (at 20 on day 1 and 08 on day 2)	4
V	12 (8/4)	18-24	52-85	Placebo, telithromycin (800 mg), montelukast (10 mg), or telithromycin and montelukast at 08 for 3 days	2
VI	12 (10/2)	21-28	57-100	Placebo or pioglitazone (30 mg) at 08 for 5 days	4

* From the total of 10 subjects, one withdrew from the study for personal reasons

Table VII. Repaglinide administration, food intake, and timing of blood samples in the studies conducted in healthy volunteers (II-VI).

Repaglinide (0.25 mg) administered at 09 with 150 mL water, following an overnight fast
Food intake after repaglinide ingestion:
15 min: a light standard breakfast (eaten in 10 min)
1 h: a standard snack (eaten in 5 min)
2 h: a standard snack (eaten in 5 min)
3 h: a personalised standard warm meal (eaten in 30 min)
7 h: a personalised standard light meal (eaten in 30 min) *
12 h: a personalised standard snack (eaten in 20 min) *
Blood samples were taken prior to, and 20, 40, 60, 80, and 100 min and 2, 2½, 3, 4, 5, 7, 9,* and 12 h* after the administration of repaglinide

* Not in studies II and III, where the study day ended 7 h after repaglinide ingestion.

2.4 Determination of drug concentrations

The concentrations of repaglinide, its metabolites and pretreatment drugs were determined using a liquid chromatography-tandem mass spectrometry (LC-MS-MS) system, HPLC with ultraviolet or fluorescence detection, and in the case of cyclosporine, a commercially available radioimmunoassay (CYCLO-Trac; Diasorin, Stillwater, Minn, USA). The mass spectrometer used was a PE SCIEX API 3000 mass spectrometer (Sciex Division of MDS Inc, Toronto, Ontario, Canada), and the ion transitions monitored for repaglinide were m/z 453 to m/z 230 (Table VIII).

Table VIII. The quantification of analytes in studies II-VI.

Study	Analytes quantified	Method used	Limit of quantification	Between-day CV [#]
II	Repaglinide	LC-MS-MS	0.05 ng/mL	<10%
	Bezafibrate	HPLC-UV	0.1 mg/mL	<9%
	Fenofibrate	HPLC-UV	0.1 mg/mL	<11%
III	Repaglinide and M1*	LC-MS-MS	0.1 ng/mL	<14%
	Trimethoprim	HPLC-UV	0.1 mg/mL	<5%
IV	Repaglinide, M1, M2, and M4*	LC-MS-MS	0.02 ng/mL	<14%
	Cyclosporine in blood	Radioimmunoassay	30 ng/mL	<5%
V	Repaglinide, M1, M2, and M4*	LC-MS-MS	0.01 ng/mL	<10%
	Montelukast	HPLC-fluorescence	5.0 ng/mL	<14%
	Telithromycin	LC-MS-MS	5.0 ng/mL	<7%
VI	Repaglinide	LC-MS-MS	0.02 ng/mL	<13%
	Pioglitazone	LC-MS-MS	0.5 ng/mL	<7%

* Limit of quantification is for repaglinide; the limit of quantification for repaglinide metabolites could not be determined because authentic reference compounds were not available. Repaglinide metabolite concentrations were given in arbitrary units (U/mL) relative to the ratio of the peak height of each metabolite to that of the internal standard in the chromatogram.

Between-day coefficient of variation (CV) was determined for low, intermediate, and high concentration control samples in the relevant concentration range; the highest of the three values is presented in the table.

2.5 Pharmacokinetic calculations

The pharmacokinetics of repaglinide were characterised by C_{max} , t_{max} , AUC from 0 to infinity ($AUC_{0-\infty}$) and $t_{1/2}$. C_{max} and t_{max} were taken directly from the original data. The terminal log-linear part of each concentration-time curve was identified visually, and k_e was determined from log-transformed data using linear regression analysis. $t_{1/2}$ was calculated by the equation $t_{1/2} = \ln(2)/k_e$. The AUC values were calculated by use of the linear trapezoidal rule for the rising phase of the plasma concentration-time curve and the log-linear trapezoidal rule for the descending phase, with extrapolation to infinity, when appropriate, by dividing the last measured concentration by k_e . The pharmacokinetics of repaglinide metabolites and pre-treatment drugs were determined similarly, but their concentrations were measured from fewer samples.

2.6 Pharmacodynamic variables

The pharmacodynamics of repaglinide were characterised by the maximum increase and maximum decrease from baseline blood glucose concentration, and by mean change in blood glucose concentration from baseline, except in study V, where maximum, minimum and mean blood glucose concentrations were used. The mean change was calculated by dividing the net area under the blood glucose concentration-time curve (from 0 to 3 h, 0 to 7 h, and 0 to 12 h) by the corresponding time interval. In study V, the mean blood glucose concentration was calculated by dividing the area under the blood glucose concentration-time curve by the corresponding time interval.

2.7 Genotyping

The subjects that participated in study IV were genotyped for selected SNPs in the genes encoding OATP1B1 and P-glycoprotein, as well as for selected *CYP2C8* and *CYP3A5* alleles.

The presence of two SNPs in the gene encoding the hepatic transporter OATP1B1, *SLCO1B1* (c.-11187G>A in the promoter region and c.521T>C [Val174Ala] in exon 5), two SNPs in the gene encoding the transporter MDR1 (P-glycoprotein), *ABCB1* (c.2677G>T/A [Ala893Ser/Thr] in exon 21 and c.3435C>T [syn] in exon 26) were analysed. The *CYP2C8* and *CYP3A5* genotypes were determined by investigating the presence of *CYP2C8**3 (c.416G>A, 1196 A>G; Arg139Lys, Lys399Arg), *CYP2C8**4 (792C>G; Ile264Met), and *CYP3A5**3 (6986A>G; nonexpressor) alleles.

2.8 In vitro-in vivo interaction predictions

In studies V and VI, the effect of montelukast and pioglitazone on the AUC of a *CYP2C8* substrate was estimated on the basis of their previously published K_i values for *CYP2C8*. The inhibitor concentration available to the enzyme in vivo ($[I]_{in vivo}$) was calculated using following equations:¹⁶

$$\text{Systemic total } C_{max}: \quad [I]_{in vivo} = C_{max}$$

$$\text{Systemic free } C_{max}: \quad [I]_{in vivo} = f_u \cdot C_{max}$$

$$\text{Estimated total portal } C_{max}: \quad [I]_{in vivo} = C_{max} + k_a \cdot F_a \cdot D / Q_h$$

$$\text{Estimated free portal } C_{max}: \quad [I]_{in vivo} = f_u \cdot (C_{max} + k_a \cdot F_a \cdot D / Q_h)$$

where f_u = the unbound fraction of the inhibitor
 k_a = absorption rate constant
 F_a = oral bioavailability
 D = dose of the inhibitor
 Q_h = hepatic blood flow

Once the $[I]_{in vivo}$ has been determined, the magnitude of potential interaction was estimated using the following estimation, where f_m = fraction of the substrate metabolised via the inhibited pathway.¹⁶

$$\frac{AUC_{inhibited}}{AUC_{control}} = \frac{1}{\frac{f_m}{1 + \left(\frac{[I]_{in vivo}}{K_i}\right)} + (1 - f_m)}$$

2.9 Statistical analysis

In the studies with two phases (III, IV and VI), the pharmacokinetic and pharmacodynamic variables from the placebo and premedication phases were compared using the paired t-test. The t_{max} values were compared using the Wilcoxon signed-rank test. When an interaction was observed, the Pearson correlation coefficient was used to investigate the possible relationships of pharmacokinetic variables of the premedication drug with the blood glucose-

lowering response and the extent of interaction. The differences were considered statistically significant when P was $< .05$. For all variables except t_{\max} , 95% confidence intervals (CI) were calculated for the mean differences between the phases.

In study IV, possible associations of the investigated SNPs and haplotypes with the degree of interaction between cyclosporine and repaglinide were investigated using ANOVA, followed by a posteriori testing with the Tukey test. The analysis was performed with the statistical programs SYSTAT for Windows (version 6.0.1) and SPSS for Windows (version 11.0) (SPSS, Chicago, USA).

In study II (3 phases), the pharmacokinetic variables were compared by repeated-measures analysis of variance (ANOVA), followed by a posteriori testing with the paired t-test with the Bonferroni correction for multiple comparisons. The Bonferroni correction was used also when analysing t_{\max} values with the Wilcoxon signed-rank test. In study V (4 phases) uncorrected P values are presented while the threshold for statistical significance with the Bonferroni correction is presented separately, as requested by the editor of *Clinical Pharmacology & Therapeutics*.

RESULTS AND DISCUSSION

1 *In vitro* studies

1.1 Metabolism of repaglinide by CYP2C8, CYP3A4 and HLM

Incubation of repaglinide with HLM, rhCYP2C8 and rhCYP3A4 resulted in time- and NADPH-dependent substrate consumption. The decline in repaglinide concentration was log-linear for at least 30 min, and the rate of metabolism was linear with respect to microsomal protein concentration up to at least 0.5 mg/mL. In the inhibition assays, preincubation with NADPH and HLM was not found to increase the observed inhibitory effect.

The K_m , V_{max} , and CL_{int} values of HLM, rhCYP2C8 and rhCYP3A4 for repaglinide metabolism are presented in Table IX. At concentrations of repaglinide up to 2 mM, the metabolism rates of the two enzymes were similar, but at higher repaglinide concentrations, CYP3A4 was more active than CYP2C8. M1, M2, and M4 metabolites of repaglinide were all formed in incubations with both rhCYP2C8 and rhCYP3A4. M1 and M2 were predominantly formed by recombinant CYP3A4, whereas M4 was formed mainly by recombinant CYP2C8.

The intrinsic clearance (CL_{int}) of repaglinide in HLM was estimated also from rhCYP CL_{int} data using the relative activity factor (RAF) calculated from Gentest product info, as well as using previously published intersystem extrapolating factors (ISEF), determined by comparing activity differences in rhCYPs and HLM. The CL_{int} values of repaglinide metabolism were similar for CYP2C8 and CYP3A4, when expressed per amount of CYP, as were the RAF-adjusted predictions of microsomal CL_{int} . Estimates based on ISEF values and CYP abundances gave different results; use of V_{max} -based ISEF values predicted CYP2C8 to be more important while CL_{int} -based ISEF values predicted CYP3A4 to be more important in the metabolism of repaglinide.

1.2 Inhibition of CYP2C8, CYP3A4 and repaglinide metabolism

The nonselective CYP2C8-inhibitor quercetin (25 μ M), and the selective CYP3A4-inhibitor itraconazole (3 μ M) both inhibited the metabolism of 2 μ M repaglinide by about 60%, their combination by about 90%. They inhibited the metabolism of 0.2 μ M repaglinide by about 60% and 70%, respectively, their combination by about 90%. Rifampicin was the most potent inhibitor of repaglinide metabolism, followed by bezafibrate, trimethoprim, gemfibrozil and fenofibrate. Bezafibrate, gemfibrozil and fenofibrate had no effect on CYP3A4-mediated midazolam 1-hydroxylation, but inhibited CYP2C8-mediated paclitaxel 6-hydroxylation in the same order of potency as they inhibited repaglinide metabolism. Rifampicin competitively inhibited both CYP3A4 and CYP2C8. Cyclosporine inhibited the formation of the repaglinide metabolites formed mainly by CYP3A4 *in vitro* (M1 and M2) in HLM, with no effect on the formation of M4 (formed mainly by CYP2C8) (Table IX).

Table IX. Summary of *in vitro* findings. The kinetic constants of repaglinide metabolism by rhCYP2C8, rhCYP3A4, and HLM (top), and inhibitory constants of studied drugs for CYP2C8-activity, CYP3A4-activity and repaglinide (2 μ M) metabolism in HLM (bottom).

Repaglinide metabolism	by rhCYP2C8	by rhCYP3A4	by HLM
K_m	2.8 μ M	15.6 μ M	24.2 μ M
V_{max}	4.9 pmol/pmol CYP/min	27.8 pmol/pmol CYP/min	3150 pmol/mg HLM/min
CL_{int}	1.7 mL/nmol CYP/min	1.8 mL/nmol CYP/min	130 mL/g HLM/min
CL_{int} (RAF)	55.1 mL/g HLM/min	64.8 mL/g HLM/min	-
CL_{int} (VISEF)	279 mL/g HLM/min	114 mL/g HLM/min	-
CL_{int} (CLISEF)	3.4 mL/g HLM/min	47.7 mL/g HLM/min	-
M1 formation	0.5 U/pmol CYP/min	8.1 U/pmol CYP/min	-
M2 formation	0.03 U/pmol CYP/min	1.0 U/pmol CYP/min	-
M4 formation	6.9 U/pmol CYP/min	0.1 U/pmol CYP/min	-

Inhibitory constants in HLM	CYP2C8 activity	CYP3A4 activity	Repaglinide metabolism
Bezafibrate	K_i 9.7 μ M	-	IC_{50} 37.7 μ M
Gemfibrozil	K_i 30.4 μ M	-	IC_{50} 111 μ M
Fenofibrate	K_i 92.6 μ M	-	IC_{50} 164 μ M
Trimethoprim	K_i 30.4 μ M *	-	IC_{50} 129 μ M **
Rifampicin	K_i 30.2 μ M	K_i 18.5 μ M	IC_{50} 13.7 μ M
Cyclosporine			
M1 formation	-	-	IC_{50} 0.2 μ M
M2 formation	-	-	IC_{50} 4.3 μ M
M4 formation	-	-	-

* The K_i of trimethoprim for CYP2C8 is from Wen *et al.*

** Repaglinide concentration used to determine IC_{50} was 0.22 μ M.

1.3 Discussion

The results from these *in vitro* studies are of theoretical and practical importance. They clarify the roles of CYP2C8 and CYP3A4 enzymes in the overall metabolism of repaglinide and the underlying mechanisms explaining the results of pharmacokinetic interaction studies with repaglinide.

The identification of CYP2C8 and CYP3A4 as the most important enzymes catalysing the oxidative biotransformation of repaglinide was reported by Bidstrup *et al.*⁷ The authors reported that the main *in vivo* metabolites of repaglinide, M1 and M2, as well as M5, were predominantly formed by CYP3A4, while M4 and M0-OH were mainly formed by CYP2C8. However, only a single repaglinide concentration of 22 μ M was used in that study, which exceeds over 100 times its therapeutic plasma concentrations.² We found that CYP2C8 and CYP3A4 metabolised repaglinide with similar reaction velocities at repaglinide concentrations up to 2 μ M. Furthermore, at supratherapeutic repaglinide concentrations, the role of CYP3A4 became greater, which may explain the findings favouring the relative importance of CYP3A4 in the metabolism of repaglinide.

Estimations of relative importance of the CYP2C8 and CYP3A4 in repaglinide metabolism based on rhCYP CL_{int} values gave different results depending on the scaling approach used. When using previously published scaling factors¹⁴⁷ based on V_{max} differences between recombinant CYP and HLM systems, the results favoured CYP2C8, but scaling factors based on CL_{int} differences favoured CYP3A4. Estimation based on the reaction velocity data provided by the rhCYP and HLM manufacturer (Gentest) suggested that the contributions of the two enzymes were similar.

The estimation of the hepatic clearance of repaglinide, based on our HLM CL_{int} results, was only 17% of the reported systemic repaglinide clearance.² This underprediction could be explained by e.g. active uptake transport of repaglinide into hepatocytes. An approximately 10-fold hepatocyte-to-plasma repaglinide unbound concentration ratio, produced e.g. by uptake transporters, would correct the estimated systemic clearance. Recently published data strongly suggest that repaglinide is a substrate of the hepatic uptake transporter OATP1B1.¹⁶⁴ *SLCO1B1* genotype (encoding OATP1B1) has been shown to influence repaglinide baseline AUC, as well as the degree of interaction between repaglinide and cyclosporine (IV), an OATP1B1 (and CYP3A4) inhibitor.^{22,187,188}

In vitro in HLM, CYP2C8 inhibitors bezafibrate, fenofibrate, gemfibrozil, and trimethoprim, CYP3A4 inhibitor cyclosporine were all shown to inhibit repaglinide metabolism. Furthermore, similar inhibition of repaglinide metabolism in HLM was observed by quercetin (CYP2C8 inhibitor) and by itraconazole (CYP3A4 inhibitor), supporting the conclusion that CYP2C8 and CYP3A4 are about equally important in the *in vitro* biotransformation of repaglinide.

Fenofibrate, bezafibrate and gemfibrozil all moderately inhibited CYP2C8 and repaglinide metabolism *in vitro*, but had no effect on CYP3A4. These similarities in their *in vitro* inhibitory characteristics do not correlate with the observed differences in their *in vivo* interaction potential, and suggest that inhibition of CYP2C8 by parent gemfibrozil does not explain its drastic interaction with repaglinide *in vivo*.⁹

A significant competitive inhibition of CYP3A4, CYP2C8 and repaglinide metabolism by rifampicin was found *in vitro*. The average rifampicin C_{max} after a dose of 600 mg corresponds to approximately 40% and 60% of our K_i values for CYP2C8 and CYP3A4, respectively. Rifampicin has also been reported to inhibit transporters, e.g. OATP1B1 and OATP1B3. The unbound fraction of rifampicin is about 30%, and during the absorption phase its concentration in the portal blood and liver is assumably higher than in peripheral plasma. Thus, if a significant amount of rifampicin is in the blood and liver, it can blunt its own observed inducing effect on a CYP2C8 and CYP3A4 substrate, such as repaglinide.

2 *In vivo* studies

The plasma concentrations of repaglinide were significantly increased by trimethoprim (repaglinide AUC +61%), cyclosporine (+144%), and telithromycin (+77%), while bezafibrate, fenofibrate, montelukast, or pioglitazone did not affect repaglinide pharmacokinetics (Figure 6, Table X). The findings from studies II-VI are presented in more detail in the following sections.

Figure 6. Effects of studied drugs on the pharmacokinetics of repaglinide.

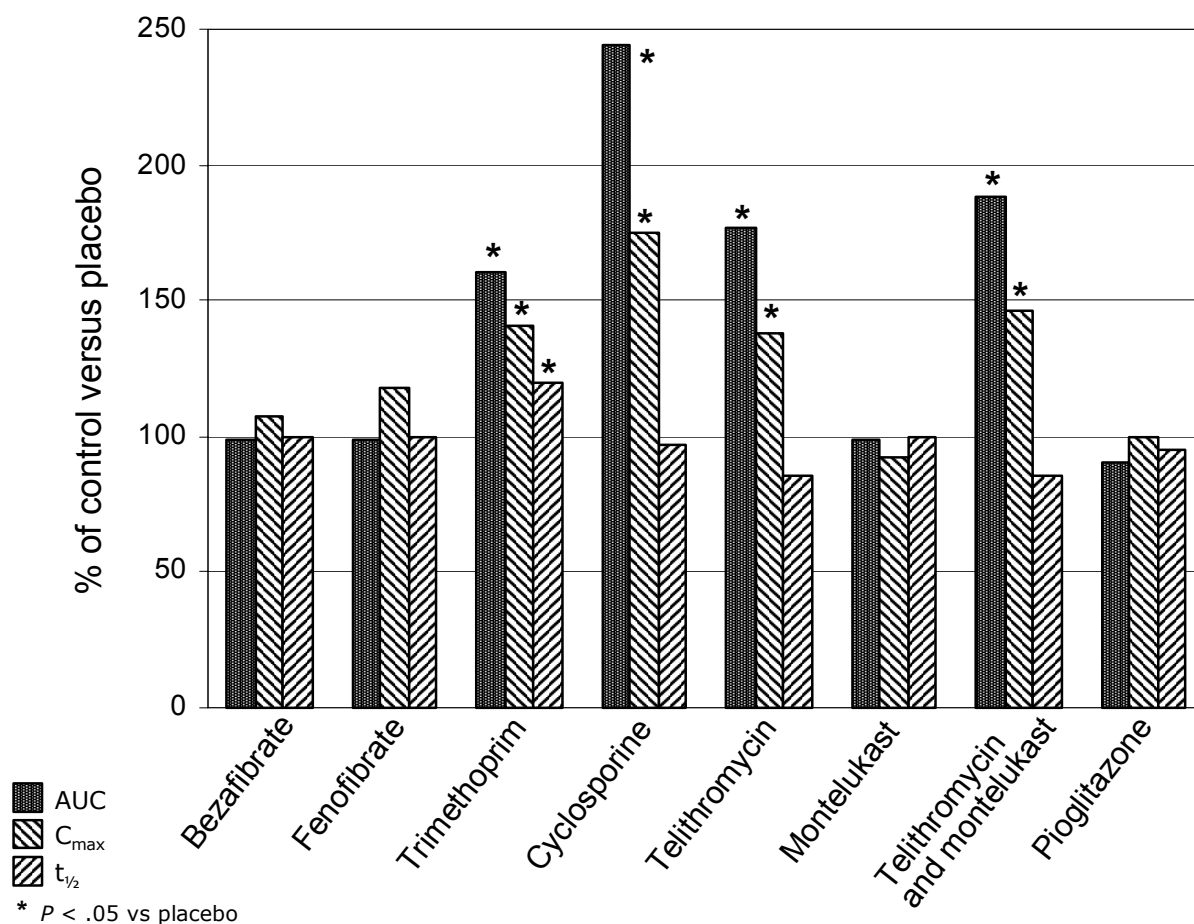


Table X. Effects of studied drugs on the blood glucose response to repaglinide (0.25mg single oral dose), compared to the respective placebo phases.

Studied drug	Mean difference in blood glucose concentration variables compared to the respective placebo phase (mmol/L).				
	Minimum concentration	Maximum concentration	Mean concentration 0 to 3 h	Mean concentration 0 to 7 h	Mean concentration 0 to 12 h
Bezafibrate (II)	0.2	0.0	0.1	0.0	-
Fenofibrate (II)	0.1	0.3	0.2	0.1	-
Trimethoprim (III)	-0.1	-0.3	-0.1	-0.1	-
Cyclosporine (IV)	-0.1	-0.1	0.0	0.0	0.0
Telithromycin (V)	-0.4	-0.7 *	-0.6 *	-0.4	-0.2
Montelukast (V)	-0.2	-0.4	-0.3	-0.2	-0.1
Combination of telithromycin and montelukast (V)	-0.4	-0.5 *	-0.4	-0.3	-0.2
Pioglitazone (VI)	0.0	-0.3	0.0	0.0	0.1

* $P < .05$ versus the placebo phase.

2.1 Effects of bezafibrate and fenofibrate (II)

Bezafibrate and fenofibrate had no statistically significant effects on any of the pharmacokinetic variables of repaglinide. In the bezafibrate and fenofibrate phases, the mean $AUC_{0-\infty}$ for repaglinide was 99.1% and 99.4% of the corresponding value during the placebo phase, respectively. The $t_{1/2}$ of repaglinide was 1.3 h in all phases.

The AUC_{0-8} values of bezafibrate and fenofibrate were 18.1 ± 6.3 mg·L/h and 52.8 ± 21.7 mg·L/h, respectively, and C_{max} values were 4.9 ± 1.4 mg/L and 8.0 ± 3.1 mg/L, respectively.

Bezafibrate and fenofibrate did not change the effect of repaglinide on the blood glucose concentrations, when compared with placebo. None of the subjects had symptomatic hypoglycaemia.

2.2 Effect of trimethoprim (III)

The plasma concentrations of repaglinide were increased by trimethoprim compared with placebo. Trimethoprim raised the $AUC_{0-\infty}$ and C_{max} of repaglinide by 61% ($P < .001$) and 41% ($P < .001$), respectively, and prolonged the $t_{1/2}$ of repaglinide from 0.9 to 1.1 h ($P = .001$). Trimethoprim had no effect on the pharmacokinetics of the M1 metabolite of repaglinide, but decreased the M1:repaglinide AUC ratio by 38% ($P < .001$).

The AUC_{0-8} and C_{max} of trimethoprim were 18.7 ± 3.8 mg·L/h and 2.9 ± 0.6 mg/L, respectively. There was a correlation between the AUC_{0-8} of trimethoprim and the increase in the AUC of repaglinide caused by trimethoprim (Pearson $r = 0.68$, $P = .04$).

No significant differences were seen in the blood glucose response between the placebo and trimethoprim phases. None of the subjects had symptomatic hypoglycaemia.

2.3 Effect of cyclosporine (IV)

The plasma concentrations of repaglinide were significantly raised by cyclosporine. Cyclosporine raised the mean C_{max} and $AUC_{0-\infty}$ of repaglinide to 175% ($P = .003$) and 244% ($P < .001$) of control values, respectively. There was no statistically significant change in the t_{max} or $t_{1/2}$ of repaglinide.

Compared with the corresponding control values, cyclosporine significantly increased the urinary excretion of unchanged repaglinide (to 265%) and its metabolites M2 (to 748%) and M4 (to 499%) but had no significant effect on the excretion of M1. However, cyclosporine significantly reduced the ratio of M1 to repaglinide (by 62%) and increased the ratio of M2 to repaglinide (to 258%) and M4 to repaglinide (to 181%) in urine. The renal clearance of repaglinide remained unchanged by cyclosporine.

The mean C_{max} and $AUC_{0-\infty}$ and median t_{max} values of cyclosporine were 664 ± 158 ng/mL, 1998 ± 636 ng/mL, and 80 min, respectively. There was a correlation between the $AUC_{0-\infty}$ of cyclosporine and the increase in the $AUC_{0-\infty}$ of repaglinide caused by cyclosporine (Pearson $r = 0.61$, $P = .034$).

In subjects with the *SLCO1B1* 521TC genotype, the increase in the $AUC_{0-\infty}$ of repaglinide by cyclosporine was 42% smaller than in subjects with the 521TT (reference) genotype ($P = .047$). No other statistically significant associations between the investigated SNPs or haplotypes and the extent of interaction or repaglinide baseline pharmacokinetics were found.

Although no statistically significant differences were observed in the mean blood glucose response between the placebo and cyclosporine phases, the blood glucose response of individual subjects correlated with the degree of pharmacokinetic interaction between cyclosporine and repaglinide. However, none of the subjects had symptomatic hypoglycaemia. The ratio of repaglinide C_{max} values between the phases correlated with the difference in the mean blood glucose change from 0 to 3 h ($P = .045$) and with the difference in maximum blood glucose increase ($P = .031$). The ratio of repaglinide $AUC_{0-\infty}$ values between the phases correlated with the difference in the mean blood glucose change from 0 to 3 hours ($P = .038$). The subject with the greatest (5-fold) increase in repaglinide $AUC_{0-\infty}$ also had the greatest enhancement of the blood glucose-lowering effect of repaglinide.

2.4 Effects of telithromycin and montelukast (V)

2.4.1 Effect of telithromycin

Telithromycin considerably increased the plasma concentrations and urinary excretion of unchanged repaglinide but did not affect its $t_{1/2}$. Repaglinide mean

C_{\max} and $AUC_{0-\infty}$ were raised to 138% ($P = .006$) and 177% ($P < .001$) of the placebo values, respectively.

Telithromycin decreased the C_{\max} and $AUC_{0-\infty}$ of the metabolite M1 but increased those of metabolites M2 and M4. The M1:repaglinide $AUC_{0-\infty}$ ratio was lowered by 68% ($P < .001$), and the M2:repaglinide and M4:repaglinide $AUC_{0-\infty}$ ratios were raised to 125% ($P = .012$) and to 153% ($P < .001$) by telithromycin, respectively. Telithromycin increased the urinary excretion of unchanged repaglinide to 229% ($P < .001$), and its metabolites M2 to 180% ($P < .001$) and M4 to 198% ($P < .001$), but reduced the excretion of M1 by 41% ($P < .001$) compared to placebo. Telithromycin also reduced M1:repaglinide ratio in urine by 80% ($P = .001$), compared to placebo.

The C_{\max} and AUC_{0-13} of telithromycin were 1180 ± 250 ng/mL and 5350 ± 1520 ng·h/mL, respectively, in the telithromycin phase. There were no significant correlations between the pharmacokinetic variables of telithromycin and the extent of interaction between telithromycin and repaglinide.

Compared with the placebo phase, the maximum (by 0.7 mmol/L, $P = .002$) and mean (0 to 3 h) blood glucose concentrations (by 0.5 mmol/L, $P = .008$) were reduced in the telithromycin phase. None of the subjects had symptomatic hypoglycaemia.

2.4.2 Effect of montelukast

Montelukast had no statistically significant effect on any of the pharmacokinetic variables of parent repaglinide or its metabolites M1, M2 or M4, compared to the placebo phase. Repaglinide C_{\max} and $AUC_{0-\infty}$ values during the montelukast phase were 92% and 99% of those during the placebo phase.

No significant differences were observed in the blood glucose concentrations between the placebo and montelukast phases. None of the subjects had symptomatic hypoglycaemia.

2.4.3 Effect of telithromycin and montelukast

The combination of telithromycin and montelukast raised the mean C_{\max} and $AUC_{0-\infty}$ of repaglinide to 146% ($P = .002$) and 188% ($P < .001$) of placebo values, respectively. There were no significant differences in any of the pharmacokinetic variables of repaglinide between the combination phase and the telithromycin phase.

The combination decreased the C_{\max} and $AUC_{0-\infty}$ of the M1 but increased those of the M2 and M4, when compared to the placebo phase. The combination lowered the M1:repaglinide $AUC_{0-\infty}$ ratio by 73%, and raised the M2 and M4 to repaglinide $AUC_{0-\infty}$ ratios to 118% and to 141%, respectively, when compared to the placebo phase. The combination increased also the urinary excretion of unchanged repaglinide to 235% ($P < .001$), and its metabolites M2 to 186% ($P = .001$), and M4 to 191% ($P < .001$), but reduced the excretion of M1 by 44% ($P < .001$). The combination decreased the M1:repaglinide, M2:repaglinide, and M4:repaglinide ratios in urine when compared to placebo.

Montelukast had no significant effect on the pharmacokinetics of telithromycin, and telithromycin had no significant effect on the pharmacokinetics of montelukast.

The maximum blood glucose concentration was reduced in the combination phase compared to the placebo phase by 0.5 mmol/L ($P = .030$). None of the subjects had symptomatic hypoglycaemia.

2.5 Effect of pioglitazone (VI)

In the pioglitazone phase, the C_{\max} and $AUC_{0-\infty}$ of repaglinide were 100% ($P = .99$) and 90% ($P = .22$) of the control values, respectively. The $t_{1/2}$ was unchanged, about 1.0 h in both phases. The median t_{\max} of repaglinide was 40 min and 20 min in the placebo and pioglitazone phases, respectively ($P = .014$). On the study day, pioglitazone mean C_{\max} and AUC_{0-13} of pioglitazone were 778 ± 186 ng/mL and 5090 ± 1320 ng·h/mL, respectively.

The 5-day pioglitazone administration did not change the baseline blood glucose concentrations (4.6 ± 0.4 mmol/l), compared to the placebo phase (4.5 ± 0.2 mmol/l). Neither the minimum, maximum, nor the mean change of blood glucose after repaglinide intake were changed statistically significantly by pioglitazone. None of the subjects had symptomatic hypoglycaemia.

2.6 Discussion

2.6.1 Effect of bezafibrate, fenofibrate, montelukast, and pioglitazone

Administration of the usual therapeutic doses of bezafibrate, fenofibrate, montelukast, and pioglitazone had no significant effect on the pharmacokinetics or pharmacodynamics of repaglinide in healthy subjects. The AUC and C_{\max} values for bezafibrate, fenofibrate, montelukast, and pioglitazone, as well as their interindividual variation, were comparable with those found in previous studies,^{18,178,180,183,212,213} and based on individual plasma concentrations, compliance to pre-treatment medication was good, indicating that the lack of interaction was not caused by a failure to take the drugs.

The lack of interaction between bezafibrate or fenofibrate with repaglinide is clearly in contrast to the potent effect of gemfibrozil on the pharmacokinetics of repaglinide; gemfibrozil has increased repaglinide AUC 8.1-fold, greatly enhancing its glucose-lowering effects and prolonging its $t_{1/2}$.⁹ Bezafibrate and fenofibrate inhibited CYP2C8 and repaglinide metabolism *in vitro* similarly as parent gemfibrozil,⁹ but the characteristics of gemfibrozil glucuronide conjugate^{214,215} probably explain the differences in the interaction potential of these three fibrates *in vivo*. *In vivo*, gemfibrozil is a potent inhibitor of CYP2C8 and it has increased the AUC of several CYP2C8 substrates (e.g. cerivastatin,¹³ rosiglitazone,²¹⁶ and pioglitazone).²⁰² It was recently demonstrated that gemfibrozil 1- β -glucuronide is a potent and selective metabolism-dependent CYP2C8 inhibitor, and also inhibitor of OATP1B1.^{214,215} Bezafibrate and fenofibrate appear not to be sufficiently potent CYP2C8 (or OATP1B1) inhibitors to influence repaglinide metabolism *in vivo*, especially as their available concentration to inhibit the enzyme is low due to high plasma protein binding (bezafibrate 95%, fenofibrate 99%).¹⁷⁸

Montelukast is a very potent and selective CYP2C8 inhibitor *in vitro* (K_i 0.0092 μM),¹⁶ and based on *in vitro* CYP2C8 inhibition data and therapeutic montelukast plasma concentrations, it has been estimated that montelukast would increase in the AUC of a CYP2C8-cleared drug from 2-fold (using unbound montelukast C_{max} , assuming a 1% free fraction) to over 100-fold (based on total montelukast C_{max}).¹⁶ The available *in vivo* and *in vitro* evidence suggests that the contribution of CYP2C8 to the metabolic clearance of repaglinide is at least 50%, and it is likely that during the phases of study V that included inhibition of CYP3A4 by telithromycin, the contribution of CYP2C8 was even higher. However, even with concomitant CYP3A4 inhibition, repaglinide pharmacokinetics and the ratio of M4 (formed almost exclusively by CYP2C8 *in vitro*)⁷ to repaglinide in plasma and in urine were unaffected by montelukast. Accordingly, the lack of effect of montelukast on repaglinide pharmacokinetics and CYP2C8-mediated metabolism suggests that the effect of montelukast on CYP2C8 *in vivo* is very weak.

The apparent discrepancy between the previously published estimates and our findings could be explained by underestimation of montelukast protein binding. The plasma protein binding of montelukast has been reported to be more than 99%, but the exact free fraction is not known.^{213,217,218} Assuming a free fraction of 1% for montelukast and that only 50% of the metabolism of repaglinide is mediated by CYP2C8, the estimated increase in the AUC of repaglinide by montelukast would yield 30%. However, an assumed free fraction of 0.1%, montelukast would be estimated to cause only a 4-10% increase in the AUC of a drug cleared 50-100% by CYP2C8. The latter estimates agree well with our *in vivo* results and suggest that the high protein binding of montelukast limits its concentration available to CYP2C8 *in vivo*.

The efficacy of pioglitazone-repaglinide combination therapy has been compared to monotherapy with pioglitazone or repaglinide in patients with type 2 diabetes.²⁰ In this 24-week parallel-group study with 246 adult patients (baseline HbA_{1c} about 9%), the effect of the combination on glycemic parameters was much better (mean change in HbA_{1c} -1.8%) than that of either repaglinide (HbA_{1c} -0.2%) or pioglitazone (HbA_{1c} +0.3%) alone, but the plasma concentrations of pioglitazone and repaglinide were not determined in that study. Pioglitazone is a relatively potent *in vitro* inhibitor of CYP2C8 (K_i 1.7 μM) and CYP3A4 (K_i 11.8 μM),¹⁹ and study VI was designed to test the hypothesis that inhibition of repaglinide metabolism by pioglitazone contributes to the observed pharmacodynamic efficacy their combination therapy.

Using the K_i for CYP2C8 and estimated total portal C_{max} ,¹⁶ pioglitazone is estimated to cause an almost 3-fold increase in the AUC of a drug cleared solely by CYP2C8, and about 1.5-fold increase in the AUC of a drug of which 50% is metabolised by CYP2C8 (such as repaglinide). However, the high plasma protein binding (97-99%)²⁰⁰ of pioglitazone probably limits its intracellular concentration available to the enzyme *in vivo*, and calculations based on the free plasma concentrations of pioglitazone estimate practically no increase in repaglinide AUC, which agrees well with the observed *in vivo* results. These results strongly suggest that pioglitazone does not inhibit repaglinide metabolism *in vivo*, and that its inhibitory effect on CYP2C8 and CYP3A4 is very weak in humans. The synergistic effect of pioglitazone-repaglinide combination therapy in patients with type 2 diabetes can be explained by a slowly developing pharmacodynamic

interaction without a pharmacokinetic component. Repaglinide reduces blood glucose concentrations by enhancing short-term insulin release,² whereas the effects of pioglitazone are seen more slowly, mediated via activation of the nuclear receptor PPAR- γ .²¹⁹

2.6.2 Effect of trimethoprim, cyclosporine, and telithromycin

Therapeutic doses of trimethoprim and telithromycin used in the treatment of acute infections, and cyclosporine at doses used e.g. in the treatment of rheumatoid arthritis were found to significantly increase repaglinide plasma concentrations.

Trimethoprim elevated the plasma concentrations of repaglinide, prolonged its half-life, and decreased the M1:repaglinide AUC ratio, indicating that it had inhibited the metabolism of repaglinide to M1 (formed by both CYP2C8 and CYP3A4 *in vitro*).⁷ Trimethoprim, a selective and moderately potent CYP2C8 inhibitor,¹⁷ also inhibited the metabolism of repaglinide *in vitro* in HLM. Itraconazole, which inhibits CYP3A4²⁰⁷ much more potently than trimethoprim inhibits CYP2C8 according to K_i values, has had a smaller effect on the AUC of repaglinide *in vivo* (a 40% increase),⁹ suggesting that the contribution of CYP2C8 in the biotransformation of repaglinide *in vivo* is important. Trimethoprim had no significant effect on the blood glucose-lowering effect of repaglinide in this low-dose study, but concomitant use of trimethoprim may increase the blood glucose-lowering effect of repaglinide and the risk of hypoglycemia, particularly if higher repaglinide doses are used.

Even short-term use of cyclosporine markedly increased the plasma concentrations of repaglinide. There was large variation in the extent of the interaction, the increase in repaglinide AUC ranged from 1.2-fold to over 5-fold even in this homogeneous group of young healthy volunteers, partly explained by genetic factors and variability in cyclosporine concentrations. The greatest increases in the plasma repaglinide concentrations were associated with the greatest increases in the blood glucose-lowering effect of repaglinide. There was also a correlation between cyclosporine AUC and the increase in repaglinide AUC by cyclosporine.

It has been reported, that cyclosporine moderately inhibits CYP3A4,²²⁰ but has no significant effect on CYP2C8.¹⁰² In addition, our *in vitro* results demonstrated that cyclosporine inhibited the mainly CYP3A4-mediated repaglinide metabolism (to M1 and M2), without an effect on M4 formed by mainly CYP2C8. The ratio of M1 to repaglinide in urine was significantly decreased by cyclosporine, suggesting that cyclosporine inhibited the formation of also M1 *in vivo*, similarly as *in vitro*. On the other hand, the ratios of M2 to repaglinide and M4 to repaglinide in urine were increased, suggesting that cyclosporine did not inhibit the formation of these major metabolites *in vivo*, but may have inhibited their hepatic (or biliary) elimination. Cyclosporine has been shown to inhibit efflux transporters (P-glycoprotein, MRP),^{221,222} expressed in the canalicular membrane of the hepatocyte.

Cyclosporine is also a potent inhibitor of OATP1B1,²² and the pharmacokinetics of repaglinide depend on the *SLCO1B1* (encoding OATP1B1) genotype.¹⁶⁴ The effect of cyclosporine on repaglinide pharmacokinetics was smaller in carriers of *SLCO1B1* 521T>C SNP than in noncarriers (wild type). Furthermore, cyclosporine

reduced the M1:repaglinide AUC ratio less than itraconazole,⁹ even though it increased repaglinide AUC much more than itraconazole or clarithromycin.^{8,9} Inhibition of CYP3A4-mediated biotransformation of repaglinide by cyclosporine is unlikely to solely explain their interaction because stronger CYP3A4-inhibitors have had a smaller effect on repaglinide AUC, and because M1 only a minor repaglinide metabolite *in vivo*. Thus, inhibition of the OATP1B1-mediated hepatic uptake of repaglinide by cyclosporine probably contributes to their interaction.

Telithromycin significantly increased the plasma concentrations and effects of repaglinide. The M1:repaglinide ratio in plasma and urine was decreased by telithromycin, indicating that telithromycin inhibited CYP3A4-catalysed biotransformation of repaglinide to M1. The increase in repaglinide AUC by telithromycin was greater than the increases previously caused by other potent inhibitors of CYP3A4 (itraconazole, clarithromycin).^{8,9} Telithromycin is a competitive inhibitor of CYP3A4 *in vitro*, and has considerably increased the AUC of CYP3A4 substrates simvastatin (9-fold) and midazolam (6-fold).¹⁸ In plasma, the AUC ratios of M2 and M4 to repaglinide were increased by telithromycin, but there was a slight (nonsignificant) decrease in the ratios of M2 and M4 to repaglinide in urine. These findings may be explained by increased renal clearance of repaglinide caused by telithromycin. The C_{max} and AUC of repaglinide were increased by telithromycin, while no effect on $t_{1/2}$ was seen. The formation of the M1 metabolite (formed primarily by CYP3A4 *in vitro*) was inhibited already before the C_{max} of repaglinide was reached. These findings suggest that telithromycin raised the plasma concentrations of repaglinide by inhibiting its presystemic CYP3A4-mediated metabolism, occurring presumably in the gut wall and the liver.

GENERAL DISCUSSION

1 Methodological consideration

1.1 *In vitro* studies

HLM and rhCYPs are widely used and reliable systems for drug-drug interaction and drug metabolism studies.⁸³ In studies I and III, the aim was to study the total elimination of repaglinide by HLM (and inhibition of its elimination), rather than analysing the formation rates of specific repaglinide metabolites. Therefore, a substrate depletion method was used,²²³ and calculations were based on the rate of disappearance of parent repaglinide in incubations. When using this method in HLM for a substrate that is metabolised by more than one enzyme, the determined apparent kinetic constants reflect the sum of several enzymatic reactions. Kinetic constants determined this way are less accurate than those determined for a single enzymatic pathway, using e.g. rhCYPs.¹³⁷

To avoid interindividual variation, pooled HLM were used in the studies instead of HLM from individual donors. Pooled HLM adequately represent the population average (Caucasian), and contain all the enzymes present in the endoplasmic reticulum of hepatocytes, with their relative abundances conserved. Using the HLM system, the contributions of different enzymes to the metabolism of a substrate can be determined using selective inhibitors, and possible metabolism-dependent inhibition can be assessed using preincubations.²²⁴ However, conjugative processes such as glucuronidation are more difficult to study in HLM, because the active site of UGTs are inside the lumen of endoplasmic reticulum in the cell and inside the microsomal micelle in HLM. The study of drug glucuronidation in HLM requires disruption of the microsomal membrane e.g. using detergents or pore-forming antifungal agent alamethicin.²²⁵

Studies in HLM were complemented by use of rhCYP2C8 and rhCYP3A4 to characterise their roles in repaglinide elimination (I), and in the formation of repaglinide metabolites M1, M2, and M4 (IV). The activity of one enzyme can be directly determined in the rhCYP system, and the formation rates of repaglinide metabolites by rhCYP2C8 and rhCYP3A4 could be directly compared. However, scaling factors are needed to compare results from rhCYPs to those obtained in HLM or to make *in vivo* predictions, and no consensus has been reached concerning different scaling approaches.¹⁴⁷

Organic solvents are needed in *in vitro* drug metabolism studies, because most drugs are so poorly soluble in water. Excess of organic solvents inhibits CYP-mediated biotransformation, and the susceptibility of individual CYPs to such inhibition differs greatly.¹³⁸ In our studies the drugs were dissolved in methanol, and a final concentration of 1% (v/v) was used. Methanol and acetonitrile have been reported to be the most suitable for *in vitro* CYP studies, and 1% methanol concentration alters the activity of CYP2C8 and CYP3A4 by <8%.¹³⁸

The choice of substrate concentrations used in *in vitro* studies is critical, especially if only a single substrate concentration is used. The contribution of different CYP isoforms to the metabolism of a substrate can differ greatly at different substrate concentrations.⁶ When studying probe substrate reactions in

HLM, the substrate concentration was near the K_m of the substrate. When determining the K_i value a range of substrate concentrations below and above the K_m were used.

The peak concentration of repaglinide after the highest recommended single dose of 4 mg is approximately $0.22 \mu\text{M}$.² Repaglinide is rapidly eliminated from the body, and *in vivo*, repaglinide concentrations are therefore typically $<0.2 \mu\text{M}$. Only about 2% of repaglinide in plasma is unbound,¹⁶¹ and the concentration available for metabolism by CYP enzymes is probably $<0.02 \mu\text{M}$. The limit of quantification method available (at low concentrations) and drug solubility (at higher concentrations) restrict the choice of substrate concentration. The range of repaglinide concentrations used to study the effects of other drugs on repaglinide metabolism and the formation of repaglinide metabolites was 0.2-2 μM , in order to reliably quantify the metabolism of repaglinide and formation of its metabolites, and inhibition of these processes.

These *in vitro* studies could have been improved by studying the effect of gemfibrozil glucuronide on repaglinide metabolism by HLM and rhCYPs. Attempts were made to determine the effect of gemfibrozil glucuronide on CYP2C8 activity in study I using preincubation in the presence of glucuronic acid and alamethicin, but variation between duplicated was large in these multi-step *in vitro* assays. In addition, *in vitro* studies determining the capacity of different transporters to carry repaglinide (and effect of possible inhibition to such processes) are lacking.

1.2 *In vivo* studies

A randomised, balanced cross-over design was used in the *in vivo* studies with healthy volunteers. In this design, the subjects serve as their own controls, limiting the effects of interindividual variation. The elimination half-lives of the pretreatment drugs and their metabolites have been reported to be no more than 24 h, and the 2 or 4 week wash-out period between the phases was sufficient to minimise possible carry-over effects. Compared with the fasted state, coadministration of food has only minor effects on the pharmacokinetic parameters of repaglinide.² Furthermore in our studies, the food intake was standardised and strictly controlled to minimise the possible effect of food ingestion on the pharmacokinetics of repaglinide.

For safety reasons, only a small, 0.25 mg dose of repaglinide was used in the studies in healthy volunteers and carbohydrate intake was frequent to avoid hypoglycaemia. Healthy subjects are more sensitive to the pharmacodynamic effects of repaglinide than diabetic patients, because insulin secretion capacity is decreased in diabetics.²²⁶ The possibility of a significant inhibitory interaction, resulting in increased concentrations and effects of repaglinide, was taken into consideration when selecting the dose. The pharmacokinetics of repaglinide are linear with respect to dose, and the presence of type 2 DM has not been associated with altered repaglinide pharmacokinetics compared to healthy volunteers.¹ Thus, it is reasonable to assume that the findings from studies with a 0.25 mg repaglinide dose can be extrapolated to normal therapeutic doses (0.5–4 mg) of the drug in diabetic patients.

The relatively modest (or absent) differences in the blood glucose-lowering effect of repaglinide despite increased plasma concentrations in the *in vivo* studies

could be explained, at least partially, by the repeated intake of food as well as by the use of a subtherapeutic dose of repaglinide. The enhancement of insulin secretion and reduction in postprandial hyperglycaemia by repaglinide (0.5-4 mg) have been reported to be dose-dependent.¹ Thus, while increases in repaglinide AUC have not caused hypoglycaemic episodes under the experimental conditions, similar increases in the AUC of therapeutic repaglinide doses may be associated with enhanced blood-glucose lowering effect of repaglinide and increased risk of hypoglycaemia.

The timing of repaglinide administration (1 h after the last pretreatment dose) was chosen to ensure the adequate absorption of pretreatment drugs before repaglinide ingestion. The compliance to pretreatment drug therapy was ensured by measuring their concentrations on the study days, and by adhering to pretreatment schedule (each dose marked taken by the subjects).

2 Pharmacokinetic drug-drug interactions of repaglinide

2.1 Role of CYP enzymes in repaglinide metabolism

Repaglinide is almost completely eliminated by metabolism and >90% of the dose is excreted into faeces.⁵ The major metabolites of repaglinide formed *in vivo* are metabolite M1 (about 4% of the dose) and metabolite M2 (about 66%).⁵ Of the CYP enzymes investigated *in vitro*, only CYP2C8 and CYP3A4 have been shown to contribute to the metabolism of repaglinide to any significant extent.⁷ Metabolism of repaglinide to M1 and M2 is catalysed by both CYP2C8 and CYP3A4, and CYP3A4 has been reported to be the principal enzyme responsible for these reactions *in vitro*.⁷

However, the total plasma concentrations of repaglinide *in vivo* are much lower than the concentrations used in the above-mentioned *in vitro* study,² and the relative contribution of CYP3A4 to repaglinide metabolism may have been overestimated, compared to the *in vivo* situation. Our *in vitro* results showed that at higher repaglinide concentrations (>2 μM), the contribution of CYP3A4 became greater than that of CYP2C8, but the reaction velocities of repaglinide metabolism by rhCYP2C8 and rhCYP3A4 were approximately equal at lower repaglinide concentrations *in vitro*. In addition, the *in vitro* CL_{int} values of rhCYP2C8 and rhCYP3A4 for repaglinide metabolism were similar, and chemical inhibition of CYP2C8 and CYP3A4 resulted in a similar reduction of total repaglinide metabolism.

In pharmacokinetic studies, the M1:repaglinide AUC ratio has been decreased by trimethoprim (CYP2C8 inhibitor), by telithromycin (CYP3A4 inhibitor), and by itraconazole (CYP3A4 inhibitor).⁹ Telithromycin and cyclosporine also decreased the M1:repaglinide ration in urine. These findings indicate that both CYP2C8 and CYP3A4 contribute to biotransformation of repaglinide to M1 *in vivo*.

The M2:repaglinide AUC ratio was increased by telithromycin, and cyclosporine increased M2:repaglinide ratio in urine. There was a slight (nonsignificant) decrease in the ratio of M2 to repaglinide in urine by telithromycin, probably explained by the increased renal clearance of repaglinide in the telithromycin phase. These findings suggest that inhibition of CYP3A4 does not affect the biotransformation of repaglinide to its main metabolite M2 *in vivo*. It has been hypothesised that the biotransformation of repaglinide to M2 would require two enzymatic steps, i.e. opening of the piperadine ring and formation of dicarboxylic acid. Though CYP3A4 is more important than CYP2C8 in the formation of M2 *in vitro*, it appears that CYP2C8 (or possibly other enzymes) mainly catalyses its formation *in vivo*.

Telithromycin has increased the M4:repaglinide AUC ratio, and cyclosporine has increased M4:repaglinide ratio in urine. There was a slight (nonsignificant) decrease in the M4:repaglinide in urine by telithromycin, but the increased renal clearance of repaglinide in the telithromycin phase probably explains this finding. These findings suggest that CYP3A4 plays only a minor role in the biotransformation of repaglinide to M4 *in vivo*, similarly as *in vitro*.⁷

The drastic gemfibrozil-repaglinide *in vivo* interaction⁹ does not involve the inhibition of CYP3A4, but probably results from simultaneous inhibition of CYP2C8 and OATP1B1. Furthermore, the effect potent CYP3A4 inhibitors on

repaglinide AUC has been smaller than (clarithromycin and itraconazole),^{8,9} or similar to (telithromycin) the effect of trimethoprim, a selective but only moderately potent CYP2C8 inhibitor.¹⁷ In addition, CYP2C8 genotype has been shown to correlate with repaglinide AUC.¹⁶⁵

Altogether, these findings suggest that the contribution of CYP2C8 to the metabolism of repaglinide is at least as important as that of CYP3A4 *in vivo*. Similarly, the contributions of the two enzymes to the metabolism of repaglinide *in vitro* appear to be about equally important, if the repaglinide concentration used is not too high.

2.2 Role of OATP1B1 in repaglinide pharmacokinetics

In a recent publication that combined the results from several pharmacokinetic studies, the AUC of repaglinide was 3-fold higher in subjects homozygous for the functionally significant *SLCO1B1* c.521T>C SNP (n=4), and 41% higher in heterozygous subjects (n=16), compared with subjects with the reference genotype (n=36), suggesting a major role for hepatic uptake transporter OATP1B1 in repaglinide pharmacokinetics.¹⁶⁴ In addition, the effect of cyclosporine on repaglinide AUC was smaller in those subjects with the *SLCO1B1* c.521T>C SNP compared to those with the wild-type *SLCO1B1* genotype, and the observed interaction was probably caused by inhibition of both CYP3A4 and OATP1B1.

Although these genetic association findings strongly suggest that repaglinide is a substrate for OATP1B1, there is no direct evidence (e.g. *in vitro* studies) about the capability of OATP1B1 to transport repaglinide. However, repaglinide has been shown to have high affinity for OATP1B1 *in vitro*, and repaglinide has inhibited OATP1B1 activity in transfected MDCKII cells recombinantly expressing OATP1B1.²²⁷

2.3 Pharmacokinetic studies with repaglinide

In studies in healthy volunteers, CYP2C8 or CYP3A4 inhibitors without an additional mechanism of interaction have caused <80% increases in repaglinide AUC (Table XI).^{8,9,12} Cyclosporine and gemfibrozil⁹ have caused the greatest increases in repaglinide AUC (2.4-fold and 8.1-fold, respectively), probably by inhibiting both CYP-mediated biotransformation and OATP1B1-mediated hepatic uptake of repaglinide.

Table XI. The effect of CYP or transporter inhibitors on the pharmacokinetics of repaglinide. Results are presented as mean changes compared to placebo (% of control) with the proposed mechanism of investigated interaction.

Drug studied with repaglinide	Repaglinide pharmacokinetic variables (% of control)			Metabolite to repaglinide ratios (% of control)			Enzyme or transporter inhibited
	C _{max}	AUC	t _{1/2}	M1 to R	M2 to R	M4 to R	
Clarithromycin ⁸	168%*	140%*	121%*	-	-	-	
Itraconazole ⁹	148%*	141%*	123%	21%	-	-	CYP3A4
Ketoconazole ¹² †	107%	115%	100%	-	-	-	
Telithromycin (V)	138%*	177%*	86%	32%*	125%*	153%*	
Trimethoprim (III)	141%*	161%*	120%*	62%*	-	-	
Bezafibrate (II)	107%	99%	100%	-	-	-	CYP2C8
Fenofibrate (II)	118%	99%	100%	-	-	-	
Montelukast (V)	92%	99%	100%	96%	104%	109%	
Pioglitazone (VI)	100%	90%	95%	-	-	-	CYP2C8 and CYP3A4
Gemfibrozil ⁹	239%*	808%*	285%*	93%	-	-	CYP2C8 and OATP1B1
Cyclosporine (IV)#	175%*	244%*	97%	38%*	258%*	181%*	CYP3A4 and OATP1B1
Itraconazole + gemfibrozil ⁹	275%*	1930%*	469%*	13%*	-	-	CYP2C8, CYP3A4, and OATP1B1
Telithromycin + montelukast (V)	146%*	188%*	87%	27%*	118%*	141%*	CYP2C8 and CYP3A4

* $P < .05$ versus the placebo phase.

† Repaglinide dose 2 mg, in other studies 0.25 mg.

Metabolite ratios determined from amounts excreted in urine, other metabolite ratios are AUC ratios.

2.3.1 Effect of CYP3A4 inhibition

In vivo, CYP3A4 inhibitors clarithromycin,⁸ itraconazole,⁹ ketoconazole,¹² and telithromycin have all been shown to increase the plasma concentrations of repaglinide (Table XI). *In vitro*, the CYP3A4 K_i values determined for itraconazole have ranged from 15 nM to 11 μ M, but the metabolites of itraconazole also contribute to the inhibitory effects of itraconazole.²⁰⁷ Ketoconazole inhibits CYP3A4 with an estimated K_i of 0.02-0.11 μ M,¹⁰² and clarithromycin is a mechanism-based inhibitor of CYP3A4.²²⁸ Telithromycin is a competitive inhibitor of CYP3A4, and it has increased the AUC of CYP3A4 substrates (e.g. midazolam 6-fold).¹⁸ Telithromycin has inhibited the biliary excretion of the P-glycoprotein and MRP2 substrate doxorubicin in rats, but its effect on OATP1B1 has not been studied.²²⁹ Because these potent CYP3A4-inhibitors have considerably increased repaglinide AUC (by 15-77%), with only moderate (or absent) effects on its t_{1/2}, it is likely that they have mainly inhibited the CYP3A4-mediated repaglinide biotransformation during the first-pass. For example, itraconazole⁹ and telithromycin both inhibited the formation of M1 already before repaglinide C_{max} was reached, indicating inhibition of first-pass metabolism.

Grapefruit juice has been shown to inhibit the activity and decrease the content of intestinal CYP3A4, and increase the plasma concentrations of orally administered CYP3A4 substrates (e.g. midazolam and simvastatin).²³⁰ Grapefruit juice has caused a 13% increase in repaglinide geometric mean AUC suggesting that grapefruit juice inhibited the CYP3A4-mediated presystemic metabolism of repaglinide in the intestine.¹⁶⁵

Though both CYP2C8 and CYP3A4 are functionally expressed in the intestinal mucosa, the intestinal CYP3A4 content exceeds that of CYP2C8, and CYP2C8 is likely to play only a minor role in intestinal drug metabolism.⁸⁹ Thus, during the first-pass metabolism of repaglinide, the contribution of CYP3A4 may be higher than that of CYP2C8.

2.3.2 Effect of CYP2C8 inhibition

Trimethoprim, a selective CYP2C8 inhibitor, inhibited repaglinide metabolism *in vitro*, and increased repaglinide AUC, prolonged its $t_{1/2}$, and decreased the M1:repaglinide AUC ratio *in vivo*. Gemfibrozil has increased repaglinide AUC 8.1-fold, greatly enhancing its glucose-lowering effects and prolonging its $t_{1/2}$.⁹ *In vivo*, gemfibrozil is a potent inhibitor of CYP2C8 and it has increased the AUC of several CYP2C8 substrates (e.g. cerivastatin,¹³ and rosiglitazone²¹⁶). It was recently demonstrated that gemfibrozil 1- β -glucuronide is a potent and selective metabolism-dependent CYP2C8 inhibitor.^{214,215}

These findings suggest that trimethoprim and gemfibrozil (and its glucuronide conjugate) increased repaglinide plasma concentrations by inhibiting its CYP2C8-mediated metabolism, but inhibition of OATP1B1 by gemfibrozil probably significantly contributes to their interaction. The effect of these CYP2C8 inhibitors on repaglinide $t_{1/2}$ has been greater than the effect of CYP3A4 inhibitors,^{8,9,12} indicating that in the hepatic clearance of repaglinide the contribution of CYP2C8 may be greater than that of CYP3A4.

2.3.3 Effect of gemfibrozil and cyclosporine

Cyclosporine (and its metabolites) inhibits CYP3A4, P-glycoprotein, and OATP1B1^{22-24,188} whereas gemfibrozil (and its glucuronide conjugate) inhibits CYP2C8 and OATP1B1.^{214,215} Both cyclosporine and gemfibrozil have increased the plasma concentrations of several statins,^{188,231-235 13,14,236-238} and OATP1B1 is involved in the hepatic uptake of (at least) pravastatin,²³⁹ cerivastatin,²² rosuvastatin²³² and atorvastatin.²⁴⁰ For example, the AUC of pravastatin, which has no significant CYP-mediated biotransformation, is increased 2-fold by gemfibrozil²³⁷ and more than 10-fold by cyclosporine.²⁴¹ The similarities in the observed drug interactions of gemfibrozil and cyclosporine are probably caused by the interaction mechanism common to both, that is, inhibition of OATP1B1.

The relative contributions of CYP and OATP1B1 inhibition in the gemfibrozil-repaglinide and cyclosporine-repaglinide interactions are difficult to estimate. Inhibition of a single CYP enzyme by specific inhibitors, without an additional mechanism, has produced only moderate (<80%) increases in the AUC of repaglinide.^{8,9,12} Increases in repaglinide AUC, 2.4-fold increase by cyclosporine and 8.1-fold by gemfibrozil,⁹ are clearly of greater magnitude. In the case of cyclosporine, the component of OATP1B1 inhibition is probably more important than CYP3A4 inhibition, because cyclosporine is a weaker CYP3A4-inhibitor²¹ than itraconazole²⁰⁷ or clarithromycin²⁴² *in vivo*, which have increased

repaglinide AUC by <40%. Gemfibrozil (and its glucuronide conjugate) are avidly taken up into hepatocytes from circulation^{243,244} and the glucuronide conjugate is a mechanism-dependent inhibitor of CYP2C8,^{214,215} probably making gemfibrozil a more potent inhibitor of CYP2C8 than trimethoprim *in vivo*. However, based on the available findings, no firm conclusions can be made about the relative importance of inhibition of CYP2C8 and OATP1B1 in the gemfibrozil-repaglinide interaction.⁹

2.3.4 Effect of rifampicin

To date, three studies have been conducted to determine the effect of rifampicin on the pharmacokinetics of repaglinide. In all these studies, the rifampicin pre-treatment dose was 600 mg once daily, administered for 5 to 7 days, and a single repaglinide dose was administered 0 to 24 h after the last rifampicin dose. When repaglinide was given together with or 1 h after the last rifampicin dose, a 48% and 31% reduction in repaglinide AUC was seen, respectively. However, when repaglinide was administered 12.5 h or 24 h after the last rifampicin dose and a 57% and 80% reductions in repaglinide AUC, respectively, were seen.¹⁰⁻¹²

Thus, rifampicin seems to act both as an inducer and inhibitor of repaglinide metabolism.¹¹ *In vitro* results confirmed that rifampicin competitively inhibited CYP2C8, CYP3A4 and repaglinide metabolism in HLM (I). It appears that when the concentration of rifampicin in the body is high, it blunts its own inducing effect and only a modest effect on repaglinide AUC is seen. Greater reductions in repaglinide AUC are seen when only small (<10% of the dose after 12 h) or negligible (<1% after 24 h) amounts of rifampicin are left in the body.

These findings affect the design of drug-drug interaction studies where rifampicin is used as an inducer of drug metabolism. The inhibitory effect of rifampicin could also influence the optimal dosing times of other drugs when they must be coadministered with rifampicin. If the inducing effect of rifampicin can be blunted even partially, by ingesting coadministered drugs soon after rifampicin, their bioavailability and therapeutic effect could be improved.

2.3.5 Pharmacokinetic studies without observed interaction

Bezafibrate, fenofibrate, montelukast, and pioglitazone had no effect on the pharmacokinetics of repaglinide *in vivo*. It appears that even though these drugs have been shown to inhibit CYP activity *in vitro*, they are not sufficiently potent CYP2C8 or CYP3A4 inhibitors to influence repaglinide metabolism *in vivo*, especially as their available concentration to inhibit the enzyme is low due to high plasma protein binding (>95% for all).¹⁷⁸

Concomitant treatment with CYP3A4 substrates simvastatin, nifedipine or oral contraceptive (ethinyloestradiol/levonorgestrel) has not significantly increased the AUC of repaglinide.^{2,12} Concomitant simvastatin, nifedipine, and cimetidine have increased repaglinide mean AUC slightly (by <20%), but the changes have not been statistically significant. It appears that these drugs are too weak CYP3A4 or CYP2C8 inhibitors to significantly influence repaglinide pharmacokinetics.

3 Clinical implications

Drug-drug interactions are an important aspect of the treatment of type 2 diabetes, because oral antidiabetic drugs often need to be combined with other drugs to treat concurrent diseases.¹⁵⁴ Use of insulin secretion enhancers, such as repaglinide, carries a risk of hypoglycemia,¹ and pharmacokinetic interactions leading to elevated drug concentrations can further increase the risk. There is considerable interindividual variation in the susceptibility to drug interactions, due to both genetic and environmental differences.⁴⁹ Furthermore, the extent of drug interactions largely depends on the dosing and timing of administration of both the substrate and the inhibitor.⁴⁹

Based on studies II-VI, it can be concluded that bezafibrate, fenofibrate, montelukast, and pioglitazone do not affect repaglinide pharmacokinetics, but that coadministration of trimethoprim, telithromycin, or cyclosporine can increase repaglinide plasma concentrations. Repaglinide is susceptible to inhibition of CYP2C8, CYP3A4, or OATP1B1, and an increase its plasma concentrations can lead to markedly increased blood glucose-lowering effect and associated hypoglycaemia. When such inhibitor is started in patient receiving repaglinide, closer monitoring or blood glucose concentrations is advisable, and repaglinide dose may need to be reduced. Correspondingly, repaglinide dose may need to be raised when the inhibitor is discontinued.

In the studies included in this thesis, a subclinical repaglinide dose was used, but the risk of hypoglycaemia is greater when higher repaglinide doses are used in the clinical practice. Simultaneous inhibition of CYP2C8, CYP3A4, and OATP1B1 (by gemfibrozil and itraconazole) has synergistically increased the plasma concentrations and effects of repaglinide,⁹ and such synergism may also occur with other drug combinations, which increase repaglinide plasma concentrations through multiple interaction mechanisms.

The prevalence of hypertriglyceridemia and low HDL cholesterol concentrations is twice as high in diabetics as in nondiabetics.^{245,246} Statins are widely used in diabetic patients, but fibrates are often chosen if the predominant lipid abnormality is hypertriglyceridemia.¹⁵⁴ The concomitant use of gemfibrozil and repaglinide is not recommended because of their potentially hazardous interaction. The present findings have important therapeutic consequences, as bezafibrate and fenofibrate can be used together with repaglinide due to the lack of interaction.

Short-term use of antimicrobial agents is common, and a course of trimethoprim or telithromycin might be necessary in patients receiving repaglinide. Concomitant use of trimethoprim or telithromycin with repaglinide may increase the risk of hypoglycaemia, particularly when higher repaglinide doses are used in diabetic patients, and it is advisable to monitor blood glucose concentrations closely in such situations, and reduce repaglinide dosage if necessary.

New-onset diabetes mellitus develops to 2-53% of renal transplant recipients,²⁴⁷ and concomitant use of repaglinide and cyclosporine is a treatment option in this patient group.²⁴⁸ Simultaneous use of cyclosporine and repaglinide may increase the risk of hypoglycaemia particularly if higher cyclosporine and repaglinide doses are used. In a recent study of management of new-onset diabetes mellitus

after renal transplantation, patients receiving cyclosporine and repaglinide showed better improvement in blood glucose concentrations than those receiving repaglinide and tacrolimus or sirolimus.²⁴⁸ Increased repaglinide concentrations may have contributed to the better treatment response in the cyclosporine group, but repaglinide concentrations were not monitored in that study. However, there was no difference in the required repaglinide dose, decrease in HbA_{1c} levels, or incidence of hypoglycaemias, suggesting that the increase in repaglinide concentrations was moderate at most.²⁴⁹ However, close monitoring of blood glucose concentrations is necessary in this patient group.

Combination of repaglinide and a glitazone can be used in diabetic patients,¹ and the combination of repaglinide and pioglitazone has been shown to be much more effective (1.8% mean reduction in HbA_{1c}), than their respective monotherapies (repaglinide HbA_{1c} -0.2%, pioglitazone +0.3%).²⁰ The synergistic effect of pioglitazone-repaglinide combination therapy can be explained by a slowly developing pharmacodynamic interaction without a pharmacokinetic component, as pioglitazone does not increase repaglinide concentrations, and the two drugs can be used together without dosage modifications.

CONCLUSIONS

STUDY I CYP2C8 and CYP3A4 were of equal importance in the metabolism of therapeutic repaglinide concentrations *in vitro*, but their predicted contributions *in vivo* were variable and depended of the scaling approach used. Inhibition of CYP2C8 by parent gemfibrozil is unlikely to explain its interaction with repaglinide *in vivo*, because it is only a moderately potent inhibitor of CYP2C8 and repaglinide metabolism *in vitro*. Rifampicin competitively inhibited CYP2C8, CYP3A4 and repaglinide metabolism *in vitro*, which presumably counteracts its own inducing properties *in vivo*.

STUDY II Bezafibrate and fenofibrate did not increase the plasma concentrations or blood glucose-lowering effect of repaglinide. This lack of interaction is clearly in contrast with the strong interaction of gemfibrozil with repaglinide, and suggests that bezafibrate or fenofibrate can be used together with repaglinide with no dosage modifications.

STUDY III Trimethoprim raised the plasma concentrations of repaglinide, most likely by inhibiting its CYP2C8-mediated biotransformation. Although no significant increase in the blood glucose-lowering effect of repaglinide was seen in this study, concomitant use of repaglinide and trimethoprim may increase the risk of hypoglycaemia, particularly if higher repaglinide doses are used.

STUDY IV Cyclosporine considerably raised the plasma concentrations of repaglinide, probably by inhibiting both the CYP3A4-catalysed metabolism and the OATP1B1-mediated hepatic uptake of repaglinide. Concomitant use of repaglinide and cyclosporine may increase the risk of hypoglycaemia, particularly if higher cyclosporine and repaglinide doses are used.

STUDY V Telithromycin increased the plasma concentrations and blood glucose-lowering effect of repaglinide, probably by inhibiting its CYP3A4-catalysed biotransformation. The possibility of an increased risk of hypoglycaemia should be considered when the two drugs are used concomitantly. Montelukast had no effect on the pharmacokinetics of repaglinide, suggesting that it does not significantly inhibit CYP2C8 *in vivo*, assumably due to its extensive binding to plasma proteins.

STUDY VI Pioglitazone did not increase the plasma concentrations of repaglinide. This indicates that pioglitazone does not significantly inhibit CYP2C8 or CYP3A4 *in vivo*, probably due to its extensive plasma protein binding. The synergistic effect of pioglitazone and repaglinide seen in patients with type 2 diabetes is unlikely to be caused by inhibition of repaglinide metabolism by pioglitazone, and can be explained by a pure pharmacodynamic interaction.

GENERAL These findings underline the importance of carrying out drug interaction studies in humans to confirm predictions based on *in vitro* data, and highlight the significance of incorporating plasma protein binding to predictions of CYP inhibition based-interactions. The results emphasise the importance of transporter inhibition and genetic variation of drug-metabolising enzymes and drug transporters as factors affecting the magnitude of drug-drug interactions.

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