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PHARMACOKINETIC INTERACTIONS OF PIOGLITAZONE

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ACADEMIC DISSERTATION

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ABBREVIATIONS

Ae	amount excreted in urine
AhR	aryl hydrocarbon receptor
Arnt	aryl hydrocarbon receptor nuclear translocator
AUC	area under drug concentration-time curve
CAR	constitutive androstane receptor
Cl _{renal}	renal clearance
C _{max}	peak concentration
CV	coefficient of variation
CYP	cytochrome P450
DDC	diethyldithiocarbamate
DDD	defined daily dose
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
EM	extensive metaboliser
FMO	flaving-containing mono-oxygenase
FXR	farnesoid X receptor
HbA _{1c}	glycosylated haemoglobin
HLM	human liver microsomes
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HPLC	high-performance liquid chromatography
IC ₅₀	inhibitor concentration producing 50% decrease in enzyme activity
IM	intermediate metaboliser
k _f	apparent formation rate constant
K _i	inhibition constant
K _m	Michaelis-Menten kinetic constant
LC-MS-MS	liquid chromatography-tandem mass spectrometry
MDR1	multidrug resistance protein 1
m/z	mass-to-charge ratio
NADPH	nicotinamide adenine dinucleotide phosphate
NSAID	non-steroidal anti-inflammatory drug
OATP	organic anion-transporting polypeptide
PAH	polycyclic aromatic hydrocarbon
PDE5	phosphodiesterase type 5
P-gp	P-glycoprotein
PM	poor metaboliser
PPAR	peroxisome proliferator activated receptor
PXR	pregnane X receptor
RXR	retinoid X receptor
SSRI	selective serotonin reuptake inhibitor
t _½	elimination half-life
t _{max}	time to peak concentration
UM	ultrarapid metaboliser
V _{max}	maximum reaction velocity

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by Roman numerals I to V.

- I Jaakkola T, Laitila J, Neuvonen PJ, Backman JT.
 Pioglitazone is metabolised by CYP2C8 and CYP3A4 *in vitro*: potential
 for interactions with CYP2C8 inhibitors.
 Basic Clin Pharmacol Toxicol 2006;99:44-51.
- II Jaakkola T, Backman JT, Neuvonen M, Neuvonen PJ.
 Effects of gemfibrozil, itraconazole, and their combination on the
 pharmacokinetics of pioglitazone.
 Clin Pharmacol Ther 2005;77:404-14.
- III Jaakkola T, Backman JT, Neuvonen M, Niemi M, Neuvonen PJ.
 Montelukast and zafirlukast do not affect the pharmacokinetics of the
 CYP2C8 substrate pioglitazone.
 Eur J Clin Pharmacol 2006;62:503-9.
- IV Jaakkola T, Backman JT, Neuvonen M, Laitila J, Neuvonen PJ.
 Effect of rifampicin on the pharmacokinetics of pioglitazone.
 Br J Clin Pharmacol 2006;61:70-8.
- V Kajosaari LI, Jaakkola T, Neuvonen PJ, Backman JT.
 Pioglitazone, an *in vitro* inhibitor of CYP2C8 and CYP3A4, does
 not increase the plasma concentrations of the CYP2C8 and CYP3A4
 substrate repaglinide.
 Eur J Clin Pharmacol 2006;62:217-23.

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Study V is also included in the academic dissertation of Lauri Kajosaari.

ABSTRACT

Pioglitazone is a thiazolidinedione compound used in the treatment of type 2 diabetes. It has been reported to be metabolised by multiple cytochrome P450 (CYP) enzymes, including CYP2C8, CYP2C9 and CYP3A4 *in vitro*. The aims of this work were to identify the CYP enzymes mainly responsible for the elimination of pioglitazone in order to evaluate its potential for *in vivo* drug interactions, and to investigate the effects of CYP2C8- and CYP3A4-inhibiting drugs (gemfibrozil, montelukast, zafirlukast and itraconazole) on the pharmacokinetics of pioglitazone in healthy volunteers. In addition, the effect of induction of CYP enzymes on the pharmacokinetics of pioglitazone in healthy volunteers was investigated, with rifampicin as a model inducer. Finally, the effect of pioglitazone on CYP2C8 and CYP3A enzyme activity was examined in healthy volunteers using repaglinide as a model substrate.

Study I was conducted *in vitro* using pooled human liver microsomes (HLM) and human recombinant CYP isoforms. Studies II to V were randomised, placebo-controlled cross-over studies with 2-4 phases each. A total of 10-12 healthy volunteers participated in each study. Pretreatment with clinically relevant doses with the inhibitor or inducer was followed by a single dose of pioglitazone or repaglinide, whereafter blood and urine samples were collected for the determination of drug concentrations.

In vitro, the elimination of pioglitazone (1 μ M) by HLM was markedly inhibited, in particular by CYP2C8 inhibitors, but also by CYP3A4 inhibitors. Of the recombinant CYP isoforms, CYP2C8 metabolised pioglitazone markedly, and CYP3A4 also had a significant effect. All of the tested CYP2C8 inhibitors (montelukast, zafirlukast, trimethoprim and gemfibrozil) concentration-dependently inhibited pioglitazone metabolism in HLM. In humans, gemfibrozil raised the area under the plasma concentration-time curve (AUC) of pioglitazone 3.2-fold ($P < 0.001$) and prolonged its elimination half-life ($t_{1/2}$) from 8.3 to 22.7 hours ($P < 0.001$), but had no significant effect on its peak concentration (C_{max}) compared with placebo. Gemfibrozil also increased the excretion of pioglitazone into urine and reduced the ratios of the active metabolites M-IV and M-III to pioglitazone in plasma and urine. Itraconazole had no significant effect on the pharmacokinetics of pioglitazone and did not alter the effect of gemfibrozil on pioglitazone pharmacokinetics. Rifampicin decreased the AUC of pioglitazone by 54% ($P < 0.001$) and shortened its dominant $t_{1/2}$ from 4.9 to 2.3 hours ($P < 0.001$). No significant effect on C_{max} was observed. Rifampicin also decreased the AUC of the metabolites M-IV and M-III, shortened their $t_{1/2}$ and increased the ratios of the metabolite to pioglitazone in plasma and urine. Montelukast and zafirlukast did not affect the pharmacokinetics of pioglitazone. The pharmacokinetics of repaglinide remained unaffected by pioglitazone.

These studies demonstrate the principal role of CYP2C8 in the metabolism of pioglitazone in humans. Gemfibrozil, an inhibitor of CYP2C8, increases and rifampicin, an inducer of CYP2C8 and other CYP enzymes, decreases the plasma concentrations of pioglitazone, which can necessitate blood glucose monitoring and adjustment of pioglitazone dosage. Montelukast and zafirlukast had no effects on the pharmacokinetics of pioglitazone, indicating that their inhibitory effect on CYP2C8 is negligible *in vivo*. Pioglitazone did not increase the plasma concentrations of repaglinide, indicating that its inhibitory effect on CYP2C8 and CYP3A4 is very weak *in vivo*.

INTRODUCTION

Patients with type 2 diabetes often use several drugs concurrently (Reunanen et al. 2000) and are therefore prone to harmful drug-drug interactions. Many clinically important drug interactions are related to cytochrome P450 (CYP) enzymes, whose activity can be suppressed (inhibition) or enhanced (induction) by different drugs. Pioglitazone is a new thiazolidinedione compound used in the treatment of type 2 diabetes. It is reported to be extensively metabolised by CYP enzymes in the liver (Eckland and Danhof 2000).

CYP enzymes are essential in oxidative drug metabolism, being involved in approximately 80% of oxidative drug metabolism and accounting for almost 50% of the overall elimination of commonly used drugs (Wilkinson 2005). The major CYP enzymes involved in human drug metabolism are CYP3A4, CYP2D6, CYP2C8, CYP2C9, CYP2C19, CYP1A2 and CYP2B6. The liver is the main organ responsible for CYP-mediated drug metabolism. However, there are significant amounts of CYP enzymes in other tissues as well. For example, the enterocytes in the small intestine are an important site of metabolism for many drugs. CYP activities can vary markedly between individuals due to genetic and environmental factors and some diseases, which can lead to major differences in drug response and adverse effects.

Inhibition of CYP enzymes can lead to increased plasma concentrations of drugs metabolised by the same enzymes, thereby enhancing their pharmacological effects and increasing the likelihood of adverse effects. Induction of CYP enzymes can reduce the plasma concentrations and effects of substrate drugs. Some prodrugs need to be metabolically activated by CYP enzymes, and inhibition of their metabolism can reduce their effects, while induction can either enhance or reduce their effects and toxicity, depending on the effects of induction on the further elimination of the active metabolite.

Pioglitazone decreases insulin resistance via its action at the peroxisome proliferator activated receptor subtype gamma (PPAR- γ) (Yki-Järvinen 2004). It is not an insulin secretagogue and does not therefore cause hypoglycaemia when used alone. Its most common clinically important adverse effect is fluid retention, which can lead to or exacerbate heart failure and pulmonary oedema (Vaughn et al. 2006).

In vitro studies have suggested that pioglitazone is metabolised by multiple CYP enzymes, mainly by CYP2C8, CYP2C9 and CYP3A4 (Eckland and Danhof 2000), but it seems that the studies on the contribution of different CYP isoforms have not been published. Moreover, manufacturer's information from different sources concerning the roles of CYP2C8 and CYP2C9 is discrepant (Actos prescribing information, Pharmacia Fennica: Actos). Furthermore, the relative contribution of different CYP enzymes *in vivo* and the effects of CYP enzyme induction and CYP2C8 inhibition on the metabolism of pioglitazone in humans are unknown. Pioglitazone is reported to

inhibit both CYP2C8 (Sahi et al. 2003, Walsky et al. 2005a) and CYP3A4 (Sahi et al. 2003) enzymes *in vitro*, but its inhibitory effect on CYP2C8 activity *in vivo* has not been investigated.

The purpose of this work was to identify the CYP enzymes mainly responsible for the elimination of pioglitazone and to investigate the effects of the *in vivo* CYP2C8 inhibitor gemfibrozil, the CYP3A4 inhibitor itraconazole, the *in vitro* CYP2C8 inhibitors montelukast and zafirlukast and the CYP inducer rifampicin on the pharmacokinetics of pioglitazone in healthy volunteers. In addition, this work investigated the effect of pioglitazone on the pharmacokinetics of repaglinide in healthy volunteers in order to characterise its potential to inhibit CYP2C8 and CYP3A enzyme activity in humans.

REVIEW OF THE LITERATURE

1. Pharmacokinetics and drug interactions

Pharmacokinetics explores the time course of a drug and its metabolites in the body (Tozer and Rowland 2006). Pharmacokinetics is often divided into absorption and disposition. Absorption describes the movement of a drug from the site of administration to the circulatory system. Orally administered drugs are absorbed from the gastrointestinal tract and carried via the portal vein to the liver before entering the systemic circulation. The term bioavailability refers to the proportion of the administered drug that reaches the systemic circulation and is available to have an effect. Disposition is divided into distribution and elimination. Distribution involves the transfer of drugs between plasma and tissues, and elimination the loss of drugs from the body. Most drugs are eliminated by the liver into bile or by the kidneys into urine.

Drug interactions are an important aspect of clinical drug treatment. Drug interactions can lead to severe side effects and such interactions have even resulted in early termination of drug development, refusal of approval and withdrawal from the market (Bjornsson et al. 2003). Therefore, in addition to clinicians, also the pharmaceutical industry and regulatory authorities have paid increasing attention to drug-drug interactions. In pharmacokinetic drug interactions, the absorption, distribution, metabolism or excretion of a drug is altered. Many clinically important pharmacokinetic drug interactions are based on inhibition or induction of CYP enzymes. The characteristics of various CYP enzymes and their involvement in the metabolism of commonly used drugs are now quite well established. Active membrane transporters have also been increasingly recognised to play an important role in pharmacokinetics and drug interactions (Ho and Kim 2005). This knowledge may provide a basis for better understanding and predictability of pharmacokinetic drug interactions.

1.1. Drug metabolism

Drugs are eliminated either unchanged by the process of excretion or via biotransformation to metabolites. Most drugs are lipid-soluble, which promotes their passage through biological membranes and enables access to their site of action. Most lipophilic compounds are, however, eliminated poorly unless they are metabolised to more polar compounds (Meyer 1996). The metabolites are usually inactive or less active than the parent drug. However, some metabolites may have enhanced activity (prodrugs) or toxic effects.

Drug biotransformation reactions can be classified into phase I functionalisation reactions or phase II conjugation reactions. Phase I reactions introduce a functional group on the parent compound by oxidation, reduction or hydrolysis reactions, many of which are catalysed by the CYP system and require NADPH as a cofactor. Phase

II reactions lead to the formation of a covalent linkage between a functional group of the parent drug or phase I metabolite and an endogenous compound and include glucuronidation, sulfation, acetylation and methylation reactions. The enzyme systems involved in phase I reactions are located mainly in the endoplasmic reticulum, while phase II conjugating enzymes are located in both the cytoplasm and the endoplasmic reticulum (Krishna and Klotz 1994).

The liver is the principal organ of drug metabolism, although other organs, such as the gastrointestinal tract, kidneys, lung and skin, can have significant metabolic capacity (Krishna and Klotz 1994). A notable portion of a drug may be metabolised in the intestine or liver before entering the systemic circulation, which can significantly limit the oral bioavailability of a drug (first-pass metabolism).

Metabolic capacity can vary markedly between individuals, leading to differences in drug response and adverse effects among patients (Wilkinson 2005). The variability in metabolic capacity is multifactorial; gender, polymorphism of drug-metabolising enzymes, smoking, dietary factors and other drugs can all affect drug metabolism.

1.2. CYP enzymes

Cytochrome P450 enzymes are a family of haeme-containing mono-oxygenases that play a major role in phase I metabolism in humans (Wrighton and Stevens 1992). In addition to participating in the metabolism of drugs and other xenobiotics, CYP enzymes have an important role in the biosynthesis and degradation of many endogenous compounds such as arachidonic acid and eicosanoids, steroid hormones, cholesterol and bile acid, vitamin D and retinoic acid (Nebert and Russell 2002). The name cytochrome P450 comes from the wavelength of light (450 nm) absorbed by the pigment (P) in the enzymes when the haeme iron is reduced and bound to carbon monoxide (Omura and Sato 1962).

In humans, 57 cytochrome P450 genes arranged in 18 families have been identified, of which only the CYP1, CYP2 and CYP3 families seem to contribute to the metabolism of drugs (Nebert and Russell 2002). CYP families are further divided into subfamilies and specific isoenzymes. All isoenzymes in the same family have at least 40% amino acid similarity, and those in the same subfamily have at least 55% amino acid similarity. Individual CYP enzymes are designated by a family number (e.g. CYP2C8), a subfamily letter (CYP2C8) and a number for an individual enzyme within the subfamily (CYP2C8)(Nelson et al. 1996).

Many *in vitro* studies of CYP-mediated drug metabolism in humans are conducted using human liver microsomes (HLM). Upon homogenisation and centrifugation of liver tissue, the endoplasmic reticulum is fragmented to microvesicles, which are referred to as microsomes. The microsomes contain several drug-metabolising enzymes, including the CYP enzymes, flavin-containing mono-oxygenases (FMO) and UDP-glucuronosyltransferases (Venkatakrishnan et al. 2001).

1.2.1. CYP1A subfamily

There are two members of the CYP1A subfamily in humans: CYP1A1 and CYP1A2. CYP1A1 is found primarily in extrahepatic tissues, most notably in the lung and placenta (Miners and McKinnon 2000). CYP1A2 is mainly a hepatic enzyme, and it accounts for about 12-18% of all CYP enzymes in the liver (Rowland-Yeo et al. 2004, Klein et al. 2006). CYP1A enzymes are inducible by xenobiotics, such as polycyclic aromatic hydrocarbons (PAH) found in cigarette smoke and grilled food, and the induction is mainly mediated by the aryl hydrocarbon receptor (AhR) (Nebert and Russell 2002). Rifampicin has increased, for example, the clearance of CYP1A2 substrate mexiletine by over 60% (Pentikäinen et al. 1982), but compared with CYP3A4, CYP1A2 seems to be only weakly induced by rifampicin in humans (Backman et al. 2006).

CYP1A2 is important in the metabolism of many drugs, including caffeine, clozapine, theophylline and tizanidine (Table 1) (Bertz and Granneman 1997, Miners and McKinnon 2000, Granfors et al. 2004). Fluvoxamine and ciprofloxacin are strong inhibitors of CYP1A2 *in vivo* (Bertz and Granneman 1997). Furafylline is used as a selective CYP1A2 probe inhibitor *in vitro* (Miners and McKinnon 2000).

Marked interindividual variability has been reported in the activity of CYP1A2 in humans (Miners and McKinnon 2000). Although the *CYP1A2* gene shows structural polymorphism (www.cypalleles.ki.se/cyp1a2.htm), its importance in explaining variability in CYP1A2 activity is unclear.

1.2.2. CYP2A6

CYP2A6 is the first form of human CYP enzyme in the CYP2 family. CYP2A6 is a predominantly hepatic enzyme, and it constitutes approximately 4-8% of the total liver CYP content (Rowland-Yeo et al. 2004, Klein et al. 2006). Coumarine and nicotine are specific substrates of CYP2A6, and methoxsalen is a potent inhibitor of CYP2A6 (Pelkonen et al. 2000). CYP2A6 is highly polymorphic and its genotype has been associated with, for example, smoking habits (Malaiyandi et al. 2005). CYP2A6 may be inducible by antiepileptic drugs (Pelkonen et al. 2000).

1.2.3. CYP2B6

CYP2B6 has recently received more attention as a clinically important enzyme in drug metabolism (Turpeinen et al. 2006). It is highly polymorphic (Lang et al. 2001), and it may represent up to 6% of the total CYP content in the liver (Stresser and Kupfer 1999). It is expressed at lower levels in some extrahepatic tissues (Ekins and Wrighton 1999). Clinically used substrate drugs for CYP2B6 include bupropion, cyclophosphamide, propofol, nevirapine and efavirenz (Hesse et al. 2000, Court et al. 2001, Ward et al. 2003, Turpeinen et al. 2006). CYP2B6 is inducible by, for example, rifampicin (Loboz et al. 2006). Clopidogrel and ticlopidine are potent inhibitors of CYP2B6 (Richter et al. 2004, Turpeinen et al. 2004, Turpeinen et al. 2005).

Table 1. Examples of substrates, inhibitors and inducers of the main drug-metabolising enzymes.

CYP1A2	CYP2C8	CYP2C9 (polymorphic) 1-3% Caucasians PMs ⁷	CYP2C19 (polymorphic) 3-5% Caucasians PMs ¹¹	CYP2D6 (polymorphic) 5-10% Caucasians PMs, 1% Caucasians UMs ¹²	CYP3A4
SUBSTRATES					
Caffeine ¹ Clozapine ² Theophylline ¹ Tizanidine ³	Amiodarone ⁴ Paclitaxel ⁴ Pioglitazone ⁵ Repaglinide ⁴ Rosiglitazone ⁴	Diclofenac ⁸ Fluvastatin ⁸ Glibenclamide ⁸ Glimipiride ⁸ Glipizide ⁸ Ibuprofen ⁸ Losartan ⁸ Phenytoin ⁸ S-warfarin ⁸	Citalopram ¹¹ Diazepam ¹¹ Lansoprazole ¹¹ Moclobemide ¹¹ Omeprazole ¹¹ Proguanil ¹¹ Propranolol ¹¹	Amiripryline ¹² Codeine ¹² Fluoxetine ¹² Fluvoxamine ¹² Haloperidol ¹² Metoprolol ¹² Oxycodone ¹² Paroxetine ¹² Risperidone ¹²	Alprazolam ¹³ Atorvastatin ¹³ Carbamazepine ¹³ Cyclosporine ¹³ Dexamethasone ¹³ Diltiazem ¹³ Felodipine ¹³ HIV protease inhibitors ¹³ Lovastatin ¹³ Midazolam ¹³ Nifedipine ¹³ PDE5 inhibitors ¹³ Simvastatin ¹³ Triazolam ¹³ Verapamil ¹³
INHIBITORS					
Ciprofloxacin ² Fluvoxamine ² Furafylline ¹	Gemfibrozil ⁴ Trimethoprim ⁴	Amiodarone ⁹ Fluconazole ⁹ Miconazole ⁹ Sulfaphenazole ⁹ Voriconazole ¹⁰	Fluoxetine ¹¹ Fluvoxamine ¹¹ Omeprazole ¹¹ Ticlopidine ¹¹	Flecainide ¹² Fluoxetine ¹² Paroxetine ¹² Quinidine ¹²	Clarithromycin ¹³ Erythromycin ¹³ HIV protease inhibitors ¹³ Grapefruit juice ¹³ Itraconazole ¹³ Ketoconazole ¹³ Voriconazole ¹⁰
INDUCERS					
Cigarette smoke ¹	Phenobarbital ⁶ Rifampicin ⁶	Phenobarbital ⁶ Rifampicin ⁶	Phenobarbital ⁶ Rifampicin ⁶	Not known ¹²	Carbamazepine ¹⁴ Phenytoin ¹⁴ Rifampicin ¹⁴ St. John's wort ¹⁵

¹Miners and McKinnon 2000, ²Bertz and Granneman 1997, ³Granfors et al. 2004, ⁴Totah and Rettie 2005, ⁵Eckland and Danhof 2000, ⁶Gerbal-Chaloin et al. 2001, ⁷Kirchheiner and Brockmüller 2005, ⁸Rettie and Jones 2005, ⁹Miners and Birkett 1998, ¹⁰Theuretzbacher et al. 2006, ¹¹Desta et al. 2002, ¹²Zanger et al. 2004,¹³Dresser et al. 2000, ¹⁴Wrighton and Thummel 2000, ¹⁵Dürr et al. 2000. PM, poor metaboliser; UM, ultrarapid metaboliser.

1.2.4. CYP2C subfamily

The human CYP2C subfamily comprises four members: CYP2C8, CYP2C9, CYP2C18 and CYP2C19. Of these, CYP2C8, CYP2C9 and CYP2C19 are of clinical importance and are collectively responsible for the metabolism of about 20% of clinically used drugs (Ingelman-Sundberg 2004, Totah and Rettie 2005). CYP2C enzymes are expressed mainly in the liver, where they account for approximately 20% of the total CYP content (Shimada et al. 1994), but they are expressed at a significant level also in the small intestine (Obach et al. 2001, L  pple et al. 2003). Each member of the CYP2C subfamily is genetically polymorphic (Daly 2003).

The importance of CYP2C8 in drug metabolism is being increasingly recognised, and it has a major role in the metabolism of a growing number of substrates, including paclitaxel, repaglinide, rosiglitazone, pioglitazone, cerivastatin, amiodarone, amodiaquine, chloroquine and arachidonic acid (Eckland and Danhof 2000, Totah and Rettie 2005). Some overlapping substrate specificity appears to exist between CYP2C8 and CYP3A4 in, for instance, the metabolism of carbamazepine, cerivastatin and repaglinide (Ong et al. 2000, Totah and Rettie 2005). CYP2C8 constitutes about 7% of total microsomal CYP content in the liver, and CYP2C8 protein has been detected in several extrahepatic tissues as well (Enayetallah et al. 2004, Totah and Rettie 2005). CYP2C8 is inducible by rifampicin, phenobarbital and dexamethasone *in vitro* (Gerbal-Chaloin et al. 2001, Madan et al. 2003). Montelukast is a very selective and potent CYP2C8 inhibitor *in vitro* (Walsky et al. 2005b). Gemfibrozil (gemfibrozil glucuronide) and trimethoprim inhibit CYP2C8 both *in vitro* and *in vivo* (Backman et al. 2002, Wang et al. 2002, Wen et al. 2002, Niemi et al. 2003b, Niemi et al. 2003c, Niemi et al. 2004a, Ogilvie et al. 2006). Although the most common variant alleles CYP2C8*2 and CYP2C8*3 show decreased enzyme activity *in vitro*, the clinical consequences of the polymorphism have not been fully determined (Totah and Rettie 2005).

CYP2C9 is the predominant CYP2C enzyme in both the intestine and the liver (L  pple et al. 2003). CYP2C9 is estimated to be responsible for the metabolism of up to 15% of all drugs that undergo phase I metabolism, and its substrates include S-warfarin, phenytoin, losartan, fluvastatin, sulphonylurea antidiabetic drugs and several NSAIDs (Rettie and Jones 2005). Sulfaphenazole is a selective and potent CYP2C9 inhibitor (Miners and Birkett 1998). Clinically significant inhibition may occur also with coadministration of amiodarone, fluconazole, miconazole, voriconazole and certain other sulphonamides (Miners and Birkett 1998, Theuretzbacher et al. 2006). CYP2C9 activity *in vivo* is inducible by rifampicin (Miners and Birkett 1998). Genetic polymorphism of CYP2C9 affects warfarin, phenytoin and sulphonylurea drug dose requirements and has been associated with an increased risk of bleeding complications during warfarin treatment (van der Weide et al. 2001, Higashi et al. 2002, Kirchheiner et al. 2005). The most common allelic variants with reduced catalytic activity are CYP2C9*2 and CYP2C9*3, and they have allele frequencies of 11% and 7%, respectively, in Caucasians (Kirchheiner and Brockm  ller 2005).

CYP2C19 metabolises proton pump inhibitors, some antidepressants, diazepam, proguanil and propranolol, among others (Desta et al. 2002). Omeprazole has been used as a probe inhibitor of CYP2C19 both *in vitro* and *in vivo* (Ko et al. 1997, Desta et al. 2002). Other drugs, such as fluoxetine, fluvoxamine, ticlopidine and isoniazid, can also inhibit the metabolism of CYP2C19 substrate drugs (Desta et al. 2002). Rifampicin and artemisinin have been identified as inducers of CYP2C19 (Desta et al. 2002). Approximately 3-5% of Caucasians and up to 20% of Asian populations are poor metabolisers of CYP2C19 substrates (Desta et al. 2002). CYP2C19 genotype has been shown to affect the efficacy of proton pump inhibitor treatments (Klotz et al. 2004).

1.2.5. CYP2D6

CYP2D6 is the only functionally active isoenzyme in the CYP2D subfamily in humans. Although it constitutes only 2-5% of total CYP content in the liver, it is responsible for up to 25% of the metabolism of known drugs (Zanger and Eichelbaum 2000, Ingelman-Sundberg 2005). At lower levels, CYP2D6 is expressed in extrahepatic tissues, including the gastrointestinal tract and brain (Zanger et al. 2004). CYP2D6 is polymorphically expressed, with four existing phenotypes that define the rate of drug metabolism by CYP2D6; poor metabolisers (PM), who lack the functional enzyme, intermediate metabolisers (IM), who have at least one partially deficient allele, extensive metabolisers (EM), who have two normal alleles, and ultrarapid metabolisers (UM), who have multiple gene copies. Up to 10% of Caucasians are poor metabolisers of CYP2D6 (Zanger et al. 2004).

CYP2D6 substrates include many tricyclic antidepressants, selective serotonin reuptake inhibitors (SSRIs), neuroleptics, beta-blockers and opiates (Zanger et al. 2004). Quinidine, paroxetine, fluoxetine, fluvoxamine, moclobemide, flecainide and terbinafine are potent inhibitors of CYP2D6 (Abdel-Rahman et al. 1999, Zanger et al. 2004). In contrast to all other CYP enzymes involved in drug metabolism, CYP2D6 is not known to be inducible (Ingelman-Sundberg 2005).

1.2.6. CYP2E1

CYP2E1 accounts for about 14-17% of hepatic CYP content (Rowland-Yeo et al. 2004, Klein et al. 2006). This isoenzyme has mainly toxicological relevance, since it can bioactivate many compounds to carcinogens and reactive metabolites (Raycy and Carpenter 2000). It takes part in the metabolism of ethanol, disulfiram, paracetamol, anaesthetics such as halothane, enflurane, isoflurane and sevoflurane, and chlorzoxazone, which serves as a probe of CYP2E1 activity (Raycy and Carpenter 2000, Anzenbacher and Anzenbacherová 2001). Ethanol and isoniazid are known inducers of CYP2E1 (Raycy and Carpenter 2000). Disulfiram is reduced by CYP2E1 to diethyldithiocarbamate (DDC) (Raycy and Carpenter 2000), which is used as an *in vitro* inhibitor of CYP2E1 (Eagling et al. 1998).

1.2.7. CYP3A subfamily

CYP3A enzymes are the most important oxidative enzymes in human drug metabolism. They have been estimated to participate in the metabolism of 40-50% of all drugs (Pelkonen et al. 1998, Wrighton et al. 2000, Ingelman-Sundberg 2004). The isoforms of CYP3A in humans include CYP3A4, CYP3A5, CYP3A7 and CYP3A43.

CYP3A4 is the most abundant CYP enzyme in both the small intestinal mucosa and the liver, where it accounts for almost 30% of total CYP content (Shimada et al. 1994, Wrighton and Thummel 2000). Its substrates include the calcium-channel blockers nifedipine, felodipine, diltiazem and verapamil, the HMG-CoA reductase inhibitors atorvastatin, lovastatin and simvastatin, the HIV protease inhibitors, the PDE5 inhibitors such as sildenafil, and the benzodiazepines alprazolam, midazolam and triazolam (Dresser et al. 2000). Hepatic and intestinal CYP3A4 can be induced by several drugs, such as carbamazepine, phenytoin and rifampicin, and St. John's wort (*Hypericum Perforatum*) (Backman et al. 1996a, Backman et al. 1996b, Dürr et al. 2000, Wrighton and Thummel 2000). There are many known potent inhibitors of CYP3A4, including the azole antifungals ketoconazole, itraconazole and voriconazole, the macrolide antibiotics clarithromycin, erythromycin and troleandomycin, the calcium-channel blockers diltiazem and verapamil, the HIV protease inhibitors and grapefruit juice (Dresser et al. 2000, Wrighton and Thummel 2000, Theuretzbacher et al. 2006). Although the activity of CYP3A4 varies greatly, its population distribution is unimodal and genetic polymorphisms do not appear to explain the interindividual variation (Wilkinson 2005).

CYP3A5 is significantly expressed in only about 10-20% of Caucasian livers (Lamba et al. 2002). It is also found in extrahepatic tissues and is the dominant CYP3A form in the human kidney (Wrighton and Thummel 2000). In individuals expressing CYP3A5, the contribution relative to the total hepatic CYP3A seems to range from 17% to 50% (Daly 2006). CYP3A7 is present primarily in foetal tissues, representing about 50% of the total CYP in foetal liver (Wrighton and Thummel 2000). It is also expressed in some adult livers (Daly 2003). The substrate specificities of CYP3A5 and CYP3A7 are, in general, similar to that of CYP3A4 (Wrighton and Thummel 2000). Variable expression of CYP3A5 and CYP3A7 may account in part for the variation in the metabolism of CYP3A4 substrates (Daly 2003). CYP3A43 is expressed in relatively high levels in the prostate and testes, but its expression in the liver is low. The functional role and substrate specificity of CYP3A43 are currently unknown (Daly 2006).

1.3. Induction and inhibition of CYP enzymes

Induction has been suggested to be an adaptive process in which prolonged exposure to drugs or other chemicals causes an up-regulation in the amount of enzymes that are capable of metabolising the inducing agent. Since induction affects the rate of protein synthesis (or degradation), a steady state with respect to induction is generally reached

in two to three weeks. The disappearance of the induction effect (wash-out period) after discontinuation of the inducing agent can also take several weeks (Wilkinson 2005). Induction of drug-metabolising enzymes may increase the elimination and reduce the bioavailability of the substrate drug, and correspondingly, decrease the drug's plasma concentration. In contrast, for drugs that are metabolised to active or reactive metabolites, induction can lead to enhanced drug effects or toxicity (Pelkonen et al. 1998).

Inhibition of drug-metabolising enzymes can lead to increased plasma concentration of the substrate drug, and thus, exaggerated and prolonged pharmacological effects. This increases the likelihood of adverse effects and drug toxicity, especially with drugs that are extensively metabolised and have a narrow therapeutic index, unless appropriate dose reductions are made. In the case of prodrugs requiring metabolic activation, inhibition can reduce the clinical efficacy of the substrate drug. Contrary to induction, inhibition may occur immediately after one or two doses of the inhibitor (Lin and Lu 1998).

1.3.1. Mechanisms of induction

Induction of drug-metabolising enzymes is mainly mediated by intracellular nuclear receptors. These include the pregnane X receptor (PXR), the constitutive androstane receptor (CAR) and the aryl hydrocarbon receptor (AhR) (Handschin and Meyer 2003).

PXR and CAR mediate the induction of CYP2 and CYP3 enzymes, but phase II conjugative enzymes and drug transporters can also be induced (Honkakoski et al. 2003). The mechanism of induction involves binding of the inducing agent to PXR or CAR. The complex then forms a heterodimer with the retinoid X receptor (RXR), which in turn binds to DNA-responsive element and enhances the transcription of the target gene (Willson and Kliewer 2002). PXR and CAR seem to have flexible and overlapping binding specificities, and they can activate each other's target genes. Human PXR is activated by a wide range of structurally diverse chemicals, such as rifampicin, ritonavir and hyperforin, and phenobarbital has been shown to activate human CAR (Honkakoski et al. 2003). Both PXR and CAR are abundantly expressed in the liver and intestine, with little expression appearing in other tissues.

Polycyclic aromatic hydrocarbon compounds found in tobacco smoke and grilled food induce drug-metabolising enzymes by binding to the AhR. This complex, together with another protein, AhR nuclear translocator (Arnt), increases enzyme expression by binding to the target gene's responsive element. This mechanism activates mainly CYP1A1 and CYP1A2, but the concentrations of glutathione S-transferase and UDP-glucuronosyltransferase enzymes are also increased (Fuhr 2000).

Other known nuclear receptors, including the farnesoid X receptor (FXR) and the peroxisome proliferator activated receptor (PPAR), have also been shown to take part in regulating the expression of drug disposition genes (Dixit et al. 2005).

Contrary to nuclear receptor-mediated induction, ethanol can induce CYP2E1 by stabilisation of the enzyme, which results in accumulation of CYP2E1 (Fuhr 2000).

1.3.2. Mechanisms of inhibition

The clinically relevant mechanisms of CYP inhibition can be divided into reversible and irreversible inhibition (mechanism-based inactivation) (Zhang and Wong 2005).

Based on enzyme kinetics, reversible inhibition can be categorised into competitive, uncompetitive, noncompetitive and mixed inhibition (Zhang and Wong 2005). In competitive inhibition, the inhibitor binds to the enzyme at the same site as the substrate and subsequently blocks the substrate binding; the substrate and inhibitor compete for the enzyme's active site. This type of inhibition can be overcome by increasing concentrations of the substrate. Competitive inhibitors often have similar structures as the substrate drugs. In uncompetitive inhibition, the inhibitor binds effectively only to the enzyme-substrate complex and makes the complex catalytically inactive. In noncompetitive inhibition, the inhibitor binds to the enzyme with the same affinity in both unbound and substrate-bound forms, reducing its catalytic activity. As a result, the extent of inhibition depends only on the concentration of the inhibitor. In mixed inhibition, the inhibitor can bind with different affinities to the enzyme or to the enzyme-substrate complex, thus interfering with the substrate binding and vice versa. High substrate concentrations can reduce but not overcome mixed inhibition.

In irreversible inhibition, drugs with reactive functional groups are metabolised by CYP enzymes to reactive intermediates that covalently modify the CYP enzyme, and inhibition cannot therefore be reversed (Lin and Lu 1998). Because metabolic activation is required, these drugs are often called mechanism-based inactivators or suicide substrates. Binding of an irreversible inhibitor to the enzyme can be prevented by competition with a substrate or a reversible inhibitor.

1.4. Drug transporters

It has become increasingly evident that active drug transport systems influence the pharmacokinetics of many drugs by controlling their movement into and out of cells (Giacomini and Sugiyama 2006). Transporters work in concert with drug-metabolising enzymes, and it is thus often appropriate to consider together the impact of CYP-mediated drug metabolism and transporter-mediated drug efflux and uptake when making assessments of drug pharmacokinetics (Ho and Kim 2005). Drug interactions can occur when induction or inhibition of drug transporters alter e.g. intestinal absorption, proximal renal-tubular excretion, biliary excretion or penetration across the blood-brain barrier of substrate drugs (Giacomini and Sugiyama 2006). The best-known drug transport systems that play a role in drug interactions are the P-glycoprotein (MDR1, multidrug resistance protein 1) and human organic anion-transporting polypeptides (OATPs) (DuBuske 2005).

The P-glycoprotein is a transmembrane protein that operates as an efflux pump to export drugs out of cells. It facilitates excretion of substances into urine, bile and intestinal lumen (i.e. reduced absorption) and prevents excess accumulation in the brain (Fromm 2003). There is overlapping specificity between CYP3A4 and P-glycoprotein substrates and inhibitors (Ayrton and Morgan 2001). Substrate drugs for P-glycoprotein include anticancer drugs, HIV protease inhibitors, steroids, digoxin, quinidine, cyclosporine and loperamide (Giacomini and Sugiyama 2006). Quinidine, erythromycin, verapamil and itraconazole are known inhibitors of P-glycoprotein (DuBuske 2005, Ho and Kim 2005). P-glycoprotein is inducible by, for example, rifampicin and St. John's wort (Fromm 2003).

OATPs are a class of transmembrane proteins that are expressed in human intestine, liver, kidney and brain tissue (Ho and Kim 2005). In contrast to the P-glycoprotein, OATPs act as drug uptake pumps, transporting drugs into cells. Substrates for members of the OATP subfamily include bile salts, hormones, HMG-CoA reductase inhibitors, digoxin and methotrexate (Ho and Kim 2005, Shitara et al. 2005).

Many agents that affect P-glycoprotein function also affect OATP activity. Coordinate activity of both drug uptake and efflux transporters may determine the net absorption and subsequent elimination of a drug (Kim 2003). In addition to drug-induced changes in P-glycoprotein and OATP activity, these transporters also exhibit genetic polymorphism (Ho and Kim 2005, König et al. 2006).

2. Pioglitazone

Pioglitazone [(±)-5-[[4-[2-(5-ethyl-2-pyridinyl)ethoxy]phenyl]methyl]-2,4-]-thiazolidinedione monohydrochloride] is a thiazolidinedione class antidiabetic drug that acts primarily by decreasing insulin resistance. The thiazolidinediones (or "glitazones") were introduced in the late 1990s as an adjunctive therapy for type 2 diabetes. The first member of the class, troglitazone (Rezulin®), was rapidly withdrawn from the market because of fulminant drug-induced hepatitis (Gale 2001). The other two compounds, pioglitazone (Actos®) and rosiglitazone (Avandia®), appear to be devoid of idiosyncratic liver toxicity (Yki-Järvinen 2004).

2.1. Pharmacodynamics and clinical use

Pioglitazone is a ligand for peroxisome proliferator activated receptor-γ (PPAR-γ), a member of the nuclear receptor superfamily. Once activated by pioglitazone, PPAR-γ forms a heterodimer with the retinoid X receptor. The heterodimer then binds to specific DNA sequences and regulates the target genes involved in the metabolism of glucose and lipids. The activation of PPAR-γ leads to increased insulin sensitivity in hepatic, fat and skeletal muscle cells, thereby inhibiting hepatic gluconeogenesis and increasing peripheral glucose uptake. However, the exact mechanism remains obscure. Pioglitazone is not an insulin secretagogue, and it is dependent on the presence of

insulin to exert its effects. In addition to its action on glucose metabolism, pioglitazone promotes the differentiation of adipocytes, which leads to redistribution of lipid from visceral to subcutaneous deposits (Waugh et al. 2006).

In Europe, pioglitazone is approved as monotherapy and in combination with metformin or sulphonylurea for treatment of patients with type 2 diabetes. Pioglitazone is taken orally once daily at a dosage of 15 or 30 mg/day, with titration to 45 mg/day if necessary (Waugh et al. 2006). At maximal dose, pioglitazone decreases glycosylated haemoglobin value (HbA_{1c}) on average by 1-1.5% (Yki-Järvinen 2004). The blood glucose lowering effect of pioglitazone develops gradually over weeks, with a maximal decrease in blood glucose being reached after 10-14 weeks (Aronoff et al. 2000).

2.2. Pharmacokinetics and drug interactions

Pioglitazone is rapidly absorbed from the gastrointestinal tract, with peak plasma concentrations (C_{\max}) observed within two hours (Eckland and Danhof 2000). Food slightly delays the time to peak concentration (t_{\max}), but does not alter the extent of absorption (Waugh et al. 2006). The mean absolute oral bioavailability of pioglitazone is 83% (Eckland and Danhof 2000). Pioglitazone is extensively (97% to over 99%) bound to plasma proteins, primarily to albumin, and has a rather small apparent volume of distribution (0.63 l/kg) (Eckland and Danhof 2000, Waugh et al. 2006).

In humans, pioglitazone is extensively metabolised by hydroxylation and oxidation to five primary metabolites (M-I, M-II, M-IV, M-V and M-VI), and by further oxidation of M-IV to a secondary metabolite M-III (Figure 1)(Eckland and Danhof 2000, Baba 2001). In addition, four minor metabolites (M-VII to M-X) have been identified *in vitro* and in animals (Shen et al. 2003). The hydroxy derivatives of pioglitazone, M-II and M-IV, and the keto derivate M-III have been shown to be pharmacologically active in animal models. M-IV and M-III are the principal metabolites found in human serum, and at steady state they comprise approximately 75-80% of the total AUC of active compounds (Actos prescribing information, Baba 2001). M-II is found at relatively low concentrations in plasma, and it does not significantly contribute to total active compounds (Eckland and Danhof 2000). M-IV and M-III are highly (>98%) bound in plasma proteins (Waugh et al. 2006). The mean elimination half-life of pioglitazone ranges from 3 to 9 hours (Eckland and Danhof 2000, Baba 2001), but the half-life of the active metabolites M-IV and M-III is considerable longer (26-28 hours), which is likely to contribute to an extended pharmacological activity that allows for once daily administration of pioglitazone (Eckland and Danhof 2000). Following oral administration, approximately 15-30% of the pioglitazone dose is recovered in the urine. However, renal elimination of the parent pioglitazone is negligible, and the drug is primarily excreted as metabolites and their conjugates (Waugh et al. 2006). The main compound excreted in urine is M-V (Eckland and Danhof 2000). Age and gender have no significant effect on the pharmacokinetics of pioglitazone (Waugh et al. 2006). Overall, the pharmacokinetics of pioglitazone in patients with type 2 diabetes is similar

to that in healthy volunteers (Eckland and Danhof 2000).

In vitro studies with HML have suggested that multiple CYP isoenzymes (1A1, 1A2, 2C8, 2C9, 2C19, 2D6 and 3A4) are involved in the metabolism of pioglitazone, with the most important isoenzymes reported to be CYP2C8, CYP2C9 and CYP3A4 (Eckland and Danhof 2000, Hanefeld 2001). CYP2C8 and CYP3A4 are reported to contribute approximately 40% and less than 20%, respectively, to the metabolism of pioglitazone and M-IV (Hanefeld 2001). However, the information on the contribution of different CYP isoforms to the metabolism of pioglitazone seems to be based on unpublished data, and the experimental systems in which these results were obtained, have not been described. In addition, information from different sources appears to be discrepant. For example, the Finnish product information has previously stated that the metabolism of pioglitazone occurs predominantly via CYP3A4 and CYP2C9 (Pharmaca Fennica: Actos), whereas the US label states that the major CYP isoforms involved are CYP2C8 and CYP3A4 (Actos prescribing information). Prior to this thesis, no published studies have been available on the effects of CYP enzyme inducers or CYP2C8 inhibitors on the pharmacokinetics of pioglitazone *in vivo*.

Pioglitazone has been reported to both inhibit (testosterone 6 β -hydroxylation, K_i 11.8 μ M) and induce CYP3A4 activity *in vitro* (Sahi et al. 2003). In healthy volunteers, pioglitazone has not altered CYP3A4 marker activity (urinary excretion ratio of 6 β -hydroxycortisol to cortisol) or affected the pharmacokinetics of CYP3A4 substrate simvastatin significantly, suggesting that pioglitazone is not a significant inducer or inhibitor of CYP3A4 *in vivo* (Prueksaritanont et al. 2001, Nowak et al. 2002). Pioglitazone has also been reported to inhibit CYP2C9 (tolbutamide hydroxylation, K_i 32.1 μ M) *in vitro*, but it has not altered the pharmacokinetics of drugs metabolised mainly by CYP2C9 (warfarin, glibenclamide, glipizide) *in vivo* (Gillies and Dunn 2000).

In vitro, pioglitazone has most potently inhibited CYP2C8 (paclitaxel 6 α -hydroxylation, K_i 1.7 μ M; amodiaquine N-deethylation, IC_{50} 11.7 μ M) (Sahi et al. 2003, Walsky et al. 2005a). However, no studies have been published on the effect of pioglitazone on the pharmacokinetics of CYP2C8 substrate drugs in humans.

Pioglitazone has been reported to inhibit OATP1B1 and OATP1B3 *in vitro*, suggesting that pioglitazone could also be a substrate for these transporters (Nozawa et al. 2004). To date, the effect of genetic polymorphism in CYP2C8 or drug transporters on pioglitazone metabolism has not been studied (Kirchheiner et al. 2005).

2.3. Adverse effects

Common adverse effects of pioglitazone include weight gain, fluid retention and plasma volume expansion, which can produce mild dilutional anaemia, peripheral oedema and can lead to or exacerbate heart failure (Waugh et al. 2006). The underlying mechanism of fluid retention has not been fully elucidated, but it appears to be a dose-related class

effect of thiazolidinediones (Macfarlane and Fisher 2006). The risk of oedema and heart failure is higher when thiazolidinediones are combined with insulin (Nesto et al. 2004). The manufacturer of pioglitazone reports an incidence of peripheral oedema of 4.8% for monotherapy and 5.9-7.2% for combination therapy with metformin or sulphonylurea, increasing to 15.3% for combination treatment with insulin (Vaugh et al. 2006). In a recently published large study, oedema in the absence of heart failure was experienced by 21% of pioglitazone recipients versus 13% of placebo recipients, and heart failure occurred significantly more often in pioglitazone recipients (11%) than in placebo recipients (8%; $P < 0.0001$) (Dormandy et al. 2005). Heart failure and insulin therapy are contraindications to the use of thiazolidinediones in Europe.

Unlike troglitazone, pioglitazone has rarely been associated with hepatotoxicity or elevated liver enzymes. There have been a few reports of liver injury with pioglitazone, but direct causality has not been established (Vaugh et al. 2006).

Recent studies have suggested that exposure to thiazolidinediones has important effects on bone. In rodent models, activation of PPAR- γ by thiazolidinediones has stimulated adipocyte formation from the mesenchymal precursor cells at the expense of the formation of osteoblasts, resulting in bone loss (Marie and Kaabeche 2006). Recent data from an observational study in older adults with diabetes suggest that thiazolidinedione use is associated with an increased rate of bone loss in women (Schwartz et al. 2006). Another randomised, controlled study found that short-term rosiglitazone treatment inhibits bone formation and accelerates bone loss in healthy postmenopausal women (Grey et al. 2007). In addition, a safety analysis of a large, controlled clinical trial revealed that significantly more female patients who received rosiglitazone experienced fractures than did females who received either metformin or glibenclamide (Kahn et al. 2006). The majority of the fractures observed in the rosiglitazone group were in the humerus, hand and foot. The number of women with hip or spine fractures was low and did not differ between the groups.

3. Drugs studied with pioglitazone *in vivo*

3.1. Rifampicin

Rifampicin is a bactericidal antibiotic drug of the rifamycin class that acts by inhibiting RNA polymerase in bacterial cells (Campbell et al. 2001). It is used mainly to treat tuberculosis (Douglas and McLeod 1999), but it has also a role in the treatment of e.g. staphylococcal infections. The dosage of rifampicin is usually between 450 and 600 mg once daily.

Rifampicin is readily absorbed, with therapeutic concentrations achieved in 2-4 hours after oral administration. Rifampicin induces its own metabolism and the half-life, which is between 2 and 5 hours on initiation of therapy, decreases to 2-3 hours after repeated administration (Douglas and McLeod 1999). Rifampicin is metabolised by

deacetylation. Both unchanged rifampicin and desacetyl-rifampicin are excreted in the bile and urine, and unchanged rifampicin is reabsorbed into enterohepatic circulation. Rifampicin is 60-80% protein bound (Dollery 1999).

The most common adverse effects of rifampicin are gastrointestinal disturbances, rash and fever, and rifampicin can cause body fluids, such as urine and tears, to become reddish in colour. Rifampicin has been shown to produce liver dysfunction, particularly in patients with chronic liver disease or in patients taking other hepatotoxic agents (Dollery 1999).

Rifampicin is a potent inducer of several drug-metabolising enzymes and the P-glycoprotein, and it can markedly (more than 10-fold) reduce the plasma concentrations of many drugs, particularly drugs with significant presystemic metabolism (Niemi et al. 2003a). Rifampicin has its greatest effects on the expression of CYP3A4 in the liver and small intestine (Niemi et al. 2003a), but it also induces CYP2C enzymes in human hepatocytes and small bowel enterocytes (Gerbal-Chaloin et al. 2001, Raucy et al. 2002, Madan et al. 2003, Glaeser et al. 2005). Drug interaction studies in humans indicate that rifampicin induces CYP2C enzymes also *in vivo* (Niemi et al. 2003a). For example, rifampicin has been found to decrease the AUCs of antidiabetics rosiglitazone (CYP2C8 and CYP2C9 substrate) and repaglinide (CYP2C8 and CYP3A4 substrate) by over 50% (Niemi et al. 2000, Niemi et al. 2004a, Park et al. 2004a).

3.2. Itraconazole

Itraconazole is a triazole antifungal agent that interferes with fungal cell membrane ergosterol synthesis by inhibiting the fungal cytochrome P450 enzyme lanosterol 14-demethylase (Grant and Clissold 1989). In addition to inhibiting the fungal enzyme, all azoles can also inhibit human drug-metabolising CYP enzymes, thereby impairing the metabolism of many drugs (Backman et al. 2000).

Itraconazole is available as an oral capsule and as a solution, with recommended daily doses ranging from 100 to 400 mg. Optimal absorption of itraconazole capsules is achieved when these are taken with a meal (De Beule and Van Gestel 2001). Itraconazole is tightly bound to plasma albumin and blood cells, leaving only 0.2% of the drug free in plasma. Itraconazole is highly lipophilic and despite high protein binding, its concentrations within tissues are considerable, with a large apparent volume of distribution (about 11 l/kg). Tissues such as skin, nails and liver accumulate itraconazole (Grant and Clissold 1989). Itraconazole seems to be metabolised by CYP3A4 (Tucker et al. 1992, Isoherranen et al. 2004). The major metabolite, hydroxyitraconazole, reaches about 2-fold higher plasma concentrations than the parent compound and has considerable antifungal activity. Elimination of itraconazole is biphasic, with a terminal $t_{1/2}$ of approximately 20 hours after a single dose. At steady state, the terminal $t_{1/2}$ increases to 30 hours, indicating that the elimination of itraconazole is a saturable process at clinical doses (Grant and Clissold 1989). The most common adverse effects

of itraconazole are gastrointestinal disturbances, dizziness, pruritus, headache and mild increase in liver enzymes (Grant and Clissold 1989).

Itraconazole and hydroxyitraconazole are potent CYP3A4 inhibitors *in vitro* (Back and Tjia 1991, von Moltke et al. 1996, Wang et al. 1999, Isoherranen et al. 2004). *In vivo*, itraconazole greatly (i.e., 4- to over 25-fold) increases the plasma concentrations of many drugs that are metabolised mainly by CYP3A4, such as midazolam and triazolam (Olkola et al. 1994, Varhe et al. 1994, Backman et al. 2000). Itraconazole also inhibits the P-glycoprotein, consequently raising the concentrations of digoxin (Partanen et al. 1996, DuBuske 2005). In addition, itraconazole has recently been reported to inhibit CYP2B6 *in vitro* (Walsky et al. 2006). There are relatively few *in vivo* studies of the effects of itraconazole on enzymes other than CYP3A4, but it seems to have no significant inhibitory effect on CYP1A2, CYP2C9 or CYP2C19 (Backman et al. 2000). In pharmacokinetic interaction studies, itraconazole has increased the AUCs of the antidiabetic drugs (partially metabolised by CYP3A) repaglinide (CYP2C8 and CYP3A4 substrate) and nateglinide (CYP2C9 and CYP3A4 substrate) by 40-50% (Niemi et al. 2003c, Niemi et al. 2005a). In addition, itraconazole has raised the AUC of loperamide, a substrate of CYP2C8, CYP3A4 and the P-glycoprotein, almost 4-fold (Niemi et al. 2006).

3.3. Gemfibrozil

Gemfibrozil is a fibric acid derivate that reduces plasma triglyceride concentrations and slightly increases HDL cholesterol levels (Frick et al. 1987), most likely due to induction of fatty acid oxidation through activation of PPAR- α (Lee et al. 2003). Gemfibrozil is used mainly in the treatment of hypertriglyceridaemia with a recommended dose of 600 mg twice daily.

The absorption of gemfibrozil is almost complete, and peak plasma concentrations are attained in 1-2 hours after administration (Todd and Ward 1988). Gemfibrozil is at least 97% bound to plasma proteins. It is eliminated mainly by metabolism with a mean elimination $t_{1/2}$ of 7.6 hours. Both unchanged gemfibrozil and its metabolites form glucuronide conjugates. The most common adverse effects of gemfibrozil are gastrointestinal symptoms and rash (Todd and Ward 1988).

Gemfibrozil is one of the most potent inhibitors of CYP2C8 in humans, and there have been several reports of clinical interactions between gemfibrozil and CYP2C8 substrates; for example, repaglinide (8.1-fold increase in AUC), cerivastatin (5.6-fold increase in AUC), rosiglitazone (2.3-fold increase in AUC) and loperamide (2.2-fold increase in AUC) (Backman et al. 2002, Niemi et al. 2003c, Niemi et al. 2003b, Niemi et al. 2006). *In vitro*, gemfibrozil has potently inhibited CYP2C9 (tolbutamide hydroxylation, K_i 5.8 μ M), whereas its inhibitory effect towards CYP2C8 has been substantially weaker (paclitaxel 6 α -hydroxylation, K_i 75 μ M) (Wen et al. 2001, Wang et al. 2002). However, in humans, the effects of gemfibrozil on CYP2C9-mediated

metabolism have been smaller or even lacking (Niemi et al. 2001b, Lilja et al. 2005, Niemi et al. 2005a). Recent studies have demonstrated that a major metabolite of gemfibrozil, gemfibrozil glucuronide, is a rather selective and more potent inhibitor of CYP2C8 (paclitaxel 6 α -hydroxylation, IC₅₀ 1.8 μ M after a 30-min preincubation) than the parent gemfibrozil, indicating that the interactions of gemfibrozil *in vivo* are, at least in part, due to the metabolite (Shitara et al. 2004, Ogilvie et al. 2006). Gemfibrozil also inhibits OATP1B1, which could partially explain the gemfibrozil-cerivastatin and gemfibrozil-repaglinide interactions (Shitara et al. 2004).

3.4. Montelukast and zafirlukast

Montelukast and zafirlukast are leukotriene receptor antagonists that are both anti-inflammatory and bronchodilatory. They are used orally as adjunctive therapy in asthma and allergic rhinitis. The recommended doses of montelukast and zafirlukast are 10 mg once daily and 20 mg twice daily, respectively. Their most common adverse effects are headache and gastrointestinal symptoms (Lipworth 1999).

The peak plasma concentrations of montelukast are achieved in 3-4 hours and its oral bioavailability is approximately 64% (Markham and Faulds 1998). Montelukast is more than 99% bound to plasma protein (Singulair prescribing information). It is extensively metabolised and its $t_{1/2}$ ranges from 2.7 to 5.5 hours (Markham and Faulds 1998). CYP2C9 and CYP3A4 are involved in the metabolism of montelukast *in vitro* (Singulair prescribing information). Montelukast has recently been found to be a very potent and selective CYP2C8 inhibitor *in vitro*, with a K_i value ranging from 0.0092 to 0.15 μ M depending on the microsomal protein concentration (Walsky et al. 2005a, Walsky et al. 2005b).

Peak plasma concentrations of zafirlukast are achieved within 3 hours of oral administration. Zafirlukast is over 99% bound to plasma proteins and has a terminal $t_{1/2}$ of approximately 10 hours. It undergoes extensive hepatic metabolism, with CYP2C9 as the major biotransformation pathway. Zafirlukast has been reported to inhibit CYP2C9 and CYP3A4 *in vitro* (Dekhuijzen and Koopmans 2002). Zafirlukast has also recently been found to inhibit CYP2C8 *in vitro*, with an IC₅₀ value of 0.388 μ M (amodiaquine N-deethylation) (Walsky et al. 2005a).

According to previous predictions based on *in vitro* inhibition data and therapeutic plasma montelukast concentrations, the expected magnitude of its effect on a CYP2C8 cleared drug has ranged from a 2-fold to over a 100-fold increase in AUC, depending on whether unbound (assuming 1% free fraction) or total montelukast concentration has been used in the prediction (Walsky et al. 2005b). The *in vitro* inhibitory potency and the circulating concentrations of zafirlukast have suggested a possibility of weak CYP2C8 inhibition *in vivo* (Walsky et al. 2005a). At the time of this work, no studies were published on the effects of montelukast or zafirlukast on the pharmacokinetics of CYP2C8 substrate drugs *in vivo*.

3.5. Repaglinide

Repaglinide is a fast-acting prandial oral antidiabetic agent belonging to the meglitinide family. It lowers blood glucose by enhancing insulin release from pancreatic β -cells (Hatorp 2002). Repaglinide is used in type 2 diabetes, with a recommended dose range of 0.5-4 mg before each meal (maximum daily dose 16 mg) (Prandin prescribing information). The most common adverse effect of repaglinide is hypoglycaemia (Culy and Jarvis 2001).

After oral administration, repaglinide is rapidly absorbed, with C_{\max} occurring within 1 hour. The mean absolute bioavailability is about 60% (Hatorp 2002), suggesting considerable first-pass metabolism. Repaglinide is highly (>98%) bound to plasma proteins. It is cleared rapidly from the circulation, with a half-life of approximately 1 hour (Hatorp 2002). Repaglinide is extensively metabolised in the liver. The most important enzymes participating in the biotransformation of repaglinide *in vitro* are CYP2C8 and CYP3A4 (Bidstrup et al. 2003, Kajosaari et al. 2005a). Genetic association data have suggested that repaglinide is a substrate for OATP1B1 (Niemi et al. 2005b).

In pharmacokinetic interaction studies in humans, CYP3A4 inhibition (by ketoconazole, itraconazole, clarithromycin and telithromycin) has modestly (approximately 15-80%) increased the AUC of repaglinide (Niemi et al. 2001a, Hatorp et al. 2003, Niemi et al. 2003c, Kajosaari et al. 2006). Cyclosporine, an inhibitor of CYP3A4 and OATP1B1, has raised the AUC of repaglinide by 2.4-fold (Kajosaari et al. 2005b). The CYP2C8-inhibiting antibacterial agent trimethoprim has raised the AUC of repaglinide by 60% (Niemi et al. 2004b). Gemfibrozil, an inhibitor of CYP2C8 and OATP1B1, has increased the AUC of repaglinide by about 8-fold, and the combination of itraconazole and gemfibrozil has increased the AUC of repaglinide by about 20-fold, greatly enhancing and prolonging its blood glucose lowering effect (Niemi et al. 2003c). Thus, both CYP2C8 and CYP3A4 seem to metabolise repaglinide *in vivo*, and the simultaneous inhibition of both metabolic routes (and OATP1B1) results in the greatest interaction. CYP enzyme induction by rifampicin has considerably decreased the plasma concentrations of repaglinide (Niemi et al. 2000).

AIMS OF THE STUDY

Pioglitazone has been reported to be extensively metabolised by mainly CYP2C8, CYP2C9 and CYP3A4 *in vitro*. However, the information is partly discrepant and based on unpublished data. Before this study, no controlled studies were available concerning the effects of CYP enzyme inducers or CYP2C8 inhibitors on pioglitazone pharmacokinetics. The aim of this work was to investigate the potential pharmacokinetic drug-drug interactions related to the use of pioglitazone.

Specific aims of the studies were:

- I To identify the CYP enzymes that metabolise pioglitazone and to examine the effects of the CYP2C8 inhibitors montelukast, zafirlukast, trimethoprim and gemfibrozil on pioglitazone metabolism *in vitro*.
- II To investigate the effects of the CYP2C8 inhibitor gemfibrozil, the CYP3A4 inhibitor itraconazole and their combination on the pharmacokinetics of pioglitazone to determine the role of these enzymes in the fate of pioglitazone in humans.
- III To investigate the effects of montelukast and zafirlukast, potent inhibitors of CYP2C8 and pioglitazone metabolism *in vitro*, on the pharmacokinetics of pioglitazone in humans.
- IV To investigate the effect of the potent CYP enzyme inducer rifampicin on the pharmacokinetics of pioglitazone in humans.
- V To investigate the effect of pioglitazone on the pharmacokinetics and pharmacodynamics of repaglinide in order to evaluate the potential of pioglitazone to interact with a CYP2C8 and CYP3A4 substrate drug in humans.

MATERIALS AND METHODS

1. In vitro study (Study I)

1.1. Microsomes and recombinant CYP enzymes

Human liver microsomes (HLM), representing a pool from 46 individuals, mostly of Caucasian origin, were purchased from Gentest Corporation (Woburn, MA, USA). Human liver tissue had been collected in accordance with all pertinent regulations, and permission from the donors' families had been obtained prior to organ collection. The organ collection procedures had been reviewed and approved by the respective institutional Human Subjects Committee. Human recombinant CYP isoforms (Supersomes™) from baculovirus-infected insect cells expressing cDNA encoding human CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5, and insect cell control supersomes lacking CYP content, were purchased from Gentest Corporation. HLM and recombinant CYP enzymes were stored at -70°C, thawed in a water bath and placed on ice before addition into the incubation mixture.

1.2. Incubation conditions

The incubations were carried out in 0.1 M sodium phosphate buffer (pH 7.4), containing 5.0 mM MgCl₂, 1 µM pioglitazone, 1.0 mM β-NADPH and 0.3 mg/ml microsomal protein or 20 pmol/ml recombinant CYP. The initial concentration of pioglitazone (1 µM, i.e. 357 ng/ml) was roughly at the same level as the therapeutically relevant plasma C_{max} in humans (Eckland and Danhof 2000). HLM concentration was chosen on the basis of preliminary experiments. Pioglitazone, solvent with or without inhibitor, buffer and HLM or recombinant enzymes were premixed, and incubations were commenced by the addition of β-NADPH. Zafirlukast stock solution was prepared in acetonitrile, the stock solutions of other drugs were prepared in methanol. Final solvent concentration did not exceed 1% and was equal in all control incubations. All incubations were conducted in duplicate at 37°C in a shaking water bath and terminated by removing an aliquot (0.5 ml), adding it to 100 µl of perchloric acid (70%) and cooling on ice. The mean values of the duplicates were used in the calculations.

1.3. Pioglitazone metabolism and inhibition experiments

First, the metabolism of pioglitazone in HLM was studied by measuring the depletion of parent pioglitazone and the formation of its metabolites as a function of time. The depletion of unchanged pioglitazone at 60 minutes and the formation of the primary main metabolite M-IV at 8 minutes after incubation with HLM or recombinant CYP isoforms were then used as the measure of pioglitazone metabolism in further experiments.

To examine the effects of different CYP inhibitors on the metabolism of pioglitazone in HLM, montelukast (1 μ M) and trimethoprim (100 μ M) were used as inhibitors of CYP2C8, gemfibrozil (100 μ M) as an inhibitor of both CYP2C9 and CYP2C8, ketoconazole (1 μ M), itraconazole (3 μ M) and troleandomycin (100 μ M) as inhibitors of CYP3A4, sulfaphenazole (10 μ M) as an inhibitor of CYP2C9, omeprazole (10 μ M) as an inhibitor of CYP2C19, furafylline (20 μ M) as an inhibitor of CYP1A2, quinidine (10 μ M) as an inhibitor of CYP2D6, diethyldithiocarbamate (DDC; 100 μ M) as an inhibitor of CYP2E1, clopidogrel (1 μ M) as an inhibitor of CYP2B6 and rifampicin (100 μ M) as an inhibitor of CYP2C8 and CYP3A4 (Baldwin et al. 1995, Newton et al. 1995, Bourri  et al. 1996, Ko et al. 1997, Eagling et al. 1998, Wen et al. 2001, Wang et al. 2002, Wen et al. 2002, Isoherranen et al. 2004, Richter et al. 2004, Kajosaari et al. 2005a, Walsky et al. 2005b). The final concentration of each inhibitor was chosen according to the aforementioned publications. Mechanism-based inhibitors troleandomycin, furafylline, DDC and clopidogrel were pre-incubated with HLM for 15 minutes, after which pioglitazone was added.

The metabolism of pioglitazone by CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5 was screened by using 20 pmol/ml recombinant CYP enzymes and the same incubation conditions as described above. The metabolism of pioglitazone by insect cell control supersomes was also studied.

1.4. Determination of IC₅₀ values of CYP2C8 inhibitors

As the metabolism of pioglitazone was predominantly CYP2C8 mediated, the effects of CYP2C8 inhibitors were studied in more detail. Pioglitazone was co-incubated with montelukast (0-3 μ M), zafirlukast (0-10 μ M), trimethoprim (0-240 μ M) and gemfibrozil (0-180 μ M) using HLM and the previously described incubation conditions. The depletion rate of unchanged pioglitazone and the formation rate of the metabolite M-IV were calculated. The inhibitor concentrations producing a 50% decrease in the original enzyme activity (IC₅₀) were determined using non-linear regression analysis with the program FigP (version 6.0, Biosoft, Cambridge, UK).

1.5. Determination of pioglitazone and metabolite concentrations

After addition of internal standard (rosiglitazone 3 μ g/ml in methanol-water 20:80 v/v), the incubations aliquots were applied to solid-phase extraction cartridges conditioned with methanol (1 ml) and water (1 ml). The cartridges were washed with 1 ml of 100 mM hydrochloric acid and 1 ml of 70% methanol, and the analytes were eluted with 1 ml of 2% ammonium hydroxide in methanol. The eluent was evaporated to dryness under nitrogen stream (50 C), and the residues were dissolved in 100 μ l of acetonitrile-water (45:55 v/v) and transferred into autosampler vials.

The concentrations of pioglitazone and its metabolites were measured by use of a PE SCIEX API 2000 liquid chromatography-tandem mass spectrometry (LC-

MS-MS) system (Sciex Division of MDS Inc., Toronto, Ontario, Canada) (Lin et al. 2003). Chromatography was performed on an XTerra RP C₁₈ column (3.9x100 mm; Waters, Milford, MA, USA) using a mobile-phase gradient consisting of 10 mM ammonium acetate (pH 9.5). The mass spectrometer was operated in positive atmospheric pressure chemical ionization mode with selected reaction monitoring. The ion transitions monitored were mass-to-charge ratio (*m/z*) 357 to *m/z* 134 for pioglitazone, *m/z* 371 to *m/z* 148 for M-III, *m/z* 373 to *m/z* 150 for M-IV, *m/z* 387 to *m/z* 164 for M-V, *m/z* 389 to *m/z* 166 for M-XI and *m/z* 358 to *m/z* 135 for rosiglitazone. These transitions represent the product ions of the [M+H]⁺ ions. The limit of quantification for pioglitazone was 0.3 nM, and the coefficient of variation (CV) was 9.1% at 0.045 µM, 4.9% at 0.6 µM and 4.4% at 2.25 µM (n=12). Because authentic standards for the metabolites were not available, their quantities are given in arbitrary units (U/ml) relative to the ratio of the peak height of the metabolite to the peak height of the internal standard. A signal-to-noise ratio of 10:1 was used as the limit of detection for the metabolites.

2. *In vivo* studies (Studies II-V)

The studies were carried out at the Department of Clinical Pharmacology, University of Helsinki. The study protocols were approved according to current regulations by the Ethics Committee for Studies in Healthy Subjects and Primary Care of the Helsinki and Uusimaa Hospital District and by the Finnish National Agency for Medicines. Before entering the studies, the volunteers had received both oral and written information and had given a written informed consent.

2.1. Subjects

A total of 40 (28 male, 12 female) healthy volunteers participated in the studies (Table 2). The subjects were ascertained to be healthy by medical history, clinical examination and routine laboratory tests. None of the subjects was using continuous medication or oral contraceptives or was a frequent smoker. Female subjects gave a negative pregnancy test before entering the studies. The use of grapefruit juice or any pharmaceuticals was prohibited 1 week prior to each study. Participation in any other trial and blood donation within 1-2 months before or after the studies were also prohibited.

Table 2. Characteristics of the subjects.

<i>Study</i>	<i>Subjects</i> n (male/female)	<i>Age (y)</i>	<i>Weight (kg)</i>	<i>Body mass index (kg/m²)</i>
II	12 (9/3)	23 ± 3	70 ± 9	22 ± 2
III	12 (8/4)	24 ± 2	72 ± 12	23 ± 2
IV	10 (7/3)	23 ± 1	69 ± 8	22 ± 2
V	12 (10/2)	23 ± 2	71 ± 12	23 ± 4

Age, weight and body mass index are mean ± SD (standard deviation).

2.2. Study design

All four studies were of randomised, placebo-controlled crossover design. Details of the studies are given in Table 3.

Table 3. Structure of the studies.

<i>Study</i>	<i>Pretreatment medication and dose</i>	<i>Duration of pretreatment</i>	<i>Wash-out period (weeks)</i>	<i>Study drug and dose</i>
II	Gemfibrozil 600 mg x 2 Itraconazole 100 mg x 2 (first dose 200 mg) Gemfibrozil and itraconazole Placebo x 2	4 days at 08:00 and 20:00	4	Pioglitazone 15 mg on day 3 at 09:00
III	Montelukast 10 mg x 1 and placebo x 1 Zafirlukast 20 mg x 2 Placebo x 2	6 days at 09:00 (day 3, 08:00) and 21:00	3	Pioglitazone 15 mg on day 3 at 09:00
IV	Rifampicin 600 mg x 1 Placebo x 1	6 days at 20:00	4	Pioglitazone 30 mg on day 6 at 09:00
V	Pioglitazone 30 mg x 1 Placebo x 1	5 days at 08:00	4	Repaglinide 0.25 mg on day 5 at 09:00

The pretreatment medication and placebos were supplied, packed and labelled according to a randomisation list for each subject by the Helsinki University Central Hospital (HUCH) Pharmacy. The study drugs (pioglitazone and repaglinide) were also supplied by the pharmacy. Repaglinide tablets (0.5 mg) were halved and weighed by the investigators. The doses of pretreatment drugs and pioglitazone were chosen to reflect current use. A small dose of repaglinide was used for safety reasons.

The study drugs were given orally with 150 ml of water after an overnight fast at 09:00. In Studies II-IV, the subjects received a standard warm meal 3 hours, and light meals 7 and 11 hours after pioglitazone intake. In Study V, each subject received a standardised breakfast (eaten in 10 minutes) precisely 15 minutes after the administration of repaglinide, 2 standardised snacks (eaten in 5 minutes) after 1 and 2 hours, a standard warm meal after 3 and 7 hours and a standard light meal after 11 hours. Food intake was identical in the different study phases. During the study days, the subjects were under direct medical supervision. During the days of repaglinide administration, blood glucose levels were monitored throughout the day. Additional carbohydrates, glucose solution for intravenous use and glucagon for intramuscular use were available, but they were not needed.

2.3. Blood and urine sampling

On the days of administration of the study drugs, a forearm vein of each subject was cannulated for blood sampling. In Studies II-IV, timed blood samples were drawn before the administration of pioglitazone and at 1, 2, 3, 4, 5, 7, 9, 12, 24 and 48 hours after (also at 72 and 96 hours in Study III). In Study V, the blood samples were collected before the administration of repaglinide and 20, 40, 60, 80 and 100 minutes and 2, 2½, 3, 4, 5, 7, 9 and 12 hours later. Blood samples (10 ml) were collected into tubes containing ethylenediaminetetra-acetic acid (EDTA). In Study V, 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 by centrifugation within 30 minutes and stored at -70°C until analysis.

In Studies II and IV, urine was collected cumulatively in fractions of 0 to 12, 12 to 24 and 24 to 48 hours after the administration of pioglitazone. After each collection period, the volume of urine was measured and an aliquot was stored at -70°C until analysis.

2.4. Determination of plasma and urine drug concentrations

2.4.1. Pioglitazone and pioglitazone metabolites

The concentrations of pioglitazone and its metabolites were measured by using PE SCIEX API 3000 and PE SCIEX API 2000 (Study IV, urine drug concentrations) liquid chromatography-tandem mass spectrometry systems (Sciex Division of MDS Inc., Toronto, Ontario, Canada) (Lin et al. 2003). Chromatography was performed on XTerra RP C₁₈ column (3.9x100 mm; Waters, Milford, MA, USA) using gradient elution. The mobile phase consisted of 10 mM ammonium acetate (pH 9.0-9.5, adjusted with 25% ammonia solution) and acetonitrile. Rosiglitazone served as an internal standard. The mass spectrometers were operated in the atmospheric pressure chemical ionization with positive ion detection mode. The ion transitions monitored were mass-to-charge ratio (*m/z*) 357 to *m/z* 134 for pioglitazone, *m/z* 371 to *m/z* 148 for M-III, *m/z* 373 to *m/z* 150 for M-IV, *m/z* 387 to *m/z* 164 for M-V and *m/z* 389 to *m/z* 166 for a previously unrecognised metabolite designated M-XI and *m/z* 358 to *m/z* 135 for rosiglitazone. These transitions represent the product ions of the [M+H]⁺ ions. In Study II, the limit of quantification for pioglitazone in plasma was 0.5 ng/ml and the day-to-day CV was 3.9% at 20 ng/ml, 1.8% at 200 ng/ml and 2.9% at 2000 ng/ml (n=12). In Study III, the limit of quantification for pioglitazone was 0.1 ng/ml and the day-to-day CV was 5.7% at 0.5 ng/ml (n=6), 9.0% at 20 ng/ml (n=6), 3.0% at 200 ng/ml (n=6) and 11.9% at 2000 ng/ml (n=5). In Study IV, the limit of quantification for pioglitazone in plasma was 0.1 ng/ml and the day-to-day CV was 3.6% at 20 ng/ml, 3.3% at 200 ng/ml and 6.3% at 2000 ng/ml (n=5). A signal-to-noise ratio of 10:1 was used as the limit of detection for pioglitazone metabolites, and their quantities are given in arbitrary units (U) relative to the ratio of the peak height of the metabolite to the peak height of the internal standard.

2.4.2. Gemfibrozil, itraconazole and hydroxyitraconazole (Study II)

Plasma gemfibrozil concentrations were determined by high-performance liquid chromatography (HPLC) with ultraviolet detection and ibuprofen as an internal standard (Hengy and Kölle 1985). Day-to-day CV was below 15% at relevant concentrations. Plasma itraconazole and hydroxyitraconazole were also determined by HPLC with CVs below 4% and 6%, respectively, at relevant concentrations (Rommel et al. 1988, Allenmark et al. 1990).

2.4.3. Montelukast and zafirlukast (Study III)

The concentrations of montelukast and zafirlukast were measured from samples taken before and at 1, 2, 4 and 12 hours after pioglitazone administration by HPLC with fluorescence detection (Bui et al. 1997, Radhakrishna et al. 2003). Day-to-day CV was below 18% for montelukast and below 10% for zafirlukast at relevant concentrations.

2.4.4. Repaglinide (Study V)

Plasma repaglinide concentrations were quantified by using a PE SCIEX API 3000 LC-MS-MS system. Reversed-phase chromatographic separation was achieved on a Symmetry C₈ column (150x2.1 mm inner diameter, 3.5 µm particle size; Waters, Milford, MA, USA) using gradient elution. The mobile phase consisted of 10 mM ammonium formate (pH 4.0, adjusted with 99% formic acid) and acetonitrile. The mass spectrometer was operated in positive TurboIonSpray mode, and the samples were analysed via selected reaction monitoring employing the transition of the [M+H]⁺ precursor ion to product ion for repaglinide. The ion transitions monitored for repaglinide were m/z 453 to m/z 230. The limit of quantification was 0.02 ng/ml, and day-to-day CV was 12.6% at 0.1 ng/ml and 5.4% at 2.0 ng/ml (n=7).

2.5. Pharmacokinetic calculations

The pharmacokinetics of the study drugs (pioglitazone, pioglitazone metabolites and repaglinide) were characterised by peak concentration in plasma (C_{\max}), time to C_{\max} (t_{\max}), elimination half-life ($t_{1/2}$), areas under the plasma concentration-time curve from time 0 to 48 hours [AUC(0-48); Studies II and IV] or infinity [AUC(0-∞)] and concentration measured at 48 hours (C_{48} ; Studies II and IV). The C_{\max} and t_{\max} values were taken directly from the original data. For each subject, the terminal log-linear part of the concentration-time curve was identified visually, and the elimination rate constant (k_e) was determined by linear regression analysis of the log-linear part of the concentration-time curve. The $t_{1/2}$ was calculated by the equation $t_{1/2} = \ln 2/k_e$. The AUC values were calculated by the linear trapezoidal rule for the rising phase and the log-linear trapezoidal rule for the descending phase, with extrapolation to infinity, when appropriate, by division of the last measured concentration by k_e . The pharmacokinetic calculations were performed with an MK-model, version 5.0 (Biosoft, Cambridge, UK).

In Study II, the $t_{1/2}$ and the $AUC(0-\infty)$ of the metabolites M-III and M-IV could not be determined reliably during the gemfibrozil and gemfibrozil-itraconazole phases because the C_{max} of the metabolites was reached very late in most subjects due to the inhibition effect. Gemfibrozil, itraconazole and hydroxyitraconazole pharmacokinetics were characterised by C_{max} , concentration measured 48 hours after pioglitazone dosing (C_{48}), AUC from 08:00 (on day 3) to 9 hours after pioglitazone dosing [$AUC(0-10)$] and AUC to 49 hours [$AUC(0-49)$].

In Study IV, the plasma concentrations of parent pioglitazone seemed to decline biphasically, particularly during rifampicin treatment. Therefore, for the first dominant elimination phase (the phase contributing most to the AUC), the elimination rate constant (k_e) was determined by linear regression analysis using the first log-linear phase of the descending plasma concentration curve, and for the slow elimination phase, starting between 9 and 12 hours, a terminal elimination rate constant ($k_{e,terminal}$) was determined by using the log-linear terminal part of the plasma concentration-time curve. The dominant and terminal half-lives for pioglitazone were calculated from the equations $t_{1/2} = \ln 2/k_e$ and $t_{1/2,terminal} = \ln 2/k_{e,terminal}$. The AUC for pioglitazone was calculated by the linear trapezoidal rule for the rising phase and the log-linear trapezoidal rule for the descending phase, with extrapolation to infinity by division of the last measured concentration by $k_{e,terminal}$. The plasma concentrations of the metabolites declined monophasically. An apparent formation rate constant (k_f) was calculated for the primary metabolite M-IV by the method of residuals from the ascending part of the metabolite concentration-time curve.

In Study V, the pharmacokinetics of parent pioglitazone were characterised by C_{max} and AUC from 08:00 (on day 5) to 12 hours after repaglinide dosing [$AUC(0-13)$].

In Studies II and IV, the cumulative 48-hour excretion [$Ae(0-48)$] of pioglitazone (ng) and its metabolites (U) into urine were determined, and the renal clearance (Cl_{renal}) of pioglitazone was calculated from the expression $Ae(0-48)/AUC(0-48)$.

2.6. Pharmacodynamic measurements

In Study V, the pharmacodynamic effects of repaglinide were characterised by the maximum increase and maximum decrease in blood glucose concentration and by mean change in blood glucose concentration (from 0 to 3 hours, 0 to 7 hours and 0 to 12 hours) after repaglinide intake. The mean change was calculated by dividing the net area under the blood glucose concentration-time curve (from 0 to 3 hours, 0 to 7 hours and 0 to 12 hours) by the corresponding time interval.

2.7. *In vitro-in vivo* interaction predictions

In Study V, the effect of pioglitazone on the AUC of a CYP2C8 substrate was estimated on the basis of pioglitazone's previously reported K_i value for CYP2C8 using the following equation:

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

,where $[I]_{\text{in vivo}}$ = inhibitor concentration available to the enzyme *in vivo*
 f_m = fraction of the substrate metabolised via the inhibited enzyme

The $[I]_{\text{in vivo}}$ values were calculated using following equations:

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

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

2.8. Statistical analysis

In Studies II and III, statistical comparisons among the phases were made with repeated-measures ANOVA, with treatment sequence as a factor, and a posteriori testing was conducted with paired t-test with the Bonferroni correction. The t_{max} values were compared with Friedman's two-way ANOVA followed by the Wilcoxon signed-rank test with the Bonferroni correction. In Studies IV and V, the pharmacokinetic and pharmacodynamic variables between the two phases were compared by using a paired t-test, or in the case of t_{max} , by the Wilcoxon signed-rank test. The level of statistical significance was $P < 0.05$ in all studies. Pharmacokinetic variables were log-transformed before the statistical analysis, when appropriate. In Study II, the Pearson correlation coefficient was used to investigate the possible relationship between the concentrations of gemfibrozil and the extent of the interaction. In Study IV, 95% confidence intervals were calculated on the mean differences between the placebo and rifampicin phases for all pharmacokinetic variables, except t_{max} . All the data were analysed with the statistical program Systat for Windows, version 6.0.1 (SPSS Inc., Chicago, IL, USA).

RESULTS

1. Metabolism of pioglitazone *in vitro* (Study I)

Incubation of pioglitazone (1 μM) with HLM resulted in time- and NADPH-dependent substrate consumption and metabolite formation. Two major metabolites, M-IV and M-III, were found. The metabolite M-XI was also detectable, but its formation was very low compared with that of M-IV. In addition, trace amounts of the metabolite M-V were observed. The decline in pioglitazone concentration was log-linear for 60 minutes, and the formation of the major primary metabolite M-IV was linear for at least 8 minutes.

The effects of chemical CYP inhibitors on pioglitazone depletion and M-IV formation in HLM are shown in Table 4. Montelukast and trimethoprim (selective CYP2C8 inhibitors), gemfibrozil (inhibits both CYP2C8 and CYP2C9), ketoconazole, itraconazole and troleandomycin (CYP3A4 inhibitors), rifampicin (inhibits both CYP2C8 and CYP3A4) and DDC markedly inhibited the metabolism of pioglitazone. Montelukast (1 μM) was the most potent inhibitor, with 63% inhibition of pioglitazone metabolism and 85% inhibition of M-IV formation.

Table 4. Percentage of inhibition of pioglitazone (1 μM) metabolism by inhibitors of a range of CYP isoforms in HLM and percentage of pioglitazone depletion by different recombinant CYP enzymes.

Target CYP	Inhibitor	% inhibited		% pioglitazone metabolised by recombinant CYP
		Pioglitazone depletion	M-IV formation	
CYP2C8	Montelukast 1 μM	63	85	56
	Gemfibrozil 100 μM	35	71	
	Trimethoprim 100 μM	37	66	
CYP3A4	Ketoconazole 1 μM	37	55	37
	Itraconazole 3 μM	28	46	
	Troleandomycin 100 μM	17	35	
CYP2C8 and 3A4	Rifampicin 100 μM	14	51	
CYP1A2	Furafylline 20 μM	4	0	15
CYP2C9	Sulfaphenazole 10 μM	5	13	3
CYP2C19	Omeprazole 10 μM	10	15	10
CYP2B6	Clopidogrel 1 μM	10	18	0
CYP2D6	Quinidine 10 μM	6	24	3
CYP2E1	DDC 100 μM	25	33	1
CYP3A5	-	-	-	15

Recombinant CYP2C8 and CYP3A4 decreased the concentrations of pioglitazone by 56% and 37%, respectively (Table 4). When incubated with recombinant CYP3A5, CYP1A2 or CYP2C19, 10-15% of pioglitazone was metabolised. Control supersomes did not metabolise pioglitazone. The formation rate of metabolite M-IV was highest in incubations with CYP2C8, while CYP3A4, CYP3A5 and CYP2C19 catalysed the formation of M-IV at a low rate. Metabolite M-III was also formed by incubation of pioglitazone with CYP2C8, CYP2C19 and CYP1A2.

All the CYP2C8 inhibitors used (montelukast, zafirlukast, trimethoprim and gemfibrozil) concentration-dependently inhibited pioglitazone metabolism in HLM. Their IC_{50} values are shown in Table 5.

Table 5. IC_{50} values of the studied CYP2C8 inhibitors for metabolism of pioglitazone (1 μ M) in HLM.

<i>Inhibitor</i>	<i>IC₅₀ values (μM)</i>	
	<i>Pioglitazone depletion</i>	<i>M-IV formation</i>
Montelukast	0.51	0.18
Zafirlukast	1.0	0.78
Trimethoprim	99	71
Gemfibrozil	98	59

2. Effects of gemfibrozil and itraconazole on pioglitazone (Study II)

2.1. Pioglitazone pharmacokinetics in plasma

Gemfibrozil raised the mean $AUC(0-\infty)$ of unchanged pioglitazone by 3.2-fold ($P < 0.001$) and prolonged its mean $t_{1/2}$ considerably, from 8.3 to 22.7 hours ($P < 0.001$), compared with placebo (Figure 2). An increase in the $AUC(0-\infty)$ (range 2.3-fold to 6.5-fold) and $t_{1/2}$ was observed in every subject. Gemfibrozil had no effect on the C_{max} or t_{max} of pioglitazone.

Itraconazole alone had no significant effect on any of the pharmacokinetic variables of pioglitazone. The gemfibrozil-itraconazole combination raised the $AUC(0-\infty)$ of pioglitazone by 3.9-fold ($P < 0.001$) and prolonged the $t_{1/2}$ from 8.3 to 40 hours ($P < 0.001$), but had no statistically significant effect on the C_{max} of pioglitazone, compared with placebo. No significant differences in pioglitazone pharmacokinetics were observed between the gemfibrozil and gemfibrozil-itraconazole phases.

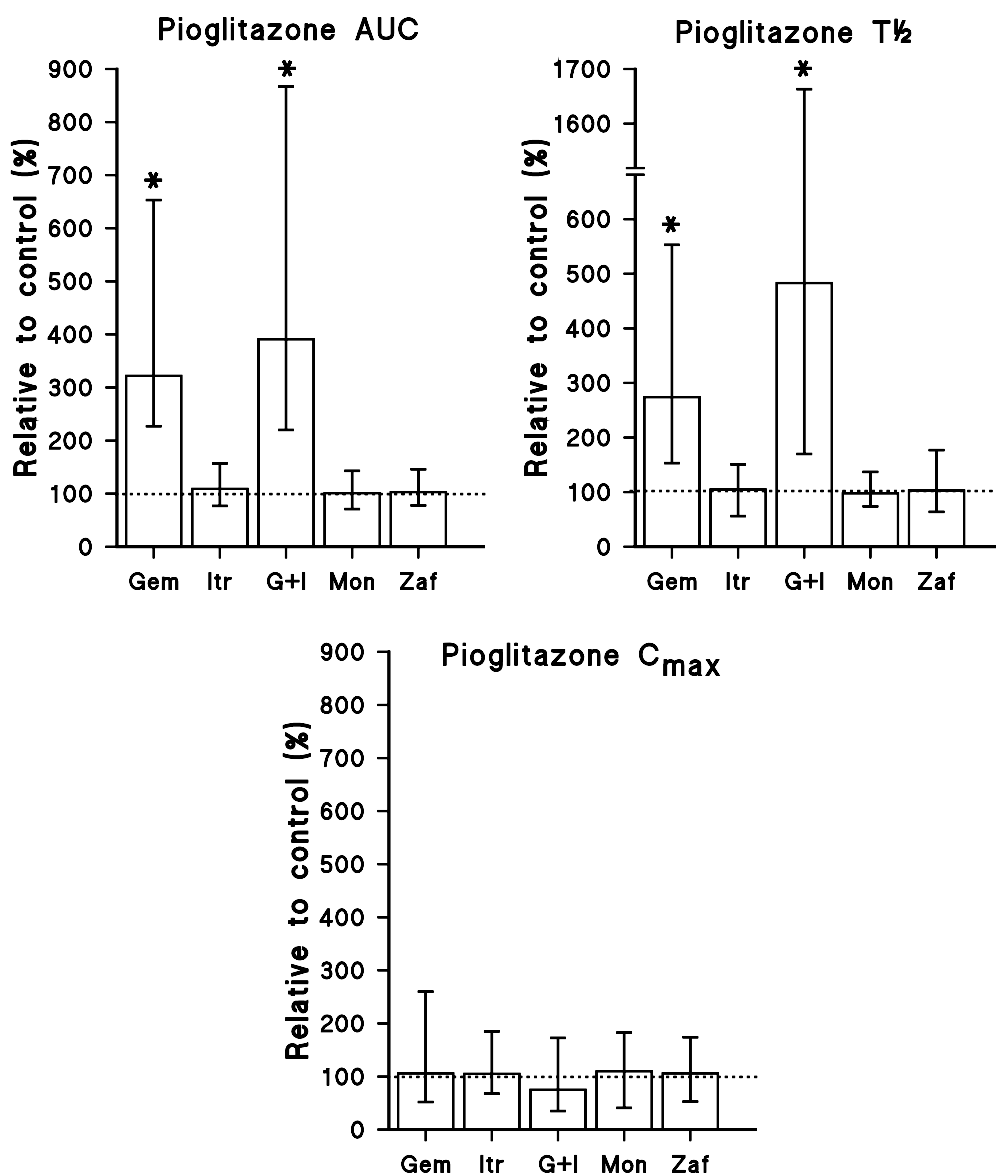


Figure 2. Effect of gemfibrozil (Gem), itraconazole (Itr), both gemfibrozil and itraconazole (G+I), montelukast (Mon) and zafirlukast (Zaf) on pharmacokinetics of pioglitazone (15 mg) in Studies II and III. Ratios of mean values and range of ratios in individual subjects of AUC(0- ∞), $t_{1/2}$ and C_{max} of unchanged pioglitazone during the inhibitor phases to those during the placebo phase (control) are presented. * $P < 0.001$ versus control.

2.2. Pioglitazone metabolites in plasma

Compared with placebo, gemfibrozil reduced the C_{\max} of the active metabolites M-IV and M-III by 49% ($P < 0.001$) and 45% ($P < 0.001$) and the AUC(0-48) by 45% ($P < 0.001$) and 42% ($P < 0.05$), respectively, but the total AUCs of the metabolites were reduced less or not at all. Gemfibrozil reduced the M-IV/pioglitazone AUC(0-48) ratio by 78% ($P < 0.001$) and the M-III/pioglitazone AUC(0-48) ratio by 77% ($P < 0.001$).

Itraconazole alone did not change the plasma concentration of M-IV or M-III, and the gemfibrozil-itraconazole combination caused similar changes in the pharmacokinetic variables of the metabolites as gemfibrozil alone.

2.3. Excretion of pioglitazone and its metabolites in urine

Compared with the placebo phase, gemfibrozil alone increased the amount of unchanged pioglitazone excreted in urine within 48 hours by 2.5-fold ($P < 0.001$) and reduced the urinary excretion of M-IV and M-III by 58% ($P < 0.001$) and 43% ($P = 0.09$), respectively. The Ae(0-48) ratios of M-IV and M-III to pioglitazone were reduced by 82% ($P < 0.001$) and 78% ($P < 0.001$), respectively, by gemfibrozil. Itraconazole had no significant effect on the excretion of pioglitazone. Gemfibrozil and itraconazole did not affect the renal clearance of pioglitazone.

The excretion of M-V in urine was reduced only insignificantly by gemfibrozil alone or itraconazole alone, but during the gemfibrozil-itraconazole phase, the 48-hour excretion of M-V was reduced by 46% ($P < 0.05$) compared with placebo. The Ae(0-48) ratio of M-V to pioglitazone was reduced non-significantly (24%; $P > 0.05$) by itraconazole, by 54% ($P < 0.05$) by gemfibrozil and by 76% ($P < 0.001$) by the gemfibrozil-itraconazole combination compared with placebo.

2.4. Gemfibrozil, itraconazole and hydroxyitraconazole concentrations

The plasma concentrations of itraconazole and hydroxyitraconazole were markedly lower during the gemfibrozil-itraconazole phase than during the itraconazole phase. Gemfibrozil reduced the mean C_{\max} and AUC(0-49) of itraconazole by 48% and 46%, respectively, and those of hydroxyitraconazole by 40% and 46%, respectively ($P < 0.001$). Gemfibrozil pharmacokinetics were not changed by itraconazole. The correlation between the AUC(0-10) of gemfibrozil and the increase in the AUC(0- ∞) of pioglitazone was not statistically significant ($r = 0.245$; $P = 0.43$).

3. Effects of montelukast and zafirlukast on pioglitazone (Study III)

3.1. Pioglitazone pharmacokinetics in plasma

Montelukast and zafirlukast had no significant effects on any of the pharmacokinetic variables of parent pioglitazone (Figure 2). In the montelukast and zafirlukast phases,

the mean $AUC(0-\infty)$ values of unchanged pioglitazone were 101% (range 71-143%) and 103% (range 78-146%) of the control, respectively. The $t_{1/2}$ values of pioglitazone were 7.6 ± 1.7 , 7.5 ± 1.5 and 7.9 ± 1.7 hours in the placebo, montelukast and zafirlukast phases, respectively.

3.2. Pioglitazone metabolites in plasma

Montelukast and zafirlukast had no effect on the pharmacokinetics of the pioglitazone metabolites M-IV, M-III, M-V or M-XI.

3.3. Montelukast and zafirlukast concentrations

On the study day, the mean C_{max} of montelukast and zafirlukast was 550 ± 210 ng/ml (range 220-900 ng/ml) and 300 ± 150 ng/ml (range 160-720 ng/ml), respectively.

4. Effect of rifampicin on pioglitazone (Study IV)

4.1. Pioglitazone pharmacokinetics in plasma

Rifampicin reduced the mean total $AUC(0-\infty)$ of pioglitazone by 54% (range 20-66%; $P < 0.001$) and shortened its dominant elimination $t_{1/2}$ from 4.9 to 2.3 hours ($P < 0.001$), compared with placebo (Figure 3). Rifampicin had no significant effect on the C_{max} , t_{max} or $t_{1/2,terminal}$ of pioglitazone.

4.2. Pioglitazone metabolites in plasma

Compared with placebo, rifampicin increased the apparent formation rate (k_f) of the primary active metabolite M-IV by 131% ($P < 0.01$). The t_{max} of M-IV and M-III was also reached significantly earlier during the rifampicin phase than during the placebo phase. In addition, rifampicin reduced the $AUC(0-\infty)$ of M-IV (by 34%; $P < 0.01$) and M-III (by 39%; $P < 0.01$), shortened their $t_{1/2}$ (M-IV by 50%; $P < 0.001$, and M-III by 55%; $P < 0.01$) and increased the $AUC(0-\infty)$ ratios of M-IV and M-III to pioglitazone by 44% ($P < 0.01$) and 32% ($P < 0.01$), respectively. The $AUC(0-48)$ values of the metabolites remained unaffected.

4.3. Excretion of pioglitazone and its metabolites in urine

Compared with placebo, rifampicin reduced the $Ae(0-48)$ of unchanged pioglitazone by 49% ($P < 0.0001$) and that of M-V by 43% ($P = 0.07$), whereas the $Ae(0-48)$ of M-IV and M-III remained unchanged. A previously unrecognised metabolite, M-XI, tentatively identified as a dihydroxymetabolite, was found in urine during both phases (Figure 4). The $Ae(0-48)$ of M-XI was increased by 44% ($P < 0.05$) by rifampicin. Rifampicin raised the M-IV/pioglitazone $Ae(0-48)$ ratio by 98% ($P < 0.01$), the M-III/pioglitazone ratio by 95% ($P < 0.01$) and the M-XI/pioglitazone ratio by 240% ($P < 0.01$). The renal clearance of pioglitazone was unaffected by rifampicin.

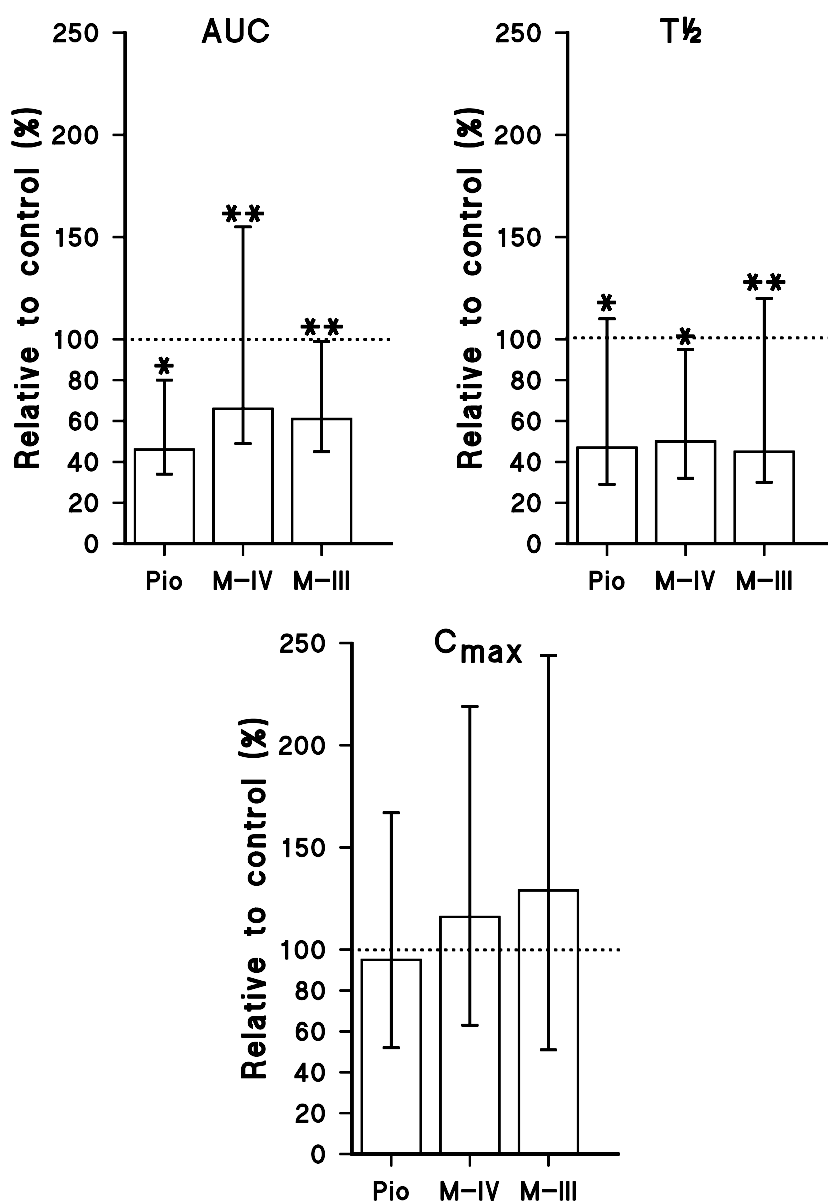


Figure 3. Effect of rifampicin on the pharmacokinetics of pioglitazone (30 mg) in Study IV. Ratios of mean values and range of ratios in individual subjects of AUC(0-∞), $t_{1/2}$ and C_{max} of unchanged pioglitazone and the active metabolites M-IV and M-III during the rifampicin phase to those during the placebo phase (control) are presented. * $P < 0.001$ versus control, ** $P < 0.01$ versus control.

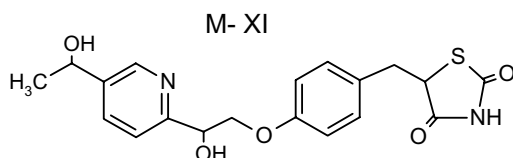


Figure 4. Suggested chemical structure for previously unrecognised dihydroxy-metabolite M-XI.

5. Effect of pioglitazone on repaglinide (Study V)

5.1. Repaglinide pharmacokinetics

Pioglitazone had no significant effects on repaglinide pharmacokinetics. In the pioglitazone phase, the C_{\max} and $AUC(0-\infty)$ of repaglinide were 100% (range 53-157%) and 90% (range 63-120%) of the control values, respectively. The $t_{1/2}$ of repaglinide remained unchanged, about 1 hour in both phases. The median t_{\max} was 40 minutes (range 20-40 minutes) and 20 minutes (range 20-40 minutes) in the placebo and pioglitazone phases, respectively ($P < 0.05$).

5.2. Effect on blood glucose

Pioglitazone did not change the baseline blood glucose concentrations (4.6 ± 0.4 mmol/l) compared with the placebo phase (4.5 ± 0.2 mmol/l). The minimum, maximum and mean change of blood glucose after repaglinide intake remained unchanged by pioglitazone. None of the subjects had symptomatic hypoglycaemia.

5.3. Pioglitazone concentrations

On the study day, the mean C_{\max} and $AUC(0-13)$ of pioglitazone were 778 ± 186 ng/ml (range 378-1040 ng/ml) and 5090 ± 1320 ng·h/ml (range 2770-7250 ng·h/ml), respectively.

DISCUSSION

1. Methodological considerations

1.1. *In vitro* study

The aim of this study was to determine the contributions of different CYP enzymes to the metabolism of pioglitazone and to evaluate the risk of drug interactions in humans on the basis of *in vitro* investigations. Human liver microsomes and recombinant CYP enzymes are widely used and well-characterised systems for drug metabolism and interaction studies (Pelkonen et al. 2005). HLMs contain all the CYP enzymes expressed in the human liver, but different CYP isoforms are present in proportion to their representation in individual donor livers. To avoid interindividual variation in enzyme activity, pooled HLM (same batch in all experiments) with defined enzyme activities adequately representing the population average, was used in the experiments. Because HLMs contain all CYP enzymes, they are suitable for studying mechanism-based inhibitors using preincubation. Studies in HLM were accompanied by recombinant CYP enzymes, which are useful in identifying the role of a specific CYP isoform in the metabolism of a drug or in a particular metabolic pathway.

Because the pharmacological efficacy of pioglitazone is due to the net effect of parent pioglitazone and its major active metabolites M-IV (primary metabolite) and M-III (secondary metabolite formed from M-IV) (Eckland and Danhof 2000), both the depletion of parent pioglitazone and the formation of primary metabolite M-IV were studied after incubations with HLM and recombinant CYPs. The formation of secondary metabolite M-III was also measured, but no IC_{50} values for M-III were calculated because two metabolic steps are involved in its formation. To assure constant metabolism, the incubation times used were within the log-linear part of pioglitazone depletion and the linear part of M-IV formation, respectively. In addition, the concentration of microsomal protein was chosen on the basis of preliminary experiments to meet the initial-rate conditions.

Most drugs are poorly soluble in water, and organic solvents are often indispensable in *in vitro* experiments. An excess of organic solvents can affect enzyme activities of different CYP isoforms to a varying degree depending on the solvent used. Methanol and acetonitrile were used in this study, and their concentrations in final incubation mixtures were kept sufficiently low (<1% v/v) to avoid significant CYP enzyme inhibition (Chauret et al. 1998, Easterbrook et al. 2001). In addition, the effects of inhibitors were compared against control incubations (% of control) containing equal concentrations of solvents. Other factors in incubation conditions, such as ionic strength and pH of the incubation medium, may also influence the outcome of *in vitro* studies (Venkatakrishnan et al. 2001). Sodium phosphate buffer (0.1 M) was used as the incubation medium, which parallels the pH and ionic strength in hepatic cytoplasm *in vivo*.

The choice of substrate concentration is crucial in *in vitro* studies, especially when only a single substrate concentration is used. For example, for a substrate metabolised by multiple CYP enzymes with different affinities, use of a high (supratherapeutic) substrate concentration will overestimate the contribution of a low-affinity isoform, and the important contribution of a high-affinity isoform (at therapeutic concentrations) may be underestimated (Venkatakrishnan et al. 2001). The concentration of pioglitazone used in this study ($1\text{ }\mu\text{M} = 357\text{ ng/ml}$) was chosen to correspond roughly to the peak plasma pioglitazone concentration in humans (at a 15-mg dose, about 300-500 ng/ml) (Eckland and Danhof 2000, Jaakkola et al. 2005). However, plasma concentration of a drug does not directly correlate with its concentration at the site of metabolic activity in a hepatocyte, and the use of single substrate concentration can be misleading.

Because reference compounds were not available, the concentrations of the metabolites were quantified as arbitrary units only. However, the linearity of the detector response of the LC-MS-MS assay could be confirmed for the metabolites within the relevant concentration range by means of a sample dilution. Accordingly, we were able to reliably determine the contribution of different CYP enzymes to the formation of metabolites as relative changes in metabolite concentrations.

The inhibitory potencies of the studied CYP2C8 inhibitors were quantified by determination of IC_{50} values. This approach has the advantage of being model independent; it does not require a kinetic analysis or a knowledge of the mechanism of inhibition (Venkatakrishnan et al. 2001). The IC_{50} values are useful when comparing inhibitory potencies of different candidate inhibitors, but they are applicable only to the specific substrate concentration used (von Moltke et al. 1998). In principle, K_i values are needed if *in vitro* data are used for quantitative *in vivo* scaling (Venkatakrishnan et al. 2001).

Altogether, the use of a single pioglitazone concentration and IC_{50} values causes limitations to the interpretation of the results; this study could have been improved by using a range of substrate concentrations, determining apparent kinetic constants (K_m and V_{\max}) and determining K_i values for the inhibitors.

1.2. *In vivo* studies

All four studies were placebo-controlled, randomised, cross-over studies with a wash-out period of 3-4 weeks. In this design, the subjects serve as their own controls, which limits the effects of interindividual variation and reduces the number of subjects needed. Based on information from previous interaction studies, the number of subjects ($n=10-12$) in each study was estimated to be sufficient to detect any clinically significant pharmacokinetic interaction (about 30% change in AUC at a statistical significance level of $P < 0.05$) without exposing an unnecessarily large group of healthy volunteers to the drugs. Balanced randomisation and adequate wash-out periods were used to minimise the risk of possible period effects or carry-over effects.

The volunteers fasted overnight before administration of study drugs, and food intake during the study days was standardised to reduce possible variation in drug absorption and blood glucose concentrations (Study V). The timing of administration of the study drugs in Studies II, III and V (1 hour after pretreatment dose) was chosen to ensure adequate absorption of the inhibitor drug before administration of the study drug. Rifampicin can act as both an inducer and an inhibitor of CYP2C8 and CYP3A4, and as an inhibitor of hepatic uptake transporters (Bidstrup et al. 2004, Kajosaari et al. 2005a, Lau et al. 2007). The decrease in plasma drug concentrations has been smaller when rifampicin and the target drug (repaglinide) have been administered at the same time (Bidstrup et al. 2004). To avoid this effect, rifampicin was administered at 20:00, i.e. 13 hours before pioglitazone, in Study IV.

Significant inhibition of CYP enzymes has been reported to occur immediately after a single dose of an inhibitor (Lin and Lu 1998). The pretreatment period in the studies with CYP enzyme inhibitors was 3-5 days, and the concentrations of the inhibitors were measured during the study days and ascertained to be at therapeutic levels. It is thus unlikely that the interaction observed with gemfibrozil would have been much greater after a longer pretreatment period. Moreover, the lack of interaction observed with montelukast, zafirlukast and pioglitazone can not be explained by inadequate plasma concentrations of the inhibitors. In Study IV, rifampicin was given for 5 days before administration of pioglitazone. Compared with enzyme inhibition, enzyme induction by rifampicin requires a longer time because new protein must be synthesised. Maximal induction of intestinal CYP enzymes and transporters is achieved in about 1 week, whereas that for hepatic induction can take longer (Lee et al. 1993, Fromm et al. 1996, Niemi et al. 2003a). Thus, it is possible that a longer treatment with rifampicin would have increased the magnitude of interaction observed with pioglitazone. A 5-day pretreatment was chosen on the basis of previous interaction studies and to prevent unnecessarily long exposure of healthy volunteers to rifampicin. Because of slow elimination of total pioglitazone, blood and urine were sampled for 48-96 hours after pioglitazone intake in Studies II-IV. To avoid underestimation of the interaction, pretreatment drugs were administered also during the sample collection period.

Because healthy subjects are more sensitive to the hypoglycaemic effects of repaglinide, only a small dose (0.25 mg) of repaglinide was used. The pharmacokinetics of repaglinide is linear with respect to dose (Hatorp 2002), and it is reasonable to assume that the findings can be extrapolated to normal therapeutic doses (0.5-4 mg) of the drug. The pharmacokinetics of pioglitazone and repaglinide in patients with type 2 diabetes is similar to that of healthy volunteers (Eckland and Danhof 2000, Hatorp 2002). Accordingly, the pharmacokinetic interactions can be presumed to be equal in both groups.

In Study II, the $t_{1/2}$ and AUC(0- ∞) of the metabolites M-IV and M-III could not be determined reliably during the gemfibrozil and gemfibrozil-itraconazole phases because of inhibition of their formation and further elimination by gemfibrozil.

Therefore, a longer sample collection period was used in Study III. The concentrations of the metabolites of pioglitazone were quantified as arbitrary units only because reference compounds were not available. However, the effects (or lack thereof) of the pretreatment drugs on the metabolite pharmacokinetics could be reliably shown, and the results are in good agreement with the pharmacokinetics of parent pioglitazone.

M-V, an inactive polar metabolite, was detected in significant amounts in urine. In addition, in Study IV, a previously unrecognised metabolite, M-XI, was found in urine. Its mass-to-charge ratio of 389 suggests that the compound is a dihydroxymetabolite, formed by further hydroxylation of M-II or M-IV. The neutral loss scan of m/z 389 resulted in the same neutral 223 amu fragment as the parent pioglitazone and its other metabolites. During the placebo phase in Study IV, the $A_e(0-48)$ of M-XI was approximately one-tenth of that of M-V, which is the main compound in urine. The plasma concentrations of the metabolites M-V and M-XI seemed to be very low compared with those of M-IV in Study III, and their rapid excretion into urine is consistent with their polar nature.

In Study IV, the plasma concentrations of unchanged pioglitazone seemed to decline biphasically, particularly during induction by rifampicin. The majority of the elimination of pioglitazone took place during the first phase, and rifampicin considerably shortened “the dominant elimination half-life” of pioglitazone associated with this phase. However, the long terminal half-life of pioglitazone remained unaffected by rifampicin. This could reflect, for example, a slow release of pioglitazone from peripheral tissues, which is not affected by induction. An example of this kind of situation is the elimination of the aminoglycoside antibiotic gentamycin, which is rapidly eliminated from the body during the first phase, before distribution equilibrium is slowly achieved during the terminal phase (Tozer and Rowland 2006). According to a review of original Japanese studies, biphasic elimination of pioglitazone has been observed also previously (Baba 2001), and both one and two elimination half-lives have been used in different studies. In the present studies, the use of two different models in the pharmacokinetic calculations is somewhat inconsistent, but it does not affect the magnitude of the observed interactions or the clinical relevance of these findings.

2. Metabolism of pioglitazone by CYP enzymes *in vitro*

The results from these *in vitro* studies clarify the roles of CYP2C8, CYP2C9 and CYP3A4 enzymes in the metabolism of pioglitazone. Our findings strongly suggest that pioglitazone *in vitro* is metabolised principally by CYP2C8 and to a lesser extent by CYP3A4. Other CYP isoforms seem to play a minor role in the biotransformation of pioglitazone.

The potent CYP2C8 inhibitors montelukast (highly selective for CYP2C8) and zafirlukast (non-selective) strongly inhibited the depletion of the parent pioglitazone

and the formation of the main primary metabolite M-IV, with IC_{50} values very close to their reported values for CYP2C8 (Walsky et al. 2005a, Walsky et al. 2005b). Trimethoprim, a moderately potent, selective inhibitor of CYP2C8 (Wen et al. 2002), and gemfibrozil, a non-selective CYP2C8 inhibitor (Wen et al. 2001, Wang et al. 2002), also markedly inhibited pioglitazone metabolism. In addition, in incubations of pioglitazone with recombinant CYP2C8, the depletion of pioglitazone was rapid and the rate of formation of M-IV was very high, consistent with a major role for CYP2C8 in the metabolism of pioglitazone.

The potent CYP3A4 inhibitors ketoconazole (1 μ M) (Baldwin et al. 1995, Bourri  et al. 1996, Eagling et al. 1998), itraconazole (3 μ M) (Back and Tjia 1991, Olkkola et al. 1994, Wang et al. 1999, Isoherranen et al. 2004) and troleandomycin (100 μ M) (Newton et al. 1995) inhibited pioglitazone metabolism by 17-37%, and the formation of M-IV by 35-55%. While itraconazole and troleandomycin are more selective inhibitors of CYP3A4, ketoconazole inhibits also CYP2C8 activity (by about 30%) at the concentration used (Ong et al. 2000). Thus, inhibition of CYP2C8 may partially explain the inhibitory effect of ketoconazole on the metabolism of pioglitazone. With recombinant CYP3A4 and CYP3A5 enzymes, the rate of pioglitazone depletion was approximately 70% and 50% of that with CYP2C8, and both these CYP3A forms catalysed the formation of M-IV at a low rate compared with CYP2C8. This finding suggests that the CYP3A forms are more important in the formation of other pioglitazone metabolites than in the formation of M-IV.

In HLM, inhibitors of CYP2C8 and CYP3A4 had a slightly greater inhibitory effect on the formation of M-IV than on the depletion of pioglitazone. This suggests that enzymes other than CYP2C8 and CYP3A4 are involved in the formation of other pioglitazone metabolites to a greater extent than in the formation of M-IV. Recombinant CYP2C19 catalysed pioglitazone depletion (10% in 60 minutes) and M-IV formation to some extent, and the non-selective CYP2C19 inhibitor omeprazole (inhibits also CYP2C9 and CYP3A4) (Ko et al. 1997) had a minor effect on the metabolism of pioglitazone. Recombinant CYP1A2 metabolised pioglitazone by 15% in 60 minutes, and catalysed the formation of a low but detectable amount of M-III. However, the potent, selective CYP1A2 inhibitor fyrafylline (Eagling et al. 1998) had practically no effect on pioglitazone elimination or metabolite formation. These findings suggest that CYP2C19 and CYP1A2 may be involved, but to a much lesser degree than CYP2C8 and CYP3A4, in the metabolism of pioglitazone.

Rifampicin, in addition to its well-known inducing properties, is a competitive inhibitor of CYP2C8 and CYP3A4 *in vitro* (Bidstrup et al. 2004, Kajosaari et al. 2005a). Rifampicin (100 μ M) inhibited pioglitazone elimination slightly (by 14%), and, particularly, the (CYP2C8-mediated) formation of M-IV (by 51%). The CYP2E1 inhibitor DDC inhibits also CYP2C8 (Ong et al. 2000) and CYP3A4 activities (Eagling et al. 1998), which explains our finding that DDC inhibited pioglitazone metabolism (by 25%), but recombinant CYP2E1 did not metabolise pioglitazone to any appreciable extent.

Similarly, the CYP2D6 inhibitor quinidine and the CYP2B6 inhibitor clopidogrel slightly inhibited pioglitazone metabolism, but the corresponding recombinant CYP did not metabolise pioglitazone. Quinidine can slightly inhibit CYP3A4 (Newton et al. 1995) and clopidogrel inhibits also CYP2C19 (Richter et al. 2004), which could explain the findings. CYP2C9 did not metabolise pioglitazone to any appreciable extent, and the CYP2C9 inhibitor sulfaphenazole had only a marginal inhibitory effect, suggesting that CYP2C9 is not significantly involved in pioglitazone elimination.

Overall, these results are consistent with a major role of CYP2C8 and a less significant role of CYP3A4 in the metabolism of pioglitazone. The formation of the major primary metabolite M-IV also seems to be predominantly catalysed by CYP2C8. By contrast, we found no evidence of significant involvement of CYP2C9 in the elimination of pioglitazone. CYP2C8 inhibitors montelukast, zafirlukast, gemfibrozil and trimethoprim markedly inhibited pioglitazone metabolism *in vitro*. Accordingly, interaction studies were warranted to evaluate their interaction potency *in vivo*.

3. Effects of CYP2C8 and CYP3A4 inhibition on pioglitazone pharmacokinetics

Inhibition of CYP2C8 by gemfibrozil increased the AUC of pioglitazone over 3-fold and considerably prolonged its $t_{1/2}$ without affecting the C_{\max} of pioglitazone. The C_{\max} , AUC(0-48) and AUC(0-48) ratios of the metabolites M-IV and M-III to pioglitazone were lower during the gemfibrozil than during the placebo phase, indicating that gemfibrozil inhibits the oxidative metabolism of pioglitazone. These effects were seen in every subject, although the extent of the interaction varied substantially between individuals. The potent CYP3A4 inhibitor itraconazole alone had no effect on the pharmacokinetics of pioglitazone, and the itraconazole-gemfibrozil combination caused similar changes in the pharmacokinetics of pioglitazone as gemfibrozil alone.

In vitro, gemfibrozil is a more potent inhibitor of CYP2C9 than of CYP2C8 (Wen et al. 2001, Wang et al. 2002). However, *in vivo*, gemfibrozil is the most potent CYP2C8 inhibitor known, and it has markedly (2.2- to 8.1-fold) increased the AUCs of several CYP2C8 substrates (Backman et al. 2002, Niemi et al. 2003c, Niemi et al. 2003b, Niemi et al. 2006), whereas its effects on CYP2C9-mediated metabolism have been much smaller or even lacking (Niemi et al. 2001b, Lilja et al. 2005, Niemi et al. 2005a). At least part of the *in vivo* inhibitory effect of gemfibrozil on CYP2C8 is caused by its glucuronide metabolite, which can probably accommodate the large active site of CYP2C8 (Schoch et al. 2004, Shitara et al. 2004, Ogilvie et al. 2006). In our study, gemfibrozil considerably increased the AUC and $t_{1/2}$ of parent pioglitazone and reduced the AUC ratios of both M-IV and M-III to pioglitazone, as well as the urinary excretion ratios of both M-IV and M-III to pioglitazone. These results indicate that, also *in vivo*, CYP2C8 has an important role in the total elimination of pioglitazone, and that gemfibrozil inhibits the CYP2C8-mediated metabolism of parent pioglitazone and the formation of M-IV and M-III. The C_{\max} of pioglitazone remained unaffected by

gemfibrozil. Thus, the effect of gemfibrozil on the systemic elimination of pioglitazone was considerable, whereas its effect on the presystemic metabolism of pioglitazone was insignificant, consistent with the low hepatic extraction ratio and negligible first-pass metabolism of pioglitazone (Eckland and Danhof 2000).

Although the half-lives of the metabolites M-IV and M-III could not be determined reliably, the elimination of the metabolites seemed to be considerably slower during the gemfibrozil and gemfibrozil-itraconazole phases than during the other two phases. Accordingly, the total AUCs of the metabolites were concluded not to be markedly reduced by gemfibrozil. This finding has later been confirmed by Deng et al. in an interaction study using a longer sample collection period; gemfibrozil (600 mg twice daily) increased the AUC of parent pioglitazone 3.4-fold and inhibited the further elimination of M-IV and M-III (prolonged the half-lives to about 2-fold) without a significant change in the total AUCs of the metabolites (Deng et al. 2005). Thus, the net effect of gemfibrozil is an increase in the total AUC of active compounds, and accordingly, enhanced efficacy and increased likelihood of concentration-dependent adverse effects of pioglitazone.

On the other hand, the potent CYP3A4 inhibitor itraconazole had no effect on the plasma concentrations of pioglitazone or M-IV and M-III or on their excretion into urine. Furthermore, itraconazole did not alter the effects of gemfibrozil on pioglitazone during the gemfibrozil-itraconazole phase. This indicates that CYP3A4 is of minor importance in the total elimination of pioglitazone *in vivo*, despite its *in vitro* contribution to pioglitazone metabolism. The excretion of M-V in urine was only slightly and non-significantly reduced by gemfibrozil alone, and by itraconazole alone, but it was reduced by 46% during the gemfibrozil-itraconazole phase. Moreover, the Ae(0-48) ratio of M-V to pioglitazone was considerably reduced during the gemfibrozil-itraconazole phase. These findings suggest that both CYP2C8 and CYP3A4 may be involved in the formation of M-V *in vivo*.

According to an update of the Actos prescribing information, ketoconazole has increased the AUC of pioglitazone by 34%. In addition to CYP3A4 inhibition, ketoconazole can inhibit CYP2C8 (Ong et al. 2000). Thus, inhibition of CYP2C8 most likely explains the effect of ketoconazole on pioglitazone pharmacokinetics. In line with this, ketoconazole has also increased the AUC of the CYP2C8 substrate rosiglitazone by 47% (Park et al. 2004b).

During the gemfibrozil-itraconazole phase, the plasma concentrations of both itraconazole and hydroxyitraconazole were considerably lower than during the itraconazole phase. This finding, also observed in two other studies (Niemi et al. 2003c, Niemi et al. 2006), could be explained by a reduction in the oral bioavailability of itraconazole caused by gemfibrozil, or by displacement of itraconazole and hydroxyitraconazole from plasma proteins by gemfibrozil, leading to increased clearance or volume of distribution.

4. Effects of montelukast and zafirlukast on pioglitazone pharmacokinetics

The usual therapeutic doses of montelukast and zafirlukast had no effect on the pharmacokinetics of pioglitazone; the C_{\max} , AUC and $t_{1/2}$ of parent pioglitazone and those of the metabolites M-IV, M-III, M-V and M-XI in plasma remained essentially unchanged. This lack of an interaction was somewhat unexpected since especially montelukast but also zafirlukast are potent inhibitors of CYP2C8 (Walsky et al. 2005a, Walsky et al. 2005b) and of pioglitazone metabolism *in vitro*. These findings strongly suggest that montelukast and zafirlukast do not significantly inhibit CYP2C8 enzyme *in vivo* in humans.

Montelukast is a very potent and selective CYP2C8 inhibitor *in vitro* (Walsky et al. 2005b). It has been reported to be more than 99% bound to plasma proteins *in vivo* (Singulair prescribing information). According to previous predictions based on *in vitro* inhibition data and on therapeutic montelukast plasma concentrations, the expected magnitude of the drug-drug interaction for a CYP2C8-cleared drug ranges from a 2-fold to a 100-fold increase in AUC, depending on whether unbound (assuming 1% free fraction) or total montelukast concentration is used in the prediction (Walsky et al. 2005b). Unexpectedly, montelukast had no inhibitory effect on the elimination of pioglitazone in humans. The apparent discrepancy between the *in vitro* data and these *in vivo* findings could be explained by the high plasma protein binding of montelukast (>> 99%) limiting the concentration available for CYP2C8 enzyme in the hepatocytes. An updated protein binding value of >99.7% for montelukast has been reported (Walsky 2006, personal communication), which yields a prediction of <1.3-fold increase in the AUC of a CYP2C8 substrate when assuming <0.3% free fraction (Table 6). When an assumed 0.1% free fraction is used, montelukast would be estimated to cause only a 10% increase in the AUC of a drug cleared by CYP2C8, which is in agreement with our *in vivo* results. Our results are also consistent with recent *in vivo* findings showing that montelukast does not affect the pharmacokinetics of the CYP2C8 substrates repaglinide and rosiglitazone (Kajosaari et al. 2006, Kim et al. 2006).

Zafirlukast is also a potent inhibitor of CYP2C8 *in vitro* (Walsky et al. 2005a). The *in vitro* inhibitory potency and the circulating concentrations of zafirlukast have suggested a possibility of some degree of CYP2C8 inhibition *in vivo* (Walsky et al. 2005a). However, also zafirlukast is extensively (>99%) bound to plasma proteins (Dekhuijzen and Koopmans 2002) and if the estimations are based on its unbound concentrations, it becomes unlikely that zafirlukast would cause inhibition of CYP2C8 *in vivo* (Walsky et al. 2005a). The lack of effect of montelukast and zafirlukast on pioglitazone pharmacokinetics suggests that these drugs have no significant inhibitory potency towards CYP2C8 *in vivo*.

Table 6. Estimated effect of montelukast on the AUC of a drug metabolised solely by CYP2C8 *in vivo*, based on inhibition of CYP2C8 activity by montelukast *in vitro*.

<i>In vivo</i> inhibitor concentration available to enzyme	<i>Assumed unbound fraction (f_u)</i>	<i>Equation used to estimate in vivo inhibitor concentration ([I]_{in vivo}) available to enzyme</i>	<i>Magnitude of drug interaction (fold increase in exposure)</i>	
			$\frac{AUC_{inhibited}}{AUC_{control}} =$	$\frac{1}{\frac{f_m}{1 + \left(\frac{[I]_{in\ vivo}}{K_i}\right)} + (1 - f_m)}$
Systemic total C _{max}	-	[I] _{in vivo} = C _{max}		99
Systemic free C _{max}	1%	[I] _{in vivo} = f _u · C _{max}		2
Systemic free C _{max}	0.3%	[I] _{in vivo} = f _u · C _{max}		1.3
Systemic free C _{max}	0.1%	[I] _{in vivo} = f _u · C _{max}		1.1

Values used for montelukast include mean peak concentration in plasma in Study III (C_{max}) (550 ng/ml = 0.9 µmol/l), unbound fraction (f_u) (0.1-1%) and inhibition constant (K_i) (0.0092 µmol/l). The fraction of the affected drug metabolised by CYP2C8 (f_m) is assumed to be unity in the expression.

5. Effect of induction on pioglitazone pharmacokinetics

Rifampicin considerably reduced the plasma concentrations of pioglitazone, shortened its dominant elimination t_½, enhanced the formation of the metabolites M-IV and M-III and increased the metabolite/pioglitazone ratios in plasma and urine, indicating that rifampicin induced the metabolism of pioglitazone during its elimination phase. The C_{max} of pioglitazone remained unaffected by rifampicin, consistent with the negligible presystemic metabolism of pioglitazone. Rifampicin also shortened the t_½ of M-IV and M-III, which can be explained by induction of the further metabolism of these metabolites.

Rifampicin is a potent inducer of many enzymes involved in drug metabolism (Niemi et al. 2003a). Although rifampicin has its greatest effect on the pharmacokinetics of CYP3A4 substrate drugs (Backman et al. 1996a, Fromm et al. 1996, Holtbecker et al. 1996, Villikka et al. 1997, Kyrklund et al. 2000), it can have a considerable effect also on the pharmacokinetics of drugs metabolised mainly by CYP2C8 (Niemi et al. 2000, Niemi et al. 2004a, Park et al. 2004a). Because itraconazole had no significant effect on the pharmacokinetics of pioglitazone, even when CYP2C8 was inhibited by gemfibrozil, induction of CYP3A4 alone can not explain the effect of rifampicin on pioglitazone pharmacokinetics. Accordingly, rifampicin seems to reduce the plasma concentrations of pioglitazone mainly by inducing its CYP2C8-catalysed biotransformation in the liver.

Rifampicin increased the apparent formation rate (k_f) of the metabolite M-IV, shortened its t_{\max} and increased the M-IV/pioglitazone ratio in plasma and urine, indicating that rifampicin enhanced the activity of this metabolic step. The further elimination of M-IV was also induced by rifampicin, as reflected by the decrease in the total AUC and $t_{1/2}$ of M-IV. The formation of the secondary metabolite M-III is dependent on the formation of its precursor M-IV. Accordingly, the increases in M-III/pioglitazone ratios were equal to the increases in M-IV/pioglitazone ratios. Furthermore, the total AUC and $t_{1/2}$ of M-III were reduced by rifampicin, indicating induction of the elimination of M-III as well. Thus, the net effect of rifampicin on pioglitazone is a substantial decrease in the total AUC of active compounds, which may deteriorate the clinical efficacy of pioglitazone.

In addition to the active metabolites, metabolites M-V and M-XI were found in urine. Rifampicin showed a tendency to reduce the excretion of M-V into urine, which could be explained by reduced plasma concentrations of parent pioglitazone. The increased excretion of M-XI during the rifampicin phase suggests that its formation is inducible.

6. Effect of pioglitazone on the CYP2C8 and CYP3A4 substrate repaglinide

Administration of pioglitazone did not increase the plasma concentrations of repaglinide; the C_{\max} , AUC and $t_{1/2}$ of repaglinide remained unchanged, and only a clinically insignificant shortening occurred in its t_{\max} . The 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 efficacy of pioglitazone-repaglinide combination therapy has been compared with monotherapy with pioglitazone or repaglinide in type 2 diabetes (Jovanovic et al. 2004). The effect of the combination on glycaemic parameters was much better than that of either drug alone or their sum, but the plasma concentrations of pioglitazone and repaglinide were not determined. Pioglitazone is a relatively potent *in vitro* inhibitor of CYP2C8 and CYP3A4 (Sahi et al. 2003, Walsky et al. 2005a), and repaglinide is susceptible to inhibition of these enzymes (Niemi et al. 2001a, Niemi et al. 2003c, Niemi et al. 2004b, Kajosaari et al. 2006). Accordingly, this study was designed to test the hypothesis that inhibition of repaglinide metabolism by pioglitazone contributes to the synergistic effect observed *in vivo*.

Calculating the maximum *in vivo* inhibitory effect of pioglitazone, using the K_i of 1.7 μM for CYP2C8 (Sahi et al. 2003) and estimated total portal C_{\max} (Eckland and Danhof 2000, Walsky et al. 2005a), pioglitazone could cause an almost 3-fold increase in the AUC of a drug cleared solely by CYP2C8, and about a 1.5-fold increase in the AUC of a drug of which 50% is metabolised by CYP2C8 (such as repaglinide). However, pioglitazone is also highly (97% to over 99%) bound to plasma proteins

(Eckland and Danhof 2000, Actos prescribing information) and calculations based on the free plasma concentrations, estimating practically no increase in AUC, agree well with these *in vivo* findings.

7. Clinical implications

Many drug-drug interactions are beneficial, such as when two oral antidiabetics are combined to yield synergistic pharmacodynamic efficacy. Because of comorbidity related to type 2 diabetes, oral antidiabetic drugs often need to be used concurrently with several other drugs, exposing diabetics also to harmful drug-drug interactions. Pioglitazone is a new antidiabetic drug, and only limited information was available to estimate its potential for drug-drug interactions when it came on the market at the start of this thesis. Although the consumption of pioglitazone is still limited, e.g. in Finland, 0.58 defined daily doses (DDD)/1000 inhabitants compared with 46 DDD/1000 inhabitants for all oral antidiabetic drugs, its use is increasing (National Agency for Medicines, drug consumption statistics 2006). From the perspective of drug safety, we consider it important to investigate potential interactions of this new drug before its wide clinical use.

Pioglitazone can cause fluid retention and peripheral oedema, which can lead to or exacerbate heart failure and pulmonary oedema (Waugh et al. 2006). The precise mechanism of this adverse reaction is not known, but it appears to be a dose-related class effect of the thiazolidinediones (Macfarlane and Fisher 2006). Gemfibrozil raised the mean AUC of pioglitazone over 3-fold, and a 6.5-fold increase was observed in one subject. It is thus possible that concomitant use of gemfibrozil with pioglitazone may increase the risk of this concentration-dependent adverse effect of pioglitazone.

The blood glucose lowering effect of pioglitazone develops gradually over a period of weeks and is dose-dependent (Aronoff et al. 2000). The AUC values of the active metabolites of pioglitazone were not reduced by gemfibrozil. Thus, the net effect of gemfibrozil is likely an increase in the efficacy of pioglitazone, although this was not investigated in the present single-dose study.

Rifampicin considerably decreased the plasma concentrations of total active compounds of pioglitazone, and concomitant use of rifampicin with pioglitazone may decrease the efficacy of pioglitazone. Other CYP2C8 enzyme inhibitors or inducers probably also could alter the plasma concentrations of pioglitazone. It is thus advisable to monitor blood glucose concentrations when starting treatment with gemfibrozil or rifampicin (or other drugs affecting CYP2C8 activity) and to adjust pioglitazone dosage as necessary. Furthermore, reducing the dosage of pioglitazone when rifampicin treatment is discontinued is important. However, further long-term studies in patients with type 2 diabetes receiving pioglitazone are needed to determine the clinical significance of these interactions.

CONCLUSIONS

The following conclusions can be drawn on the basis of the results of Studies I-V:

1. Pioglitazone is metabolised mainly by CYP2C8 and to a lesser extent by CYP3A4 *in vitro*. CYP2C9 appears to be insignificant in the total elimination of pioglitazone. Montelukast, zafirlukast, gemfibrozil and trimethoprim markedly inhibit pioglitazone metabolism *in vitro*.
2. Gemfibrozil elevates the plasma concentrations of pioglitazone, most likely by inhibiting its CYP2C8-mediated metabolism. Concomitant use of gemfibrozil with pioglitazone may increase the efficacy and concentration-related adverse effects of pioglitazone. CYP3A4 appears to be of minor importance in pioglitazone metabolism *in vivo*.
3. Montelukast and zafirlukast do not increase the plasma concentrations of pioglitazone, indicating that their inhibitory effect on CYP2C8 is negligible *in vivo*, despite their strong inhibitory effect on CYP2C8 *in vitro*.
4. Rifampicin caused a substantial decrease in the plasma concentrations of pioglitazone, probably by induction of CYP2C8. Concomitant use of rifampicin with pioglitazone may decrease the efficacy of pioglitazone.
5. Pioglitazone does not increase the plasma concentrations of repaglinide, indicating that the inhibitory effect of pioglitazone on CYP2C8 and CYP3A4 is very weak *in vivo*. The synergistic effect of pioglitazone and repaglinide seen in patients with type 2 diabetes is unlikely to be caused by pharmacokinetic interaction.
6. These findings highlight the importance of *in vivo* interaction studies and the incorporation of relevant pharmacokinetic properties of potentially interacting drugs, including their plasma protein binding, into *in vitro* – *in vivo* interaction predictions.

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