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Inhibition of CYP1A2-mediated drug metabolism *in vitro* and in humans

With special emphasis on rofecoxib and other NSAIDs

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ACADEMIC DISSERTATION

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Helsinki 2008, Yliopistopaino

To Jesse
To my family
To Ali

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ABBREVIATIONS

ABC-carrier	ATP-dependent carrier
Ae	Amount excreted into urine
AhR	Aromatic hydrocarbon receptor
ANOVA	Analysis of variance
AUC	Area under the concentration/effect-time curve
BCRP	Breast cancer resistance protein
b.i.d.	Twice a day
CAR	Constitutive androstane receptor
CI	Confidence interval
CL	Clearance
C_{max}	Peak concentration
COX	Cyclo-oxygenase
CV	Coefficient of variation
CYP	Cytochrome P450
DSST	Digit symbol substitution test
EM	Extensive metabolizer
F_a	Fraction absorbed from the gastrointestinal tract
FDA	U. S. Food and Drug Administration
$f_{m,CYP}$	Fraction metabolized by a certain CYP enzyme
f_u	Unbound fraction
GI-tract	Gastrointestinal tract
GST	Glutathione transferase
HLM	Human liver microsomes
HPLC	High performance liquid chromatography
IC_{50}	Inhibitor concentration producing 50% inhibition of enzyme activity
i.m.	Intramuscular administration
i.v.	Intravenous administration
k_a	Absorption rate constant
k_{deg}	Enzyme degradation constant
k_e	Elimination rate constant
K_i	Inhibition constant for reversible inhibitor
K_I	Mechanism-based inhibitor concentration producing half maximal rate of inactivation
k_{inact}	Maximal rate of inactivation for mechanism-based inhibitor
K_m	Michaelis-Menten kinetic constant
M-	Tizanidine metabolites
MRP	Multidrug resistance-associated protein
m/z	Mass/charge ratio
NADPH	Nicotinamide adenine dinucleotide phosphate
NAT	N-acetyltranferase

NSAID	Nonsteroidal anti-inflammatory drug
NTCP	Na^+-dependent taurocholate co-transporting polypeptide
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
PM	Poor metabolizer
p.o.	Oral administration
PXR	Pregnane X receptor
Q_h	Hepatic blood flow
rhCYP	Recombinant human CYP enzyme
s.c.	Subcutaneous administration
SD	Standard deviation
SEM	Standard error of mean
SLC	Solute carrier family
SSRI	Selective serotonin reuptake inhibitor
SULT	Sulfotransferase
$t_{½}$	Elimination half-life
t.i.d.	Three times a day
t_{max}	Time to peak concentration
UDP	Uridine diphosphate
UGT	UDP-glucuronosyltransferase
UM	Ultrarapid metabolizer
VAS	Visual analog scale
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 Backman JT, **Karjalainen MJ**, Neuvonen M, Laitila J and Neuvonen PJ. Rofecoxib is a potent inhibitor of cytochrome P450 1A2: studies with tizanidine and caffeine in healthy subjects. Br J Clin Pharmacol 2006;62:345-57.

II **Karjalainen MJ**, Neuvonen PJ and Backman JT. Rofecoxib is a potent, metabolism-dependent inhibitor of CYP1A2: implications for *in vitro* prediction of drug interactions. Drug Metab Dispos 2006;34:2091-6.

III **Karjalainen MJ**, Neuvonen PJ and Backman JT. Tolfenamic acid is a potent CYP1A2 inhibitor *in vitro* but does not interact *in vivo*: correction for protein binding is needed for data interpretation. Eur J Clin Pharmacol 2007;63:829-36.

IV **Karjalainen MJ**, Neuvonen PJ and Backman JT. Celecoxib is a CYP1A2 inhibitor *in vitro* but not *in vivo*. Eur J Clin Pharmacol 2008;64:511-9.

V **Karjalainen MJ**, Neuvonen PJ and Backman JT. *In vitro* inhibition of CYP1A2 by model inhibitors, anti-inflammatory analgesics and female sex steroids: Predictability of *in vivo* interactions. Basic Clin Pharmacol Toxicol (In press)

ABSTRACT

Background and aims The cytochrome P450 (CYP) enzymes are responsible for the elimination of numerous xenobiotics, and CYP1A2 is one of the major members of this family, constituting about 12-20% of the total CYP content. The widely-used muscle relaxant tizanidine was recently found to be metabolized mainly by CYP1A2, and it has proven to be a selective and sensitive substrate for *in vivo* interaction studies. Rofecoxib is a nonsteroidal anti-inflammatory drug (NSAID) which was thought to modestly inhibit CYP1A2. Case reports of concomitant use of tizanidine and rofecoxib suggested a strong interaction between them. However, the mechanism was unknown. Other NSAIDs are also often used in combination with muscle relaxants, but little was known about their effects on CYP1A2 activity. In addition, the underlying mechanism of the interaction between tizanidine and oral contraceptives containing ethinylestradiol and gestodene has been unclear. The purpose of this study was to investigate the effect of rofecoxib, several other NSAIDs and female sex steroids on CYP1A2 activity *in vitro* and also to some extent *in vivo*, and to evaluate the predictability of *in vivo* inhibition based on *in vitro* data.

Methods In the *in vitro* experiments in Studies II, III, IV and V, the effects of several NSAIDs, female sex steroids and model inhibitors of CYP1A2 on phenacetin O-deethylation (CYP1A2 activity) was studied in human liver microsomes (HLM), with and without preincubation. The inhibitor concentration causing 50% inhibition of enzyme activity (IC_{50}) and the inhibition constant (K_i) were determined for apparent inhibitors, and inactivation constants (K_I and k_{inact}) for rofecoxib. The *in vivo* studies reported in Studies I, III and IV were performed using a randomized, placebo controlled, two-phase, cross-over design. After pretreament with the inhibitor (rofecoxib 25 mg once daily for 4 days (Study I), tolfenamic acid 200 mg t.i.d. for 3 days (Study III) or celecoxib 200 mg b.i.d for 4 days (Study IV)) or placebo, the subjects ingested a single dose of tizanidine (4 mg in Studies I and III, and 2 mg in Study IV). Plasma (Studies I, III and IV) and urine (Study I) concentrations of tizanidine and its metabolites were measured, and the pharmacodynamic effects were recorded. A day before tizanidine ingestion, a caffeine test was performed.

Results *In vitro*, fluvoxamine, tolfenamic acid, mefenamic acid and rofecoxib potently inhibited CYP1A2 (IC_{50} < 10 µM). Ethinylestradiol, celecoxib, desogestrel and zolmitriptan were moderate (IC_{50} 20-200 µM), and etodolac, ciprofloxacin, etoricoxib and gestodene were weak inhibitors of CYP1A2 (IC_{50} > 200 µM). At 100 µM, other tested NSAIDs and steroids inhibited CYP1A2 less than 35%. Preincubation increased the inhibitory effect of rofecoxib, progesterone and desogestrel. Rofecoxib was discovered to be a mechanism-based inhibitor of

CYP1A2 with a K_I of 4.73 µM and a k_{inact} of 0.070 min^{-1}. *In vivo*, rofecoxib greatly increased the peak plasma concentration (C_{max}) (6.1-fold), area under the concentration-time curve (AUC) (13.6-fold), half-life ($t_{½}$) (from 1.6 h to 3.0 h) and the pharmacodynamic effects of tizanidine. The metabolism of caffeine was also impaired by rofecoxib. Despite their relatively strong *in vitro* CYP1A2 inhibitory effects, tolfenamic acid and celecoxib did not have a significant effect on tizanidine pharmacokinetics or pharmacodynamics or the caffeine test *in vivo* in humans. Tolfenamic acid slightly decreased the AUC of the tizanidine metabolite M-4. Celecoxib, on the other hand, increased the C_{max} of M-3. A competitive inhibition model and the free portal C_{max} of the inhibitor predicted well the effect of fluvoxamine and the lack of effect of tolfenamic acid and celecoxib on tizanidine pharmacokinetics in humans. With this method, mefenamic acid was predicted to increase the AUC of tizanidine slightly. A mechanism-based inhibition model and the free portal C_{max} explained the effect of rofecoxib on tizanidine pharmacokinetics in humans. However, the previously described *in vivo* effects of ciprofloxacin and oral contraceptive steroids could not be predicted from the *in vitro* data.

Conclusions Rofecoxib is a potent mechanism-based inhibitor of CYP1A2, and it greatly increases the plasma concentrations of tizanidine. Thus, care should be taken if rofecoxib is used in combination with CYP1A2 substrates. Tolfenamic acid and celecoxib did not have significant effects on tizanidine pharmacokinetics or pharmacodynamics. Mefenamic acid has some inhibitory effect *in vitro*, but the clinical significance of this finding remains to be studied. Consideration of the mechanism of inhibition and use of the free plasma concentration of the inhibitor allows many but not all of the CYP1A2 interactions to be relatively well predicted from *in vitro* data. For example, the previously observed effects of the *in vivo* inhibitors ciprofloxacin and oral contraceptives are underpredicted, and their mechanisms of inhibition warrant further investigations.

INTRODUCTION

The cytochrome P450 (CYP) enzymes are responsible for the elimination of numerous xenobiotics. Inhibition or induction of CYP enzymes by other compounds may significantly alter plasma concentrations of drugs that are substrates for these enzymes. CYP1A2 is one of the major metabolizing enzymes, and it accounts for 12-20% of the total CYP content of the liver.[48,111] Inter-individual variation in CYP1A2 activity is due mainly to genetic factors.[137,158] The active site of CYP1A2 is surrounded by several aromatic residues, and that is why its substrates are usually small, lipophilic and planar molecules,[114] including caffeine, clozapine, phenacetin, theophylline, tizanidine and zolmitriptan.[12,13,41,69,204,211] Fluvoxamine, ciprofloxacin and furafylline are model inhibitors of CYP1A2,[22,59,116] whereas, for example, polycyclic aromatic hydrocarbons in tobacco smoke and carbamazepine are thought to be inducers.[75,97]

The widely-used muscle relaxant tizanidine was recently discovered to be metabolized mainly by CYP1A2. Thereafter, it has proven to be a selective and sensitive probe substrate for *in vivo* interaction studies.[8,69-72] The first-pass metabolism of tizanidine is extensive, and its oral bioavailability is only about 10-30%.[72,196] CYP1A2 inhibitors such as fluvoxamine, ciprofloxacin and oral contraceptives containing ethinylestradiol and gestodene greatly increase the plasma concentrations and pharmacodynamic effects of tizanidine.[70-72]

Rofecoxib is a nonsteroidal anti-inflammatory drug (NSAID). Its metabolism has been considered to be mainly non-CYP mediated.[177] According to the manufacturer, rofecoxib might be a modest inhibitor of CYP1A2, as 25 mg of rofecoxib raised the area under the concentration-time curve (AUC) of theophylline and *R*-warfarin by 51% and 38%, respectively.[6,172,177] However, there have been several cases reported to the U. S. Food and Drug Administration (FDA) where concomitant use of tizanidine and rofecoxib led to heightened adverse effects e.g. dizziness, drowsiness and hypotension.[53] Prior to the present study, the mechanism of this interaction was unknown.

NSAIDs and muscle relaxants are often used in combination.[178] However, at the beginning of this thesis work, little was known about the effects of a number of NSAIDs on CYP1A2 activity. The purpose of this study was to investigate the effect of rofecoxib, several other NSAIDs and female sex steroids on CYP1A2 activity *in vitro* and to some extend *in vivo*, and to determine whether *in vivo* inhibition can be predicted based on *in vitro* data.

REVIEW OF THE LITERATURE

1. Drug metabolism and transport

Once a drug enters the body, it is processed in several phases (absorption, distribution, metabolism and excretion) until it leaves the body (Figure 1). This thesis is focused on metabolism, which is an important step in the elimination of most drugs. The purpose of metabolism is to turn a lipophilic drug into a hydrophilic one, which can then be excreted into urine or feces by passive or active processes. Orally administered drugs can undergo first-pass metabolism, which occurs in the intestine or liver prior to entrance of the drug into systemic circulation.

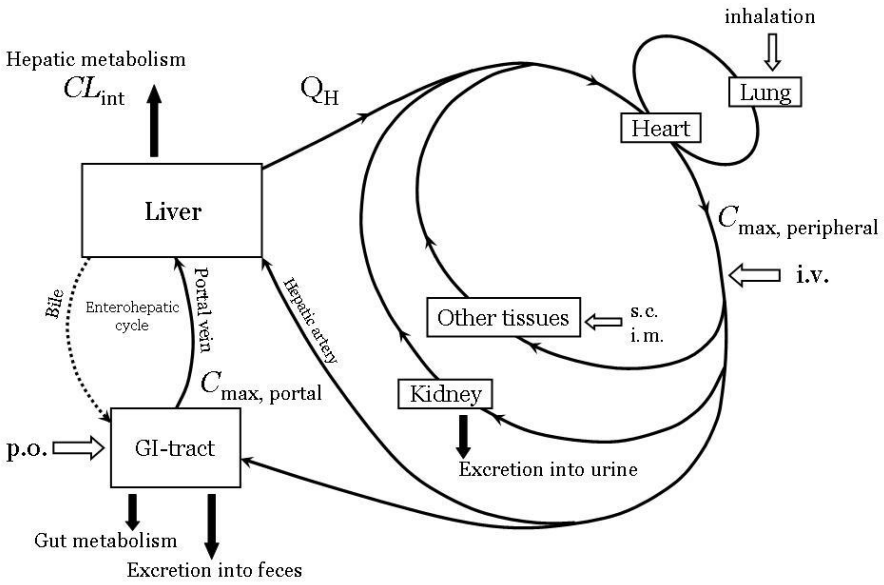


Figure 1. Basic concepts of drug absorption, distribution, metabolism and excretion. CL_{int} is the intrinsic hepatic clearance of the drug, C_{max} is the peak plasma concentration, GI-tract is the gastrointestinal tract, Q_H is the hepatic blood flow, p.o. is oral, s.c. subcutaneous, i.m. intramuscular and i.v. intravenous administration.

Drug metabolism takes place mostly in the liver, but metabolizing enzymes are also expressed in extrahepatic tissues such as in the gut epithelium, lungs and kidneys. Drug metabolizing enzymes include phase I enzymes, which are responsible for functionalization, and phase II enzymes, which are responsible for conjugation. Despite the nomenclature, the reactions can occur in reverse order as well. However, in most cases a functional group, e.g. a hydroxyl group, is attached to the drug by a phase I enzyme, and then a phase II enzyme adds a conjugate to this functional group. Movement of molecules across biological membranes into and out

of cells can occur by passive diffusion or by active transport via influx and efflux transporters, respectively.

Drug metabolism can lead to inactivation of an active compound, activation of an inactive compound i.e. prodrug, formation of an active metabolite from an active compound or toxification of an active or inactive compound. Thus, changes in the activity of a drug-metabolizing enzyme can have different consequences depending on the properties of the compound. Moreover, a decrease in the enzyme activity leads to increased plasma concentrations of the substrate drug, which usually means increased effects and toxicity, and *vice versa*, an increase in enzyme activity leads to decreased plasma concentrations of the substrate, which most often reduces the effectiveness of the drug. Alterations in enzyme activity are most relevant in the case of drugs with a narrow therapeutic window.

1.1. Phase I reactions and enzymes

CYP enzymes are the most important phase I enzymes. They most importantly catalyze oxidation, but also e.g. reduction and hydrolysis. CYP families are groups of CYP enzymes with 40% homology in amino acid sequence, and each different family is designated by an Arabic numeral. A subfamily is a group of CYP enzymes with 55% homology, and it is designated with a letter. Finally, there are individual enzymes, each of which is the product of a single gene and is designated by a final Arabic numeral.[128] Enzyme families 1, 2 and 3 have been shown to be responsible for the metabolism of xenobiotics. Other CYP enzymes metabolize endogenous compounds such as fatty acids, steroids and cholesterol.[125]

The substrates of the CYP enzymes vary greatly, even within the same enzyme subfamily. In addition, a compound can be metabolized by several CYPs. Some examples of CYP substrates, inhibitors and inducers are given in Tables 1 and 2. CYP enzymes are distributed throughout the body, especially at locations which come into contact with the environment such as the intestine, skin and nasal epithelia. The liver and intestines are the most important locations for drug metabolism.

1.1.1. The CYP1 family

CYP1A1 is expressed mainly in extrahepatic tissues such as the lung, mammary glands, lymphocytes and placenta.[148] It can be induced by polycyclic aromatic hydrocarbons like those in cigarette smoke. Its substrate specificity overlaps that of CYP1A2.

Table 1. Examples of inhibitors, substrates, inducers and *in vitro* probe reactions of CYP1A2.

Inhibitors	Substrates	Inducers	*In vitro* probe reactions
Ciprofloxacin[1]	Caffeine[4]	Broccoli[10]	Caffeine N_3-demethylation[12]
Enoxacin[1]	Clozapine[1]	Charcoal grilled meat[1]	7-ethoxyresorufin O-dealkylation[12]
Fluvoxamine[1]	Melatonin[5]	Cigarette smoke[1]	Phenacetin O-deethylation[12]
Furafylline[2]	Paracetamol[6]	Omeprazole[11]	
Rofecoxib[3]	Phenacetin[2]	Rifampicin[1]	
	R-warfarin[7]		
	Theophylline[1]		
	Tizanidine[8]		
	Zolmitriptan[9]		

1. Hersh and Moore, 2004, 2. Pelkonen et al., 1998. 3. Karjalainen et al., Studies I and II. 4. Bertz and Granneman, 1997. 5. Härtter et al., 2001. 6. Lin and Lu, 1998. 7. Kaminsky and Zhang, 1997. 8. Granfors et al., 2004a. 9. Wild et al., 1999. 10. Hakooz and Hamdan, 2007. 11. Ma and Lu, 2007. 12. Madan et al., 2002.

CYP1A2 is the most important enzyme of the 1A subfamily. Its proportion of the total drug metabolizing CYP enzymes in the liver has been suggested to be about 12-20%.[48,111,169,174,175] CYP1A2 is mostly a hepatic enzyme, but some expression has been observed in, for instance, the lung, where it is inducible.[40] Substantial inter-individual variation in the activity of CYP1A2, e.g. the metabolism of caffeine, has been found. It has been suggested that this variation is caused by genetic factors, although actual polymorphisms have not been found.[91,128,137,158] The variant allele *CYP1A2*1K* is associated with a remarkably decreased enzyme activity, but it is very rare.[91] CYP1A2 has a relatively small active site due to the several surrounding aromatic residues, and therefore, its substrates and inhibitors are usually small, lipophilic and planar molecules.[14,114] Examples of CYP1A2 substrates, inhibitors, inducers and *in vitro* probe reactions are presented in Table 1. The substrates of CYP1A2 include caffeine, clozapine, melatonin, phenacetin, *R*-warfarin, theophylline, tizanidine and zolmitriptan.[12-14,41,69,82,101,204,211] *In vitro*, caffeine and phenacetin are the most frequently used probe substrates,[128] and caffeine, melatonin and tizanidine have proven to be suitable *in vivo* probe compounds.[9,10,26,48,69,71,72,81,82] CYP1A2 is highly inducible at the mRNA and enzyme levels by environmental factors, such as polycyclic aromatic hydrocarbons in cigarette smoke, dietary agents in, for example, cruciferous vegetables, and pharmaceutical agents such as omeprazole.[78,85,118,127] However, the inducibility of CYP1A2 *in vivo* seems to be much lower than that of, for example, CYP3A4, and there seems to be large inter-individual variability in its inducibility by, for example, omeprazole.[8,10,127] The structures of CYP1A2 inhibitors seem to be fairly similar to those of substrates. Furafylline, which is structurally very similar to theophylline, is a potent mechanism-based inhibitor of CYP1A2.[148] Some fluoroquinolones such as enoxacin and ciprofloxacin are relatively weak inhibitors of CYP1A2 *in vitro*, but *in vivo* they significantly impair the metabolism of several CYP1A2 substrates.[59,62,143] The serotonin selective reuptake inhibitor (SSRI)

fluvoxamine is a potent inhibitor of CYP1A2 both *in vitro* and *in vivo*.[21,22,148] For example, it has been found to increase the dose-adjusted concentration of clozapine 5- to 10-fold, and the peak plasma concentration (C_{max}) of tizanidine 12-fold.[71,97]

1.1.2. The CYP2 family

CYP2A6 is mainly a hepatic enzyme (it accounts for 4-8% of the total hepatic CYP content)[169,174], but there is some expression in extrahepatic tissues such as the nasal mucosa and bronchial epithelial cells.[149,159] It does not have many known substrates due to its restricted substrate binding site which contains several hydrophobic amino acids.[157,159,182] For example, coumarin and nicotine are metabolized by CYP2A6, and its inhibitors are thought to be potential aids in the cessation of smoking.[157,159,182] Possession of an amine group and an aromatic ring is thought to be important in order for a molecule to be an effective inhibitor of CYP2A6.[157] Smoking, methoxalen and grapefruit juice seem to inhibit CYP2A6, and some antiepileptic drugs appear to induce it.[149] Several functional polymorphisms of CYP2A6 have been found, and the inter-individual variability in the activity of CYP2A6 can be up to 300-fold.[91,157]

CYP2B6 has been acknowledged to be an important drug metabolizing enzyme in recent years, and it represents about 1-10% of the total hepatic CYP content.[191] CYP2B6 is mainly a hepatic enzyme, but some extrahepatic expression has been observed, for example in the brain, intestine and lung.[66] Its expression has been reported to vary considerably between individuals,[191] and a couple of variant alleles have been found to be associated with clinically significantly lower CYP2B6 activity, and also an allele with higher activity has been found.[91] CYP2B6 substrates are usually non-planar, neutral or weakly basic and fairly lipophilic molecules.[191] Bupropion is an established probe substrate *in vitro* and *in vivo*, and other substrates include cyclophophamide, propofol and efavirenz.[191,192] The antiplatelet agents ticlodipine and clopidogrel are potent mechanism-based inhibitors of CYP2B6, but they are somewhat unselective.[164,190-192] Thiotepa is a slightly less potent inhibitor than ticlodipine, but it is more selective.[190] Rifampicin and phenobarbital have been found to induce CYP2B6.[192]

CYP2C8 appears to be involved in the biotransformation of at least 5% of the xenobiotics cleared by phase I processes, and its proportion of the total microsomal CYP content in the liver is about 5-7%.[169,187] In addition to the liver, CYP2C8 is located in several other tissues including the kidney, intestine, adrenal gland, brain and female reproductive organs.[112,187] The active center of CYP2C8 is relatively large, and it shares many substrates with CYP3A4. However, the reactions are usually different due to structural differences at the active site. Typical CYP2C8

substrates are quite large, mildly acidic, basic or neutral, and they include cerivastatin, paclitaxel, repaglinide and rosiglitazone.[187] CYP2C8 inhibitors include gemfibrozil, trimethoprim and montelukast. However, montelukast does not inhibit CYP2C8 *in vivo* despite strong inhibition *in vitro* due to its low free concentration in plasma.[99] CYP2C8 has been found to have at least two genetic polymorphisms (*2 and *3) that lead to altered enzyme activity.[187] The clinical consequences of variant alleles have been found to be substrate specific.[91] For example, *CYP2C8*3* seems to decrease the metabolism of *R*-ibuprofen, but increase that of repaglinide.[131,139]

CYP2C9 appears to be one of the most abundant of the drug metabolizing CYPs in the liver – 17-35% of the total.[111,169,175] In addition to the liver, CYP2C9 has been found in the adrenal gland, prostate, kidney, testes and ovaries.[112] Many NSAIDs and *S*-warfarin are CYP2C9 substrates, which are typically acidic and contain constituents with the potential to hydrogen bond.[14,148,163] *S*-warfarin, tolbutamide and diclofenac are the best *in vitro* probe substrates, and tolbutamide, flurbiprofen and phenytoin are the most commonly used *in vivo* probe substrates of CYP2C9.[148,163] Azole antifungals and sulfaphenazole are potent CYP2C9 inhibitors.[141,148] CYP2C9 is polymorphically expressed, and the variant alleles *CYP2C9*2* and *CYP2C9*3*, which are present mainly in Caucasians, are associated with significantly lower CYP2C9 activity than the wild type *CYP2C9*1*.[64,91]

CYP2C19 accounts for about 3% of the total liver CYP content,[169] and some extrahepatic expression has been observed in the intestine.[40,112] CYP2C19 substrates include compounds with variable structural or physicochemical properties, e.g. moclobemide, proguanil, diazepam, omeprazole and imipramine.[148] In drug interaction studies, the most widely used CYP2C19 probe substrates have been omeprazole and mephenytoin, but diazepam and imipramine can also be used. Many SSRIs are inhibitors of CYP2C19, fluvoxamine and fluoxetine having the most potent, though not selective, effect.[148] The CYP2C19 gene is also polymorphic. The poor metabolizer (PM) variant alleles are more abundant in Oriental (13 to 23% PMs) than Caucasian (1 to 8% PMs) populations.[64,91] PMs lack CYP2C19 activity completely.

CYP2D6 represents about 2 % of the total liver microsomal CYP content.[169,174] In addition to the liver, it is also located in, for example, the brain and epithelial tissues such as the lung.[25] Its substrates usually contain a basic nitrogen atom, which is ionized at physiological pH, and can therefore interact with an aspartic acid residue in the active site of CYP2D6. In addition, many have an extended hydrophobic region, and the ability to accept hydrogen bonds.[14] Of the many CYP2D6 substrates, including some tricyclic antidepressants, beta-blockers, SSRIs and analgesics,[182] the most commonly used probes in interaction studies in humans are

debrisoquine, dextromethorphan and metoprolol.[20,148] The basic nitrogen atom is a common structure for CYP2D6 inhibitors as well although it is not required. Quinidine is one of the most potent inhibitors of CYP2D6,[148] and terbinafine and some SSRIs also inhibit the enzyme considerably.[1,85] The first of the CYP polymorphisms to be found were those for CYP2D6, and they are clinically relevant, as about 25% of drugs are metabolized by CYP2D6 and the metabolism of 50% of them is affected by these polymorphisms.[91] There are null alleles, such as *CYP2D6*4* and **5*, and common alleles with significantly reduced activity, such as **10*, **17* and **41*.[91] Approximately 7% of Caucasians and 1% of Orientals are PMs of CYP2D6 substrates.[64] In addition, gene duplication of active alleles can lead to ultra rapid metabolism.[91]

CYP2E1 contributes about 7-15% to the total CYP content of the liver,[111,169,174] and it has also been found in, for example, the lung and the intestine.[40] Many of its substrates are carcinogens, but several anesthetics are also metabolized by this enzyme.[148] Disulfiram is probably the best known inhibitor of CYP2E1. Many organic solvents also act as CYP2E1 inhibitors, which could interfere with *in vitro* testing.[148]

1.1.3. The CYP3 family

CYP3A4 is known to be involved in the metabolism of over 50% of xenobiotics, more than any other CYP, and its proportion of the total drug metabolizing CYPs in the liver is about 22-36%.[148,169,174,175] CYP3A4 is mainly located in the liver, but substantial amounts can be found in the intestinal wall as well.[14] In addition, it is expressed in the lung, kidney, brain and placenta. The active site of CYP3A4 is large, and its substrates are often lipophilic compounds, which are bound to the enzyme mainly by hydrophilic forces. These forces are weak and allow the substrates to occupy different positions in the active site, and therefore, allow formation of different metabolites. Midazolam, testosterone and nifedipine can be used as CYP3A4 substrates in drug-drug interaction studies, and the use of at least two different substrates for phenotyping is recommended.[148,197] Many of the azole antifungal agents, macrolides and HIV protease inhibitors are potent CYP3A4 inhibitors. CYP3A4 can be induced by several factors, including dietary agents, hormones and drugs.[148] Although functionally significant polymorphisms for CYP3A4 have not been found, there is substantial inter-individual variation in its activity.[14,91]

CYP3A5 is a minor enzyme in the liver, but there is marked inter-individual variation in its amount.[148] It has been found in the lung, adrenal glands, GI-tract and kidney.[33] Significant polymorphisms for the enzyme have been observed, and most

Caucasians have an allele associated with severely impaired CYP3A5 activity.[91] **CYP3A7** has been found mostly in fetuses, and its amount drastically drops after birth.[148]

Table 2. Examples of inhibitors, substrates and inducers of CYP2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4.

CYP2B6	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP2E1	CYP3A4
Inhibitors						
Clopidogrel[1] Ticlodipine[1]	Gemfibrozil[2] Trimethoprim[2]	Amiodarone[5] Fluconazole[3] Fluvoxamine[5] Miconazole[5] Sulfaphenazole[4]	Fluconazole[3] Fluvoxamine[5] Ketoconazole[5] Omeprazole[5]	Celecoxib[5] Fluoxetine[5] Paroxetine[5] Quinidine[5] Terbinafine[7]	Disulfiram[5]	Clarithromycin[5] Grapefruit juice[4] HIV protease inhibitors[5] Azoles[5]
Substrates						
Bupropion[1] Cyclophosphamide[1] Efavirenz[1] Ketamine[1] Nevirapine[1] Propofol[1] Selegiline[1]	Cerivastatin[2] Ibuprofen[2] Paclitaxel[3] Repaglinide[2] Rosiglitazone[2]	Celecoxib[5] Diclofenac[5] Ibuprofen[5] Losartan[5] Naproxen[5] Phenytoin[5] S-warfarin[5] Tolbutamide[5]	Citalopram[5] Diazepam[5] Proguanil[3] Proton pump inhibitors[5] R-warfarin[3] S-mephenytoin[6]	Amitriptyline[5] Codeine[5] Dextromethorphan[8] Fluoxetine[3] Fluvoxamine[3] Haloperidol[5] Metoprolol[5] Tramadol[5]	Caffeine[5] Theophylline[6] Paracetamol[5]	Alprazolam[5] Cyclosporine[5] Erythromycin[5] HIV protease inhibitors[5] Midazolam[5] Simvastatin[5]
Inducers						
Phenobarbital[1] Rifampicin[1]	Phenobarbital[4] Rifampicin[4]	Phenobarbital[5] Phenytoin[3] Rifampicin[5]	Carbamazepine[5] Phenobarbital[4] Rifampicin[5]	Unknown	Isoniazid[5] Ethanol[5]	Carbamazepine[5] Phenobarbital[5] Phenytoin[5] Rifampicin[5]

1. Turpeinen et al., 2006. 2. Totah and Rettie, 2005. 3. Bertz and Granneman, 1997. 4. Pelkonen et al., 1998. 5. Hersh and Moore, 2004. 6. Lin and Lu, 1998.
7. Abdel-Rahman et al., 1999. 8. Broly et al., 1989.

1.2. Phase II reactions and enzymes

Phase II enzymes aid in transforming a lipid-soluble compound into a polar agent, which can easily be excreted from the body in urine or feces. In many cases these conjugation reactions occur after phase I reactions, and are thus called phase II reactions. However, conjugation can also occur with an untransformed drug. Conjugations include addition of a glucuronide, sulfate, acetyl or methyl groups, glutathione or an amino acid.

Glucuronidation Glucuronidation is an important step in the elimination of many compounds, but it can also be the activator of a compound in some cases.[55] UDP-glucuronosyltransferases (UGTs) catalyze the addition of UDP-glucuronic acid to a compound.[201] There are about 20 different UGT enzymes, and they are divided into two families, UGT1 and UGT2, each family member having at least 50% sequence homology with each other.[201] UGT families are further categorized into subfamilies (UGT1A, UGT2A and UGT2B),[126] and eventually given a final number to indicate each individual enzymes. UGTs are located in, among other places, the liver, kidney, intestine, adrenal glands, lung and brain. The distribution of UGTs is enzyme specific, but the factors that regulate tissue-specific expression are unidentified. The substrates of UGTs can vary greatly, and this is the most common phase II reaction for both xenobiotics and endogenous compounds like bile acids, steroid hormones, biogenic amines and thyroid hormones. In the liver, the most abundant UGT isomer seems to be UGT1A1.[201] It is the main enzyme responsible for glucuronidation of bilirubin. Other specific substrates include some phenols and ethinylestradiol. There is great inter-individual variation in the expression of UGT1A1, and it is inducible by, for instance, phenytoin.[201] Gilbert's syndrome is caused by a deficiency of UGT1A1. UGT2B7 and UGT1A4 along with UGT1A1 appear to be responsible for the glucuronidation of a large number of frequently prescribed drugs.[205] UGT1A6 and UGT1A7 seem to catalyze biotransformation of paracetamol (acetaminophen), perhaps protecting the body from its hepatotoxic effect.[201]

Sulfation Besides being involved in the transformation of different compounds in the phase II conjugation pathway, sulfation is also important in the synthesis and metabolism of steroids, and the generation of reactive electrophilic forms of various compounds.[50,68,188,200] Sulfation is catalyzed by sulfotransferases (SULTs), which are mainly cytosolic enzymes.[200] SULTs are divided into families, and family members share 45% sequence homology.[200] The substrates of phenol SULTs (SULT1) are usually small phenolic compounds like paracetamol and dopamine, and those of hydroxysteroid SULTs (SULT2) are steroids such as dehydroepiandrosterone and pregnenolone.[50,188] However, there is considerable overlap in the substrates. SULT families are further divided into subfamilies, the

members of which share 60% sequence homology. SULT1E1 seems to play a major role in the sulfation of ethinylestradiol.[210]

Glutathione conjugation Nucleophilic attack by reduced glutathione is catalyzed by glutathione transferases, historically called glutathione-S-transferases, thus the abbreviation GST.[83] Halogenonitrobenzenes, arene oxides, quinones and α,β-unsaturated carbonyls are among their substrates.[83] There are three protein families with GST activity: the soluble cytosolic and mitochondrial GSTs and the membrane-bound microsomal GST.[83] Addition of glutathione to an exogenous compound can lead to its detoxification or, more rarely, to its bioactivation. GSTs can also detoxify, degrade, synthesize and inactivate endogenous compounds.

Acetylation Acetyl-CoA-dependent N- or O-acetylation of arylamine and hydrazine drugs is catalyzed by arylamine-N-acetyltransferases (NAT).[133] Two isoenzymes have been identified - NAT1 and NAT2 - which both are genetically polymorphic.[64,133] Individuals with two variant alleles have impaired acetylation activity.[133] NATs are responsible for drug detoxification and carcinogenic activation of various environmental and therapeutic chemicals. The two NATs have their own substrate specificities, but some overlap occurs, e.g. isoniazid, sulfonamides and caffeine are metabolized by NAT2, p-aminosalicylic acid by NAT1 and 2-aminofluorene by both.[133] Some of the NAT substrates are products of oxidative metabolism by, for example, CYP enzymes.

1.3. Drug transporters

Drug transporters may control the access of a drug to metabolizing enzymes. Lipophilic drugs can easily pass through biological membranes, but many hydrophilic drugs need to be transported.[153] Uptake transporters deliver drugs to the enterocytes, hepatocytes, renal tubular cells etc., and efflux transporters transport compounds out of cells into the blood stream, bile or urine.[153] Like drug metabolizing enzymes, transporters can also be inhibited and induced by various compounds.[7] Many transporter genes have polymorphisms that affect activity, which can alter the pharmacokinetics of the substrate drugs and affect the efficacy of drug therapy. In addition, they can also be the mechanism behind a disease.[153]

1.3.1. Uptake transporters

The solute carrier family (SLC) is a family of uptake transporters. They transport compounds into the cells from the blood via the basolateral membrane in the liver and kidney, or via the luminal membrane in the intestine.[153] The SLC family

includes the previously identified OATP (organic anion transporting polypeptide) transporter family, organic anion transporters (OAT) and organic cation transporters (OCT).[153] OATPs transport both endogenous compounds and xenobiotics, which can be weak organic acids, neutral compounds and even some cationic compounds. OAT family transporters can move both negatively and positively charged compounds, and OCTs are specialized in carrying organic cations.[153]

Sodium-dependent taurocholate co-transporting polypeptide (NTCP) is the main transporter responsible for hepatic uptake of bile acids, which is an important part of their enterohepatic recirculation.[108] It appears that NTCP activity regulates activities of CYP enzymes and other transporters, as bile acids are ligands for some orphan nuclear hormone receptors.[108]

1.3.2. Efflux transporters

ATP-dependent carriers (ABC-carriers) pump compounds out of the cells, protecting them from xenobiotics. As such, these transporters can cause resistance to drug therapy. Inhibition of efflux transporters can lead to increased systemic exposure and decreased excretion of the drug.[54]

ABCB1, previously known as P-glycoprotein, is the most well known ABC-carrier. ABCB1 is encoded by multi-drug resistance genes.[153] It is expressed especially in some cancers, but also in normal tissue like the liver, kidney, small intestine, colon, adrenal glands, brain and testes.[54] Substrates of ABCB1 are lipophilic, neutral or cationic and usually non-conjugated compounds which include cytotoxic chemotherapeutic agents, digoxin, fexofenadine, verapamil, HIV-protease inhibitors, quinidine and erythromycin.[54,108,153] Many but not all of ABCB1 substrates are also substrates of CYP3A4, which needs to be considered when exploring the mechanisms behind pharmacokinetic interactions.[54,108] There is also overlap between inhibitors and inducers of ABCB1 and those of CYP3A4. Examples of inhibitors include cyclosporin A, itraconazole and quinidine, and at least rifampicin and St. John's-wort have been described to induce ABCB1.[54]

ABCC1 (previously known as the multidrug resistance-associated protein, MRP1) transports glutathione conjugates, organic anions and lipids, and is widely spread in the body.[54]

ABCC2 (previously MRP2) transports water-soluble drug conjugates (sulfate-, glucuronide- and glutathione conjugates) out of the cells.[54,153] It is located in the liver, kidney and intestine. Organic anions and some β-lactam antibiotics are also

transported via ABCC2. Dubin-Johnson syndrome is caused by a deficiency of this transporter.[153]

ABCG2 (previously MXR, BCRP, ABCP) is located in the placenta, intestine, breast, liver, veins and capillaries, and transports e.g. anthracyclines and mitoxantrone.[54]

1.4. Pharmacogenetics

Consideration of pharmacogenetics has become popular during the recent years. In the 1950s genetics were already being discovered to influence drug responses, for example, as seen in the slow acetylation of isoniazid.[89,133] The development of molecular biology and the Human Genome Project have made it possible to explore gene sequences and their effect on the function of different proteins.[64] Pharmacogenetics along with genotyping could be a valuable tool in the determination of the appropriate dose of a drug for each individual in the future. In fact, some enzyme genotyping is already in clinical use, e.g. for thiopurine methyltranferase.[51]

A number of drug metabolizing enzymes and drug transporters are genetically polymorphic. A genetic mutation is considered to be a polymorphism if there are at least two variant alleles which are both present in the population with reasonable frequency (generally accepted as over 1%).[64] Variation in the gene coding for a metabolizing enzyme can abolish, reduce or increase the expression and activity of the enzyme. The individuals carrying two normal alleles (homozygous wild type) are extensive metabolizers (EM), those with one normal and one variant allele (heterozygous) are intermediate metabolizers (IM), and those with two variant alleles are PMs.[64] Duplication or multiplication of the gene may lead to ultra rapid metabolism (UM), which has been described only for CYP2D6. Alterations in enzyme activity can lead to treatment failure or increased adverse effects, thereby affecting drug efficacy and toxicity. If the enzyme is responsible for inactivation of a drug, the dosage might need to be lowered for PMs and increased for UMs. Consideration of pharmacogenetics is especially important for drugs with a narrow therapeutic window and concentration-dependent adverse effects, such as the CYP2C9 substrate warfarin.[64] Genetic polymorphisms also affect susceptibility to inhibition and induction of the metabolic pathway. The consequences of alterations in enzyme activity are more prominent in EMs due to the higher baseline activity.[121] Polymorphisms have been described at least for CYP2A6, CYP2B6, CYP2D6, CYP2C8, CYP2C9 and CYP2C19, NATs, GSTs, thiopurine methyltransferase and several transporters such as ABCB1 and OATP-C.[54,64,83,91,153]

1.5. *In vitro* systems for studying drug metabolism

During recent decades, *in vitro* experiments have become an important tool for studying drug metabolizing enzymes. First, they were used to explain interactions observed *in vivo* in humans, and then they became a valuable tool for determination of the pharmacokinetic properties and the inhibition potential of new chemical entities.[167] Mathematical methods have been developed to predict interactions *in vivo* in humans based on the results of *in vitro* studies.

In vitro systems for studying enzyme kinetics and inhibition include human liver microsomes (HLM), recombinant human CYP enzymes (rhCYP), hepatocytes and liver slices, which are all commercially available.[128] All have their advantages and disadvantages, and the appropriate system should be chosen to answer the questions under consideration.

HLMs include all human drug metabolizing CYP enzymes in the proportions that they appear in nature.[128] However, there is extensive inter-individual variation in the quantity of different CYPs. Thus, pooled HLMs are made from several human livers to represent the average population. All CYPs can be studied with HLMs, which are also suitable for studying metabolism-dependent inhibition. The use of enzyme specific substrates and inhibitors is necessary. The protein and lipid concentrations may cause problems due to non-specific binding of the drugs.

When rhCYPs are used, unspecific substrates and inhibitors can also be used.[128] However, studying metabolism-dependent inhibition can be misleading because rhCYPs have also been observed to be inactivated in the presence of NADPH only.[194] It is also impossible if there is an inhibitory metabolite which is formed by another enzyme. In the rhCYP systems, the enzyme kinetics may vary, as all the additional contents of a hepatocyte may not be present, they may have variable activity or they might be inactive.

Hepatocytes and liver slices are complex systems which offer few advantages to the above mentioned systems but have many disadvantages.[128] In addition to metabolism and inhibition, hepatocytes give an opportunity to study induction *in vitro*. Michaelis-Menten kinetics rarely apply to them. Hepatocytes also include transporters and phase II enzymes, which might be considered an advantage, but they complicate the interpretation of the mechanisms behind an interaction. Hepatocytes in general are not suitable for studying inhibition with a marker substrate; instead, they require the use of the specific drugs involved in the proposed interaction. Studies made with hepatocytes are rarely repeatable due to the unique nature of the source.

The contribution of different enzymes to the metabolism of a drug and to the formation of specific metabolites can be evaluated using HLMs with specific inhibitors and/or rhCYPs. In addition, HLMs and rhCYPs are used to evaluate the intrinsic clearance of the drug (CL_{int}) i.e. the metabolic capacity of the liver to eliminate it.[121] The intrinsic clearance is the ratio of the maximal velocity of metabolite formation (V_{max}) to the substrate concentration needed to achieve half of that velocity i.e. the Michaelis-Menten constant (K_m):

$$CL_{\text{int}} = \frac{V_{\max}}{K_m} \quad (1)$$

Hepatic clearance of the drug (CL_H) can be obtained from the following equation:[121]

$$CL_H = Q_H \cdot E = Q_H \cdot \left(\frac{f_u \cdot CL_{\text{int}}}{Q_H + f_u \cdot CL_{\text{int}}} \right) \quad (2)$$

where Q_H is the hepatic blood flow (1500 ml/min), E is the hepatic extraction ratio and f_u is the unbound fraction of the drug. For low clearance drugs the metabolic capacity of the enzyme is the rate limiting step in elimination, i.e. $f_u \cdot CL_{int} << Q_H$, thus, CL_H approaches $f_u \cdot CL_{int}$. For high clearance drugs, the metabolic capacity is high and the hepatic blood flow is the rate-limiting step, i.e. $f_u \cdot CL_{int} >> Q_H$, thus, CL_H approaches Q_H.[121]

Most CYPs follow simple Michaelis-Menten kinetics, which assumes that there is only one binding site for the substrate at the active site of the enzyme, and that metabolite formation follows a saturating hyperbolic kinetic model. The formation rate of a product (v) with different substrate concentrations ([S]) in the absence of inhibitors is used as a tool to determine the K_m and the V_{max} according to the following equation:[212]

$$v = \frac{V_{\max}}{1 + \frac{K_m}{[S]}} \quad (3)$$

When the enzyme is saturated ([S] >> K_m) the V_{max} is reached. Determination of the kinetics of the substrate is important for the determination of appropriate conditions for *in vitro* inhibition studies.

2. Inhibition of drug metabolism

Inhibition of drug metabolism leads to an increased plasma concentration of the parent compound. The effect is particularly large if alternative elimination pathways are not available. Usually inhibition of drug metabolism leads to enhanced efficacy and toxicity. Inhibition is normally almost instantaneous as the inhibitor enters the cell containing the enzyme. The underlying mechanism behind most drug-drug interactions is inhibition of CYP enzymes.[100]

2.1. Reversible inhibition

Reversible inhibition occurs without biotransformation of the inhibitor, and the binding of the inhibitor to the enzyme is non-covalent.[128] Therefore, the inhibition is dose-dependent, and the normal activity of the enzyme is regained after the inhibitor is eliminated from the body. Reversible inhibition includes competitive, noncompetitive, uncompetitive and mixed inhibition. Reversible inhibition can be characterized by IC_{50} (the concentration of inhibitor producing 50% inhibition) or K_i (the inhibitor constant, which defines the inhibitors affinity for the enzyme).[128] IC_{50} is convenient for rapid screening of a number of compounds. However, it is less precise than K_i because it is dependent on factors, such as the substrate concentration, microsomal protein content and incubation time. Furthermore, K_i is reproducible from one laboratory to the next. Different forms of reversible inhibition affect the kinetic constants of metabolism in different ways. However, whether the inhibition increases the K_m or decreases the V_{max}, the net result is a decrease in the CL_{int}.[128] Furthermore, when the first-pass metabolism of a compound is inhibited by a competitive inhibitor, it increases the oral bioavailability (F) of the compound according to the following equation:[121]

$$F = 1 - E = \frac{Q_H}{Q_H + f_u \cdot CL_{\text{int}}} \qquad (4)$$

Competitive inhibition. Competitive inhibition is the most common form of inhibition. Substrate and inhibitor compete for the same binding site on the enzyme, so that the inhibitor can bind only to unbound enzyme. The V_{max} of the substrate remains unchanged but the K_m increases.[128,182] Competitive inhibition can be overcome by addition of more substrate.

Noncompetitive inhibition. In noncompetitive inhibition, the inhibitor and substrate bind to different locations on the enzyme. The inhibitor may bind to both unbound and bound enzyme. The V_{max} decreases but the K_m is unaffected.[128,182] Therefore, addition of substrate does not affect the inhibition potency.

Uncompetitive inhibition. In uncompetitive inhibition, the inhibitor can bind to the enzyme-substrate complex only. This inhibition mechanism leads to the decrease of both V_{max} and K_m.[128]

Mixed inhibition. Mixed inhibition includes both competitive and noncompetitive inhibition. Thus, the inhibitor can bind to the same site as the substrate, but also to another location, or to the same location but without blocking the binding of the substrate.[128]

2.2. Quasi-irreversible and irreversible inhibition

Quasi-irreversible and irreversible inhibition are metabolism-dependent, and they require the inhibitor to go through a time-dependent metabolic step. However, time-dependent inhibition can also be caused by the formation of an inhibitory metabolite, and can therefore be reversible.[194] In quasi-irreversible inhibition, a metabolic intermediate complex is formed. This means that the product of drug metabolism binds covalently to the enzyme, and works as an inhibitor. Although the metabolic intermediate complex does not affect enzyme structure and is basically reversible, the complex is so stable and long-lasting *in vivo* that drug metabolism by this enzyme is no longer possible.[100,121,194] Macrolides and calcium channel blockers are examples of drugs that form metabolic intermediate complexes.[100] In irreversible or mechanism-based inhibition, the enzyme is inactivated by covalent bonding of the inhibitor to e.g. the apoprotein or the prosthetic heme.[194,212] The criteria for mechanism-based inhibition for CYPs are time-dependent inactivation requiring NADPH, irreversibility of the inactivation by, for example, dialysis, formation of a reactive intermediate and saturating kinetics of the inactivation.[176] Typical mechanism-based inhibitors include, among others, acetylenes, furans, thiophenes, conjugated structures, terminal alkenes, di- and trichloroethylenes, and tertiary and secondary amines.[56,100] Although the inhibition is not mechanism-based in both quasi-irreversible and irreversible inhibition, the inhibition is reversed only after formation of new enzymes, thus they can also be called irreversible. To investigate irreversible inhibition *in vitro*, the inhibitor must be preincubated with the enzyme and NADPH in the absence of the substrate, and then the residual activity measured.[128,194] This inhibition is dependent both on the inhibitor concentration and the inactivation time.[121]

2.3. Structures of typical CYP1A2 inhibitors

The active site of CYP1A2 is rather small. Hence CYP1A2 inhibitors are typically relatively small molecules, and a planar structure favors inhibition. Certain

substitutions such as methyl, ethyl and chloro also increase the inhibition potential of certain scaffolds.[114] The most common scaffolds of CYP1A2 inhibitors are thiophene, pyrrole, pyridine and pyrazine, and other probable structures are pyrazole, piperazine and imidazole (Figure 2).[106]

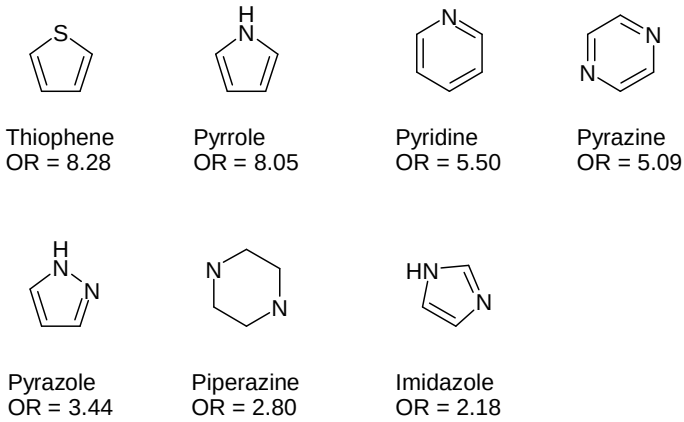


Figure 2. Scaffolds most likely to be included in the structure of CYP1A2 inhibitors (odds ratios (OR) for being CYP1A2 inhibitors compared to the rest of the scaffolds).[106]

3. Induction of drug metabolism

Nature has created the ability to induce metabolism in order to eliminate a xenobiotic faster from the body during long-term exposure. Usually, enzyme induction leads to lower drug efficacy due to the enhanced elimination of an active compound.[121] But in some cases, nature works against itself, and induction can lead to increased efficacy or even toxicity of a pro-drug. It is thought that induction of a metabolizing enzyme leads to an increased amount of the enzyme, not to changes in the activity of existing enzymes.[148] Induction usually takes place at the transcription site. Inducers of drug metabolism can enhance protein synthesis or they can stabilize the RNA, both of which lead to increased formation of the active protein. In some cases, the inducer can stabilize the protein, thus prolonging its lifespan. As induction is such a complex process, it takes time, and induction can usually be seen only days or even weeks after the exposure to the inducer has begun.[148] Common inducers include polycyclic aromatic hydrocarbons, antiepileptic drugs, corticosteroids and rifampicin.[121,148]

Among others, CYP1A, CYP2C and CYP3A enzyme families are susceptible to induction.[79] This activation is mediated by nuclear receptors, which include the constitutive androstane receptor (CAR), the pregnane X receptor (PXR) and the aromatic hydrocarbon receptor (AhR). CYP2C and CYP3A are induced by phenobarbital- and rifampicin/dexamethasone-like inducers, which activate CAR and PXR, respectively.[88] However, these two receptors have overlapping substrate and target gene specificities. Induction of CYP1A by polycyclic aromatic hydrocarbons is mediated by AhR.[79] AhR forms a heterodimer with the AhR nuclear translocator in the presence of an inducer.[185] This dimer can bind to xenobiotic response elements of the *CYP1A* gene in the proximal or distal promoter regions. The perixisome proliferator activated receptor has also been observed to induce metabolizing enzymes.[79] Ethanol has been discovered to directly stabilize CYP2E1.[79] In addition to CYPs, conjugating enzymes and transporters have also been found to be induced by nuclear receptors.[185,207]

4. *In vitro-in vivo* prediction

As *in vitro* techniques for studying enzyme inhibition are developed, methods to extrapolate these results to *in vivo* situations are also needed. The most elaborate methods take into account the inhibitor concentration at the enzyme active site ($[I]_{in\ vivo}$), the fraction of the substrate eliminated by the enzyme in question (f_m) and the mechanism of inhibition.[7,24,143,150] However, the predictive methods also make many simplifications, and assume that the absorption of the substrate from the gastrointestinal (GI) tract is complete and unaffected by the inhibitor, that the well-stirred pharmacokinetic model describes the hepatic clearance of the substrate, that metabolism occurs in the liver only, and that the pharmacokinetics of the substrate are linear.[132,209]

To predict the degree of *in vivo* inhibition caused by a reversible inhibitor, most strategies are based on the ratio of the $[I]_{in\ vivo}$ to the K_i.[7,93,105] In general, if the $[I]_{in\ vivo}$ to K_i ratio is below 0.1, an *in vivo* interaction is not expected; if it is between 0.1 and 1, an interaction is possible, and when it is above 1, an interaction is very likely.[7,24,93] However, there are exceptions to this rule, and some weak *in vitro* inhibitors can cause clinically significant interactions *in vivo*.[143]

Prediction of *in vivo* interactions of a mechanism-based inhibitor is more complex than for other types of inhibitors due to the concentration- and time-dependent nature of the inactivation.[194] Instead of the $[I]_{in\ vivo}$ to K_i ratio, two ratios are needed to determine the *in vivo* interaction potential of a mechanism-based inhibitor. The ratio of the $[I]_{in\ vivo}$ to the inhibitor concentration needed to give the half-maximal rate of inactivation (K_I) represents the inactivation potency of the inhibitor, and the ratio of the maximal rate of inactivation with a saturating concentration of the inhibitor (k_{inact}) to the degradation rate of the enzyme (k_{deg}) determines the inactivation rate.[142,194] Thus, a clinically relevant interaction is possible even if the $[I]_{in\ vivo}$ to K_I ratio is low, if the k_{inact} to k_{deg} ratio is high enough. Determination of the above mentioned *in vitro* inactivation constants used in the predictions requires more steps than the determination of inhibitor constants, as a preincubation step with various preincubation times needs to be included.[142,194]

5. Compounds

Basic pharmacokinetic properties of the compounds discussed below are presented in Table 3.

5.1. Rofecoxib

Rofecoxib is a selective inhibitor of cyclo-oxygenase-2 (COX-2). It was used for pain caused by rheumatic diseases and arthritis.[63] Rofecoxib was one of the first and most widely used coxibs until its withdrawal from clinical use.

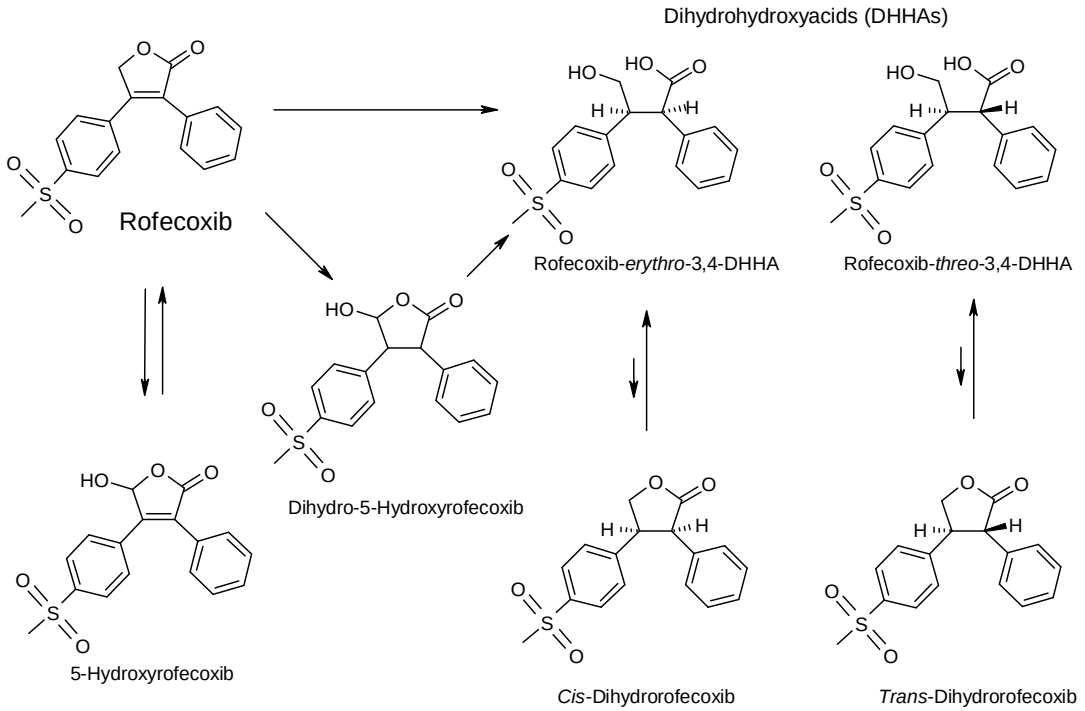


Figure 3. Metabolic pathways of rofecoxib[177]

Pharmacokinetics Rofecoxib is rapidly absorbed, and the C_{max} is reached in 2 to 3 h.[63] The daily dose of rofecoxib ranged from 12.5 to 50 mg, and the drug could be taken once daily due to its long elimination half-life ($t_{½}$) of about 17 h.[63,134] Its oral bioavailability is about 93% and it is 85% bound to plasma proteins.[134] Rofecoxib is extensively metabolized in the liver, and the primary pathways have been identified to be the formation of different 3,4-dihydrohydroxy acid derivatives by enzymes other than CYPs (Figure 3).[63,134,177] CYP1A2 and 3A4 seem to catalyze the formation of the minor metabolite 5-hydroxyrofecoxib, which can be reduced back to rofecoxib.[177]

Adverse effects Although rofecoxib has fewer adverse GI-tract effects than non-selective COX-inhibitors, some heartburn, upper abdominal pain, dyspepsia, nausea and diarrhea have been described during treatment.[63,134] It can cause fluid retention and elevation of blood pressure. Overall, rofecoxib was well-tolerated, but it was seen to increase the risk of cardiovascular events. Due to this serious adverse effect it was withdrawn from clinical use in September 2004.

Interactions Rofecoxib was suggested to moderately inhibit CYP1A2, since it raised the plasma concentrations of theophylline and R-warfarin by about 50%.[6,172]

5.2. Tolfenamic acid

Tolfenamic acid is an NSAID which has been used for migraine, dysmenorrhea and rheumatic pain (Figure 4).[77,136,151,161] In addition to non-selective COX inhibition, tolfenamic acid seems to reduce inflammation by inhibiting leukotriene synthesis and leukotriene B_4-induced chemotaxis of leukocytes.[102,122,193]

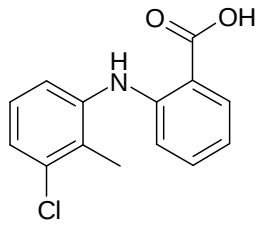


Figure 4. Chemical structure of tolfenamic acid

Pharmacokinetics The usual daily dose of tolfenamic acid is 600 mg divided in three dosages. Its oral bioavailability is about 60 to 75%, and it is highly bound to plasma proteins (99.7%).[147,151,195] Tolfenamic acid reaches its C_{max} in about 1 to 2 h.[147,151,195] The $t_{½}$ of tolfenamic acid is about 2 to 2.5 hours,[147,151,195] and it is excreted mainly (90%) into urine as glucuronide conjugates of its metabolites.[147,151] The enzymes responsible for its phase I metabolism are unknown.

Adverse effects Like other NSAIDs tolfenamic acid has mild adverse GI-tract effects including upper abdominal pain, nausea and diarrhea.[92] It can color urine yellow and cause dysuria, which is reversible and can be treated by increasing fluid intake.[92]

Interactions Tolfenamic acid has been observed to enhance the effects of oral anticoagulants and lithium, and decrease the effects of loop-diuretics.[45]

5.3. Celecoxib

Celecoxib is also a COX-2 selective NSAID which is used for pain caused by rheumatic diseases and arthritis (Figure 5).[63] Its daily dose is 400 mg divided in two dosages. The treatment should be temporary and as short as possible due to an increased risk of adverse cardiovascular effects during long term use. The molecular structure of celecoxib includes a pyrazole ring, which is thought to favor CYP1A2 inhibition (Figures 2 and 5).

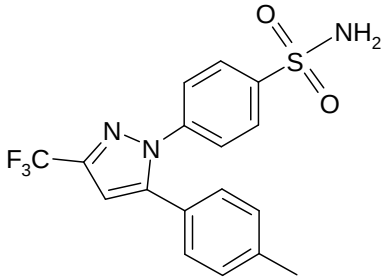


Figure 5. Chemical structure of celecoxib

Pharmacokinetics Celecoxib reaches its C_{max} in about 2 to 4 h, and has a $t_{½}$ of about 11 hours.[37,52,63] Due to the lack of an intravenous form of celecoxib, actual bioavailability studies have not been conducted, but the relative bioavailability is thought to be 99%.[37,52] Celecoxib is eliminated mainly by metabolic processing in which CYP2C9 has an important role.[37,52,63] This enzyme is polymorphic, and PMs may have significantly increased plasma concentrations of celecoxib. Celecoxib is highly bound (97%) to plasma proteins.[37,52,63]

Adverse effects Celecoxib is well-tolerated at treatment doses. It causes mild adverse GI-tract effects like abdominal pain and diarrhea, but these are milder than those caused by traditional NSAIDs.[63] Eczema, allergic reactions, fluid retention, peripheral edema and even Stevens Johnson syndrome have been described during administration of celecoxib.[37,63]

Interactions *In vitro*, celecoxib was found to be a potent inhibitor of CYP2D6 (K_i 4.2 μM).[52] Inhibition of CYP2C9, CYP2C19 or CYP3A4 was not observed.[52] Despite its high plasma protein binding, and therefore, low free concentration in plasma, celecoxib has also been found to raise plasma concentrations of the CYP2D6 substrates dextromethorphan (136%) and metoprolol (65%) *in vivo* in humans.[46,202]

5.4. Tizanidine

Tizanidine is a centrally acting muscle relaxant, and it acts as an α-adrenoreceptor agonist. Tizanidine is used particularly in spastic pain episodes in neuromuscular disorders such as multiple sclerosis, but it is also used in common tension-type headache, migraine and musculoskeletal pain.[196]

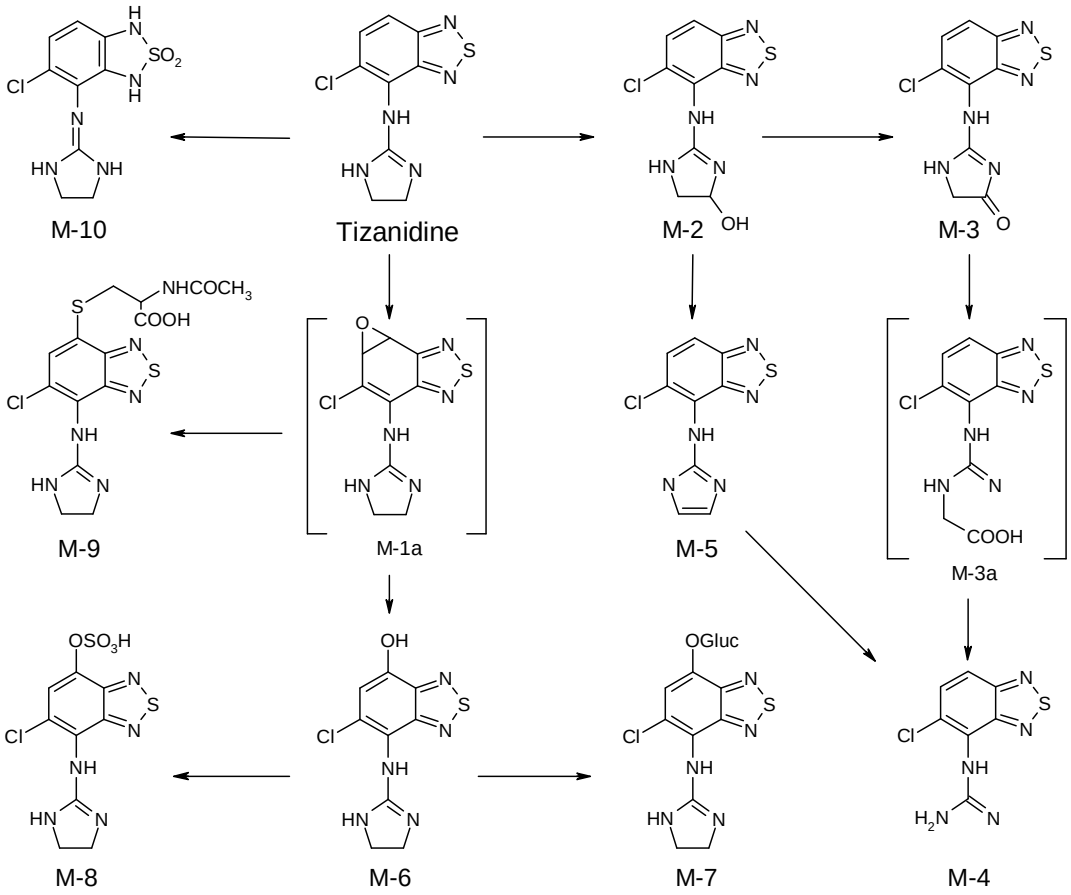


Figure 6. Metabolic pathways of tizanidine[113]

Pharmacokinetics Tizanidine is readily absorbed from the GI-tract. However, it undergoes extensive first-pass metabolism, and its oral bioavailability is about 10 to 30%.[72,196] Tizanidine is metabolized through several pathways (Figure 6),[113] and CYP1A2 catalyses its elimination both *in vitro* and *in vivo*.[69,71] Tizanidine is 30% bound to plasma proteins. Its $t_{½}$ is about 1.5 h, and it is excreted into urine mainly as metabolites.[71,72]

Adverse effects Tizanidine causes hypotension, bradycardia, somnolence, dizziness, dryness of mouth and mild adverse GI-tract effects like nausea.[196]

Interactions Tizanidine has been found to be almost exclusively metabolized by CYP1A2.[69] Since the first-pass metabolism of tizanidine is extensive, changes in CYP1A2 activity by inhibitors may increase its bioavailability remarkably. In fact, ciprofloxacin was found in particular to inhibit the first-pass metabolism of tizanidine, increasing its AUC 10-fold,[72] and fluvoxamine was found to inhibit both first-pass and systemic metabolism, resulting in a 33-fold increase in the AUC of tizanidine.[71] Oral contraceptives containing ethinylestradiol and gestodene also increased the AUC of tizanidine by 3.9-fold.[70]

5.5. Caffeine

Caffeine is a widely used substance found in coffee, various other beverages, and some over-the-counter drugs (Figure 7). It causes stimulation of the central nervous system and elevated mood, a decrease in fatigue and an increased capacity to work. It may also have mild analgesic effects.[26,103]

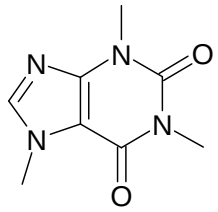


Figure 7. Chemical structure of caffeine

Pharmacokinetics Caffeine is rapidly and well absorbed from the GI-tract. It does not have significant first-pass metabolism, and it reaches its C_{max} in about 0.5 - 1.5 h.[26,138] The $t_{½}$ of caffeine is about 3 to 6 hours,[26,138] and it is excreted into urine mainly as metabolites. Caffeine is metabolized via both CYP and non-CYP mediated pathways, and more than 25 metabolites have been identified.[26] The main pathway is considered to be N^3-demethylation to 1,7-dimethylxanthine (paraxanthine).[60] This reaction is nearly exclusively catalyzed by CYP1A2. Also N^1- and N^7-demethylations of caffeine occur partly via CYP1A2. Overall, CYP1A2 accounts for 95% of the primary metabolism of caffeine. Paraxanthine is eventually converted to 5-acetylamino-6-formylamino-3-methyluracil by NAT2, and to 1-methyluracil by xanthine oxidase.[26]

Adverse effects Caffeine can have adverse cardiovascular effects like elevated or lowered heart rate and elevated blood pressure.[26,138] Decreased CYP1A2 activity has been suggested to increase the adverse cardiovascular effects of caffeine.[30,31]

Interactions CYP1A2 inhibitors decrease the formation of paraxanthine.[26]

5.6. Other drugs

Ciprofloxacin is a fluoroquinolone antimicrobial agent. It is eliminated mainly unchanged in urine and feces, but some elimination occurs via metabolism. It is 20% bound to plasma proteins and diffuses well into tissues.[206] The molecular structure of ciprofloxacin includes a piperazine ring, which is thought to favor CYP1A2 inhibition (Figure 2).[106] In fact, ciprofloxacin is a potent inhibitor of CYP1A2 *in vivo*, and it has been observed to increase the plasma concentrations of several CYP1A2 substrates such as tizanidine and theophylline.[44,62,72]

Desogestrel is a progestogen used alone or in combination with ethinylestradiol in oral contraceptives. Desogestrel is nearly completely metabolized into the active metabolite etonogestrel.[44] The oral bioavailability of etonogestrel is 62 to 81%.[44] Etonogestrel is metabolized via sulfation and glucuronidation. Finally desogestrel is excreted into urine or feces as etonogestrel, its metabolites or their conjugates.[44] Inducers of drug metabolizing enzymes may decrease the concentrations of desogestrel/etonogestrel.[44]

Diclofenac is an NSAID with possibly extensive first-pass metabolism (60%) although some reports suggest high oral bioavailability (90%).[35] Diclofenac is metabolized primarily to 4-hydroxydiclofenac by CYP2C9, and it is excreted into urine mainly as glucuronides or hydroxylated metabolites.[35,44,195] Diclofenac raises the plasma concentrations of methotrexate and possibly lithium.[35]

Ethinylestradiol is a synthetic steroid used in oral contraceptives. Its daily dose is usually 20 to 40 µg. The elimination of ethinylestradiol is biphasic, and the terminal $t_{½}$ is on average 17 h.[210] Ethinylestradiol is eliminated via metabolism (CYP3A4, SULT1E1, UGT1A1), and excreted into urine and feces.[44,210] Ethinylestradiol strongly inhibits CYP3A4, 2B6, 2C9 and 2C19 *in vitro*.[210] *In vivo*, oral contraceptives containing ethinylestradiol raise plasma concentrations of the CYP1A2 substrates tizanidine, theophylline and caffeine.[70,210]

Etodolac is a chiral NSAID, and the *S*-enantiomer is solely responsible for COX inhibition.[19] It is metabolized by hydroxylation and conjugation to, for instance, acyl-glucuronides, and excreted mainly into urine.[19,44] As NSAIDs are known to affect the plasma concentrations of cyclosporine, digoxin, methotrexate, lithium and warfarin, the manufacturer advices that care is warranted while administering these drugs along with etodolac.[170] The molecular structure of etodolac includes a pyrrole ring, which might favor CYP1A2 inhibition (Figure 2).[106]

Etoricoxib is a COX-2 selective NSAID.[130] It is excreted mainly into urine as metabolites formed primarily by CYP3A4.[4] Etoricoxib has been found to slightly increase the plasma concentrations of ethinylestradiol.[135] It inhibits SULTs, specifically SULT1E1,[135] which might explain the interaction with ethinylestradiol. The molecular structure of etoricoxib includes two pyridine rings, which are thought to favor CYP1A2 inhibition (Figure 2).[106] However, it has been found not to strongly inhibit CYP1A2, 2C9, 2C19, 2D6, 2E1 or 3A4 (IC_{50} values > 100 µM).[104,135]

Fluvoxamine is an SSRI. It is eliminated mainly by metabolism.[44,152] CYP2D6 has an important role in the metabolism of fluvoxamine, but PMs do not have significantly elevated plasma concentrations of the drug. Fluvoxamine is a potent inhibitor of CYP1A2 and CYP2C19, and a moderate inhibitor of CYP2C9, CYP3A and CYP2D6.[22,44,144,152]

Gestodene is a progestogen used in combination with ethinylestradiol in oral contraceptives. It is completely metabolized prior to excretion into urine and bile.[44] Mechanism-based inhibition of CYP3A4 by gestodene has been observed, but even high concentrations have been found not to have any effect on CYP1A2.[74] Oral contraceptives containing gestodene and ethinylestradiol have been found to raise plasma concentrations of tizanidine.[70]

Ibuprofen is one of the most widely used traditional NSAIDs. It has two enantiomers, *S*- and *R*-ibuprofen, of which the *S*-enantiomer is mainly responsible for the anti-inflammatory activity.[34] Ibuprofen is excreted into urine mainly as metabolites.[44,195] CYP2C9 has a major role in the 2- and 3-hydroxylations of both ibuprofen enantiomers, and CYP2C8 also participates in the metabolism.[34] Ibuprofen has been found to increase the plasma concentrations of digoxin and phenytoin.[34]

Ketoprofen is another widely used traditional NSAID. Ketoprofen also has two enantiomers, and only *S*-ketoprofen is capable of inhibiting prostaglandin synthesis.[11] Ketoprofen is excreted mainly into urine as acyl-glucuronides.[44,95,195] Concomitant use of ketoprofen and warfarin has been found to lead to increased prothrombin time, but the mechanism is unclear. Ketoprofen also increases plasma methotrexate concentrations, probably due to impairment of renal elimination.[95]

Ketorolac is also an NSAID with two enantiomers. *S*-ketorolac is much more active than *R*-ketorolac.[18] Ketorolac undergoes extensive metabolism into hydroxy and glucuronide metabolites, and it is excreted mainly into urine as an acyl-glucuronide.[18,44] The pyrrole ring of the molecular structure of ketorolac might favor CYP1A2 inhibition (Figure 2).[106] Ketorolac has been found to slightly

decrease the plasma concentrations of warfarin, but this has not been found to have any pharmacodynamic effect.[18]

Mefenamic acid is an NSAID of the fenamate group like tolfenamic acid. It is eliminated mainly into urine unchanged or as one of its metabolites. Some mefenamic acid is excreted into feces, mainly as an unconjugated carboxyl derivate.[195]

Meloxicam is an NSAID which shows a preference for COX-2 inhibition. Meloxicam is excreted into urine and feces, mainly as metabolites. CYP2C9 is the major metabolizing enzyme of meloxicam and CYP3A4 has a minor role.[29] Interaction potential with some often co-prescribed drugs has been studied, and slight elevations of *S*-warfarin caused by meloxicam has been observed, probably due to a shared metabolism pathway.[38]

Naproxen is also one of the traditional NSAIDs. It is highly (>99%) bound to plasma proteins, and it may displace other compounds such as warfarin from plasma proteins.[36,195] Naproxen is metabolized principally by dealkylation to O-desmethylnaproxen by CYP1A2 and CYP2C9, and it is excreted mainly into urine as a glucuronide [36,195] Naproxen slightly increases the clearance of valproic acid and warfarin, probably by displacing them from plasma protein. Methotrexate concentrations are increased by naproxen, but the mechanism for this is unclear. Naproxen may also increase plasma lithium concentrations.[36]

Progesterone is used in doses from 100 to 200 mg. It has an extensive first-pass metabolism.[44] About 95% of the oral dose of progesterone is excreted into urine as glucuronide conjugates of its metabolites.[44]

Zolmitriptan is a serotonin receptor agonist used in the treatment of migraine. It is used in oral or nasal doses of 2.5 or 5 mg. Zolmitriptan has three major metabolites, N-desmethyl, N-oxide and indoleacetic acid.[43] N-desmethyl-zolmitriptan is biologically active, and its formation is mediated mainly by CYP1A2.[173,184,204] Zolmitriptan is eliminated mainly by urinary excretion of the metabolites.[43] The zolmitriptan molecular structure includes a pyrrole ring, which is thought to favor CYP1A2 inhibition (Figure 2).[106]

Table 3. Pharmacokinetic properties of the studied drugs.

Drug	**C_{max} (µg/ml)**	**f_u**	**D (mg)**	**t_{max} (h)**	**$t_{½}$ (h)**
Celecoxib[37](Study IV)	0.61	0.03	200	2-4	8-12
Ciprofloxacin[32,44,72]	1.4	0.8	500	1-2	4-6
Desogestrel[180]	$4.2 \cdot 10^{-5}$	-	0.15	-	-
Diclofenac[35,44,195]	1-1.5	0.003	50	2	1-2
Ethinylestradiol[44,115,210]	$8 \cdot 10^{-5}$	0.017	0.02	1-3	8-24
Etodolac[19,44]	21	0.01	400	1-2	6-8
Etonogestrel[44]	0.002	0.025	-	1.5	30
Etoricoxib[3,4,130]	3.6	0.08	120	1-2	22
Fluvoxamine[44,71,152]	0.072	0.23	100	3-8	13-15
Gestodene[115]	0.004	0.006	0.075	1-2	12-20
Ibuprofen[34,44]	20-40	0.01	400	1-2	2
Ketoprofen[44,95,195]	10	0.013	100	0.5-2	1-4
Ketorolac[18,44]	2.7	0.01	30	0.5-1	4-6
Mefenamic acid[44,195]	10	0.01	1000	2-4	2
Meloxicam[38,189]	1-3	0.005	15	5-11	20-24
Naproxen[36,44,195]	50	<0.01	250	0.5-3	12-15
Progesterone[44]	$4.3 \cdot 10^{-5}$	0.05	100	2	19-95 min
Rofecoxib[63](Study I)	0.30	0.15	25	2	20
Tolfenamic acid[151](Study III)	3.44	0.01	200	2	2
Zolmitriptan[42,173]	0.023	0.75	15	3.75	2-3

C_{max}, peak plasma concentration; f_u, free fraction; D, average single dose; t_{max}, time to C_{max} after oral administration; $t_{½}$, elimination half-life.

AIMS OF THE STUDY

CYP1A2 is one of the major drug metabolizing enzymes. The muscle relaxant tizanidine has been found to be metabolized almost exclusively by CYP1A2. NSAIDs are often used in combination with muscle relaxants, but little was known about their effect on CYP1A2 prior to this study. In addition, ciprofloxacin and oral contraceptives containing ethinylestradiol and gestodene have been found to increase the plasma concentrations of tizanidine, but the mechanism is not yet fully clear.

The specific aims of the study were:

I To investigate the effect of rofecoxib on tizanidine pharmacokinetics and pharmacodynamics and on caffeine metabolism *in vivo* in humans.

II To investigate the effect of rofecoxib on CYP1A2 activity *in vitro* in order to clarify the mechanism behind the tizanidine – rofecoxib interaction.

III To investigate the effect of tolfenamic acid on CYP1A2 activity *in vitro* and *in vivo* in humans, and also to clarify the importance of protein binding by comparing the *in vitro* inhibitory potency of tolfenamic acid in the presence and absence of albumin.

IV To investigate the effect of celecoxib on CYP1A2 activity *in vitro* and *in vivo* in humans.

V To investigate and compare the effects of several NSAIDs, female sex steroids and model inhibitors on CYP1A2 activity *in vitro*, and to predict their effects on tizanidine pharmacokinetics *in vivo*.

VI To evaluate whether *in vivo* CYP1A2 interactions can be predicted based on *in vitro* data.

MATERIALS AND METHODS

1. *In vitro* studies

1.1. Microsomes

Pooled HLMs were obtained from BD Biosciences (Woburn, MA, USA). Human liver tissue had been collected in accordance with all pertinent regulations, and permission from donors' families had been obtained prior to organ collection. The procedures of organ collection had been reviewed and accepted by the respective institutional Human Subjects Committee. Each batch contained the pooled HLMs from 20 different donors. The HLMs were stored at -70°C, thawed in a warm water bath and kept on ice prior to addition to the incubation medium.

1.2. Incubation conditions

The effect of different compounds on the *in vitro* activity of CYP1A2 in the model reaction (phenacetin O-deethylation to paracetamol) was studied using HLMs.[41,183] All incubations were performed in duplicate (all the data were within 15% of the mean) in a shaking water bath at 37ºC. The incubations were carried out in 0.1 M sodium phosphate buffer (pH = 7.4) containing 5.0 mM $MgCl_2$, 1.0 mM NADPH, phenacetin and HLMs in a total volume of 1000 µl. The incubation mixture components, except NADPH, were premixed, and the resulting mixture was kept at room temperature for 3 minutes. The incubations were commenced by addition of NADPH. After the incubation, aliquots of 200 µl were removed, the reactions were stopped with 100 µl of acetonitrile (including the internal standard hydrochlorothiazide) and cooling on ice. Rofecoxib and etoricoxib were dissolved in acetonitrile, ciprofloxacin in 0.05 M sodium hydroxide and all other compounds in methanol. The final solvent concentration in the incubations was 1% for acetonitrile and methanol, and 0.01% (v/w) for sodium hydroxide. Control incubations contained equal amount of the solvent.

1.2.1. Screening, and IC_{50} and K_i determinations.

For screening tests, prior to addition of NADPH, potential inhibitor was added to the incubation mixture to give a final concentration of 10 µM or 100 µM, and for the IC_{50} determinations, sufficient potential inhibitor was added to give an appropriate final concentration. Phenacetin was added to give a final concentration of 10 µM and incubated for 30 min with the HLM mixture (0.1 mg/ml) and NADPH. The drugs studied were ciprofloxacin, celecoxib, desogestrel, diclofenac,

ethinylestradiol, etonogestrel, etodolac, etoricoxib, fluvoxamine, gestodene, ibuprofen, ketoprofen, ketorolac, mefenamic acid, meloxicam, naproxen, progesterone, rofecoxib, tolfenamic acid and zolmitriptan. The effect of albumin on the inhibitory effect (IC_{50}) of tolfenamic acid was studied by adding different concentrations of human serum albumin to the buffer solution (final albumin concentrations in the incubations 0.3, 1, 3 or 10 mg/ml). In the determination of the K_i, phenacetin (5 µM, 10 µM, 20 µM and 50 µM) was incubated with various concentrations of the inhibitor for 30 min.

1.2.2. Evaluation of metabolism-dependent inhibition.

To evaluate the potential for metabolism-dependent inhibition, incubation mixtures were prepared as described earlier, but without the substrate. Preincubations including the inhibitor were started by addition of NADPH. At 30 min, 20 µM phenacetin was added to the mixtures, and the incubations were allowed to proceed for 20 min. This sort of simple experimental design is suitable for screening purposes only.[142] As the inhibitory effect of rofecoxib and desogestrel was observed to increase with preincubation, a more elaborate experiment was carried out, in which the IC_{50} values without and with preincubation were determined under these conditions.

The inactivation constants K_I and k_{inact} were determined for rofecoxib. The preincubation mixture was prepared as described above, except using a microsomal protein concentration of 1 mg/ml. Rofecoxib concentrations of 1-48 µM were used. The mixture was preincubated for 0 to 25 min, and an aliquot of 100 µl was then transferred to another incubation tube containing phenacetin (20 µM) and NADPH, in a final volume of 1000 µl, to determine residual CYP1A2 activity. Thus, rofecoxib was diluted to one tenth of its original concentration in order to minimize the effect of "direct" inhibition of CYP1A2.

To determine the effects of trapping agents and the alternative competitive inhibitor fluvoxamine on CYP1A2 inactivation by rofecoxib, the preincubation mixture was prepared as for the k_{inact} and K_I determinations. Preincubations were carried out with and without glutathione (2 mM), superoxide dismutase (1000 U/ml), mannitol (1 mM) and fluvoxamine (0.01, 0.1 and 1 µM) both in the absence (control) and presence of 12 µM rofecoxib. The mixture was preincubated for 30 min, and after the ten-fold dilution step the residual activity was measured as described above.

To evaluate the stability of the rofecoxib-enzyme complex, dialysis was performed by preincubating HLMs with or without 12 µM rofecoxib for 30 min as described earlier (1 mg/ml of microsomal protein). The samples were immediately dialyzed

against 0.1 M sodium phosphate buffer (pH = 7.4) containing 1% solvent (3 x 2 h in a volume of 2 l) at 4ºC, followed by 10-fold dilution and measurement of phenacetin O-deethylation activity.

1.3. Measurement of drug concentrations

Paracetamol and hydrochlorothiazide were extracted with 5.0 ml of ethyl acetate for 20 min. After centrifugation, 4.2 ml of the supernatant was evaporated to dryness under nitrogen, and the residue was reconstituted with 100 µl of the mobile phase (ammonium acetate 6 mM : acetonitrile, 78 : 22). Thereafter, the paracetamol concentration was determined by high-performance liquid chromatography (HPLC) with UV detection (230 nm).[39,155] A Hewlett-Packard 1100 System (Waldbrunn, Germany) and Waters Symmetry C8 reversed-phase column, 4.6 x 150 mm (Milford, MA, USA) were used. The limit of quantification for paracetamol was 25 nM. Other drugs studied did not interfere with paracetamol determinations.

1.4. Data analysis

The IC_{50} values were determined by nonlinear regression analysis with Sigma Plot 8.0 (SPSS Inc., Chicago, IL, USA). The K_i values were calculated with the Sigma Plot 8.0 Enzyme Kinetic Module. In studies III and V, the K_i values for some compounds were estimated using IC_{50} data and assuming competitive inhibition according to the following equation:[186]

$$K_i = \frac{[I]}{\left(\frac{V_0}{V} - 1\right)\left(1 + \frac{[S]}{K_m}\right)} \qquad (5)$$

where V_0 and V are the metabolite formation rates in the absence and presence of inhibitor, respectively, [I] is the inhibitor concentration and [S] is the substrate concentration used in the incubation. A K_m of 25 µM was used for phenacetin.[120]

For estimation of the inactivation constants, preincubation-time dependent loss of CYP1A2 activity in the absence of rofecoxib was accounted for by adjusting the observed rate of metabolism with reference to the respective (vehicle) control incubation at each preincubation time. The initial rate constant for the inactivation of CYP1A2 activity by each inhibitor concentration (K_{obs}) was determined by linear regression analysis of the natural logarithm of the percentage of activity remaining versus preincubation time data.[109] Thereafter, the K_{obs} values were used to determine the K_I and the k_{inact}. Initial estimates of K_I and k_{inact} were obtained from a double-reciprocal plot of the K_{obs} (y-axis) versus inhibitor concentration [I] (x-

axis).[109] When extrapolated to the axes, the reciprocal of the intercept of the y-axis gives k_{inact}, and the negative reciprocal of the intercept of the x-axis gives K_I, as demonstrated in Figure 8.

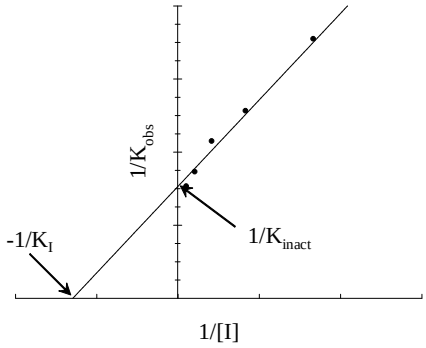


Figure 8. Determination of the inactivation constants.

Then, the K_I and k_{inact} were estimated by nonlinear regression using the following equation:[98]

$$K_{obs} = \frac{k_{inact} \cdot [I]}{K_I + [I]} \quad (6)$$

2. *In vitro-in vivo* predictions

The total and free peripheral ($C_{max,\ peripheral}$), portal ($C_{max,\ portal}$) and 10-times the portal peak plasma concentrations of the inhibitor were used as $[I]_{in\ vivo}$ in the predictions. The portal vein concentration was calculated as follows:

$$C_{\text{max, portal}} = C_{\text{max, peripheral}} + \frac{k_a \cdot F_a \cdot D}{Q_h} \quad (7)$$

where k_a is the absorption rate constant, F_a is the fraction of the substrate absorbed from the GI-tract and D is the dose. The k_a was estimated based on the time to the peak concentration (t_{max}) of the inhibitor in plasma and its $t_{½}$ from the expression $t_{max} = (\ln(k_a/k_e))/(k_a - k_e)$, where k_e is the elimination rate constant.[24,105]

2.1. Competitive inhibitor

The effect of a competitive inhibitor on *in vivo* pharmacokinetics of a CYP1A2 substrate primarily eliminated by metabolism was predicted as follows:[167,168,209]

$$\frac{AUC_{inhibited}}{AUC_{control}} = \frac{1}{\frac{f_m}{1+\frac{[I]_{\text{in vivo}}}{K_i}} + (1 - f_m)} \quad (8)$$

In study III, the predictions were made either with the total peak plasma concentration of tolfenamic acid *in vivo*, using the K_i at different *in vitro* albumin concentrations, or with the free tolfenamic acid concentration (the actual unbound fraction of 0.3% and two higher values, 1% and 5%), using the K_i obtained in the absence of albumin.

2.2. Mechanism-based inhibitor

In study II, *in vitro* data describing mechanism-based inhibition (the $[I]/K_I$ and k_{inact}/k_{deg} ratios) was used to evaluate the potential clinical impact of the inhibition according to the following equation:[123]

$$\frac{AUC_{inhibited}}{AUC_{control}} = \frac{1}{\frac{f_m}{1+\left[k_{inact}/K_I \cdot [I]/k_{\text{deg}}\right]} + 1 - f_m} \quad (9)$$

In our simulations, a CYP1A2 half-life of 38.6 h (i.e. k_{deg} = 0.0003 min^{-1}) was assumed, based on a previous estimate of the *in vivo* CYP1A2 half-life.[47,67] This value is similar to the *in vitro* half-life estimate of 36 h determined for CYP1A2 in liver slices.[162]

3. Studies *in vivo* in humans

3.1. Subjects

A total of 31 healthy non-smoking volunteers participated in the studies (Table 4). Prior to accepting the subjects, they were ascertained to be healthy by physical examination and by taking blood samples for routine laboratory tests. In study I, an electrocardiogram was also performed. Subjects with systolic blood pressure below 110 mmHg were excluded for safety reasons. Subjects provided a written informed consent prior to initiating the study. The use of any additional medications including oral contraceptives was not allowed one week prior to and during the study. Drinking of grapefruit juice and tobacco smoking were not allowed for 1 week before each study day. Alcohol and drinks containing caffeine were not permitted on the study days. The subjects were not allowed to participate in other clinical studies or donate blood for one month prior to the beginning of the studies until one month after the end of the studies.

Table 4. Characteristics of the subjects.

Study	Number of subjects (male/female)	Age years (range)	Weight kg (range)
I	9 (9/-)	22 (20-25)	79 (64-87)
III	10 (10/-)	23 (21-34)	76 (63-88)
IV	12 (8/4)	25 (19-37)	72 (59-85)

3.2. Study design

Studies I, III and IV were performed at the Department of Clinical Pharmacology in the University of Helsinki. The study protocols were approved by the Ethics committee for Studies in Healthy Subjects (Studies I and III) or the Coordinating Ethics Committee (Study IV) of the Hospital District of Helsinki and Uusimaa and the Finnish National Agency for Medicines. The studies had a randomized, two-phase cross-over, placebo-controlled design with a 2-4 week wash-out period (Table 5). On study days, the subjects ingested a single oral dose of tizanidine (Sirdalud, Novartis, Espoo, Finland) with 150 ml of water at 9.00 h. Standard meals were served 3 and 7 h after tizanidine ingestion.

Subjects were under close observation by a medical doctor during both study days. Fluids for intravenous infusion were available for immediate use, but they were not needed.

Table 5. The structure of the *in vivo* studies in Studies I, III and IV.

Study	Studied drug and dose	Duration of pretreatment and daily doses	Tizanidine dose	Wash-out period (weeks)
I	Rofecoxib 25 mg x 1 or Placebo x 1	4 days at 8.00 h	4 mg on day 4 at 9.00 h	4
III	Tolfenamic acid 200 mg x 3 or Placebo x 3	3 days at 8.00 h, 16.00 h and 22.00 h	4 mg on day 3 at 9.00 h	2
IV	Celecoxib 200 mg x 2 or Placebo x 2	4 days at 8.00 h and 20.00 h	2 mg on day 4 at 9.00 h	4

A caffeine test was performed a day before tizanidine administration. The subjects took a tablet containing 100 mg of caffeine (Cofi-Tabs 100 mg tablet, Vitabalans, Hämeenlinna, Finland) at 9.00 h, after having abstained from caffeine intake for at least 12 h, and a blood sample for analysis of plasma caffeine and paraxanthine levels was taken from each subject 6 h after caffeine intake.[60,110,179]

3.3. Sampling

On the days of tizanidine administration, a forearm vein of each subject was cannulated with a plastic cannula and kept patent with an obturator. Timed blood samples were drawn before the administration of tizanidine and at 20, 40, 60, and 90 minutes and 2, 3, 4, 5, 7, 9, (11 in Study IV), 12, and 24 h after its administration. Blood samples (10 ml each) were taken into ethylenediaminetetra-acetic acid–containing tubes. Plasma was separated within 30 minutes and stored at -40°C until analysis. In Study I, urine was collected cumulatively in 2 fractions, 0 to 12 h and 12 to 24 h. The samples were stored at -40ºC for later analysis. A 12-lead electrocardiogram was recorded prior to and at 2 h after tizanidine intake (Study I).

3.4. Determination of drug concentrations in plasma and urine

3.4.1. Tizanidine and its metabolites

In Studies I, III and IV, plasma and urine tizanidine concentrations were quantified using an API2000 liquid chromatography–tandem mass spectrometry system (MDS Sciex, Toronto, Ontario, Canada). Chromatography was performed on an XTerra RP C18 column (3.9 × 100 mm; Waters Corp.; Milford, MA, USA) using gradient elution. The mobile phase consisted of 10 mM ammonium acetate (pH 9.5, adjusted with 25% ammonia solution) and acetonitrile. The mass spectrometer was operated in the atmospheric pressure ionization mode with positive ion detection. The ion transitions monitored were mass-to-charge ratio (*m/z*) changes of 254 to *m/z* 44 for

tizanidine, *m/z* 268 to *m/z* 211 for M-3, *m/z* 228 to *m/z* 211 for M-4, *m/z* 252 to *m/z* 216 for M-5, *m/z* 415 to *m/z* 286 for M-9, *m/z* 288 to *m/z* 188 for M-10 and *m/z* 230 to *m/z* 44 for the internal standard, clonidine. These transitions represent the product ion of the $[M+H]^+$ ion. The limit of quantification of tizanidine was 0.05 ng/ml, and the day-to-day coefficient of variation (CV) was 5.6%-12.9% at 0.096 ng/ml, 3.1%-9.5% at 0.96 ng/ml and 4.6% at 9.6 ng/ml (n = 4-7). A signal-to-noise ratio of 10 : 1 was used as the limit of detection for tizanidine metabolites and their concentrations are given in arbitrary units. The detector response for each metabolite was confirmed to be linear by means of sample dilution. The CVs were between 3-11% for the metabolites at relevant concentrations (n = 8). Other studied drugs did not interfere with the determination of plasma tizanidine.

3.4.2. Rofecoxib, tolfenamic acid and celecoxib

In Study I, the plasma concentrations of rofecoxib were determined by HPLC with etoricoxib as the internal standard.[28] The limit of quantification was 5 ng/ml, and the day-to-day CV values were 0.92% at 20 ng/ml, 4.1% at 100 ng/ml and 0.63% at 400 ng/ml (n = 6).

In Study III, the plasma concentrations of tolfenamic acid were determined by HPLC with flufenamic acid as the internal standard.[140] The limit of quantification was 50 ng/ml, and the day-to-day CV was 13.5% at 300 ng/ml and 7.4% at 3000 ng/ml (n = 6).

In Study IV, the plasma concentrations of celecoxib were determined by HPLC with rofecoxib as the internal standard.[28] The limit of quantification for celecoxib was 10 ng/ml, and the day-to-day CV was 1.8% at 70 ng/ml, 5.7% at 400 ng/ml, 5.4% at 800 ng/ml and 1.4% at 1500 ng/ml (n = 3).

Tizanidine and its metabolites did not interfere with the determination of plasma rofecoxib, tolfenamic acid or celecoxib.

3.4.3. Caffeine and paraxanthine

In Studies I, III and IV, plasma caffeine and paraxanthine concentrations were determined by HPLC, with β-hydroxy-ethyltheophylline as the internal standard.[87,154] The day-to-day CV of caffeine and paraxanthine was less than 6% at relevant concentrations (n = 7).

3.5. Pharmacokinetic method

The pharmacokinetics of tizanidine were characterized by the C_{max}, t_{max}, AUC from 0 to infinity ($AUC_{0-\infty}$) and $t_{½}$. The terminal log-linear part of the concentration-time curve was visually identified for each subject. The k_e was determined with the use of linear regression analysis of the log-linear part of the plasma concentration-time curve. The $t_{½}$ was calculated using the following equation: $t_{½} = \ln(2)/k_e$. The AUC values were calculated by use of the linear trapezoidal rule for the rising phase of the plasma tizanidine concentration-time curve and 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 amount of tizanidine and its metabolites excreted into urine (Ae) was determined (Study I).

The pharmacokinetic parameters of rofecoxib and tolfenamic acid were characterized by C_{max}, t_{max}, $t_{½}$ and AUC from 0 to 25 h (AUC_{0-25}) and to infinity ($AUC_{0-\infty}$) after the last dose of rofecoxib, and from 0 to 8 h (AUC_{0-8}) after the last morning dose of tolfenamic acid. The pharmacokinetics of celecoxib were characterized by C_{max} and AUC from 0 to 12 h (AUC_{0-12}) after the last morning dose of celecoxib. The pharmacokinetic calculations were performed with the program MK-model, version 5.0 (Biosoft, Cambridge, UK)

3.6. Pharmacodynamics

Pharmacodynamic variables were monitored immediately after each blood sample until 24 h (Study I) or 12 h (Studies III and IV) after tizanidine intake. Systolic and diastolic blood pressure and heart rate were measured with an automatic oscillometric blood pressure monitor (HEM-711; Omron Healthcare GmbH, Hamburg, Germany). Subjects were in a sitting position during measurements, which were performed from their forearm twice each time. The average was used for analysis. The subjects were trained to perform 3 psychomotor tests. In the Digit Symbol Substitution Test (DSST), the number of digits correctly substituted in 2 minutes was recorded.[181] Subjective drowsiness and drug effects were measured with a 100-mm-long horizontal visual analog scale (VAS).[16] For each pharmacodynamic variable, the maximum response was recorded and the area under the effect versus time curve from 0 to 12 h (AUC_{0-12}) was calculated using the trapezoidal rule.

3.7. Statistical analysis

The results are given as mean ± SD, except t_{max} is as median with range. The pharmacokinetic and pharmacodynamic variables after the two pretreatments were compared by repeated-measures ANOVA with treatment sequence as a factor or, in the case of t_{max}, with the Wilcoxon signed-rank test. In Study IV, logarithmic transformation was used for C_{max} and AUC. For all pharmacokinetic variables except t_{max}, 95% confidence intervals (CI) were calculated on the relative to control (Study I and III) or the percent of control (Study IV) during the inhibitor phase. For the pharmacodynamic variables, 95% confidence intervals were calculated on the mean differences between the placebo and inhibitor phases. The Pearson correlation coefficient was used to investigate possible relationships between the pharmacokinetic variables of tizanidine, rofecoxib and the caffeine/paraxanthine ratio (Study I). All data were analyzed with the statistical program Systat for Windows (SPSS Inc, Chicago, Ill, USA). $P < 0.05$ was taken to indicate statistical significance.

RESULTS

1. *In vitro* studies

1.1. Inhibition of phenacetin metabolism (Studies IV and V)

The effect of the studied drugs on CYP1A2 activity (phenacetin O-deethylation to paracetamol) are presented in Table 6. Fluvoxamine, tolfenamic acid, mefenamic acid and rofecoxib potently inhibited CYP1A2. Ethinylestradiol, celecoxib, desogestrel and zolmitriptan had a moderate effect, and etodolac, ciprofloxacin, etoricoxib and gestodene were weak inhibitors. Other tested NSAIDs and steroids inhibited CYP1A2 less than 35% at 100 µM.

1.2. The effect of preincubation on the inhibition potential (Studies II and V)

A 30-min preincubation increased the inhibitory effect of rofecoxib, progesterone and desogestrel, but had no obvious effect on the potencies of other tested drugs (Table 6). The effect of preincubation on the inhibitory potential of rofecoxib and desogestrel was further studied. With a 20-min incubation time and phenacetin concentration of 20 µM, rofecoxib and desogestrel inhibited CYP1A2 activity with IC_{50} values of 23.0 µM and 85 µM, respectively, in HLMs without preincubation. A 30-min preincubation of rofecoxib or desogestrel in the presence of NADPH increased the inhibition of CYP1A2 considerably, resulting in IC_{50} values of 4.18 µM and 59.3 µM, respectively, suggesting metabolism-dependent inhibition of CYP1A2.

1.3. Inactivation of CYP1A2 by rofecoxib (Study II).

The inhibition of CYP1A2 activity by rofecoxib was preincubation time- and concentration-dependent. The inactivation of CYP1A2 was characterized by a K_I of 4.78 µM and a k_{inact} of 0.070 min^{-1}. Thus, 7% of CYP1A2 is inactivated each minute when a saturating concentration of rofecoxib is incubated with HLMs.

The trapping agents superoxide dismutase, glutathione and mannitol, had no significant effect on CYP1A2 inactivation caused by rofecoxib, and dialysis could not restore CYP1A2 activity. Fluvoxamine diminished the inactivation of the enzyme caused by rofecoxib in a concentration-dependent manner. For example, 1.0 µM fluvoxamine decreased the inactivation caused by 12 µM rofecoxib from 63% to 6%.

Table 6. Inhibition of CYP1A2 activity (phenacetin-O-deethylation). Phenacetin at 10 µM (screening and IC_{50} determination) or 5-50 µM (K_i determination) was incubated with various concentrations of the inhibitors for 30 min. The effect of preincubation on the inhibitory potency of the drugs was studied by incubating the inhibitor (at a selected concentration) for 30 min with NADPH. Phenacetin at 20 µM was then added and the incubation was allowed to proceed for 20 min.

Drug	% of control activity				Concentration in preincubation study (µM)	% of control activity	
	10 µM	100 µM	IC_{50} (µM)	K_i (µM)		No preincubation	30-min preincubation
Ciprofloxacin	79	55	220	145	200	64	54
Celecoxib	84	3.2	34	25.4	20	75	85
Desogestrel	93	20	55.2	39.4*	50	82	46
Diclofenac	74	67	-	-	100	93	89
Ethinylestradiol	55	12	24.0	10.6	20	66	69
Etodolac	94	42	237	170*	200	63	71
Etonogestrel	97	84	-	-	-	-	-
Etoricoxib	92	51	240	170*	100	67	59
Fluvoxamine	0	0	0.029	0.011	-	-	-
Gestodene	83	68	~ 1000	710*	100	79	79
Ibuprofen	98	103	-	-	-	-	-
Ketoprofen	89	67	-	-	100	97	103
Ketorolac	108	91	-	-	100	90	84
Mefenamic acid	30	3.1	3.36	3.47	2	77	84
Meloxicam	98	71	-	-	100	112	111
Naproxen	94	69	-	-	100	89	94
Progesterone	103	85	~ 1000	710*	300	59	24
Rofecoxib	31	19	8.7	6.2*	10	63	34
Tolfenamic acid	22	4.5	1.5	1.39	0.6	76	96
Zolmitriptan	76	33	122	60.1	100	68	72

IC_{50}, the inhibitor concentration causing 50% inhibition of enzyme activity; K_i, the inhibitor constant. * The K_i was estimated based on the IC_{50} value according to the equation: $K_i = [I]/(V_0/V - 1)(1 + [S]/K_m)$, where V_0 and V are the metabolite formation rates in the absence and presence of inhibitor, respectively, [I] is the inhibitor concentration and [S] the substrate concentration used in the incubation. For the Michaelis-Menten constant K_m, a value of 25 µM was used.[120]

1.4. The effect of albumin on the inhibitory effect of tolfenamic acid (Study III)

The addition of albumin to the incubation medium decreased the CYP1A2 inhibitory effect of tolfenamic acid in a concentration-dependent manner. The IC_{50} values were 1.8 µM, 3.6 µM, 13.5 µM, ~50 µM and ~160 µM with 0 mg/ml, 0.3 mg/ml, 1 mg/ml, 3 mg/ml and 10 mg/ml of albumin, respectively.

2. *In vitro-in vivo* predictions (Studies II, III, IV and V)

2.1. Competitive inhibition model (Studies III, IV and V)

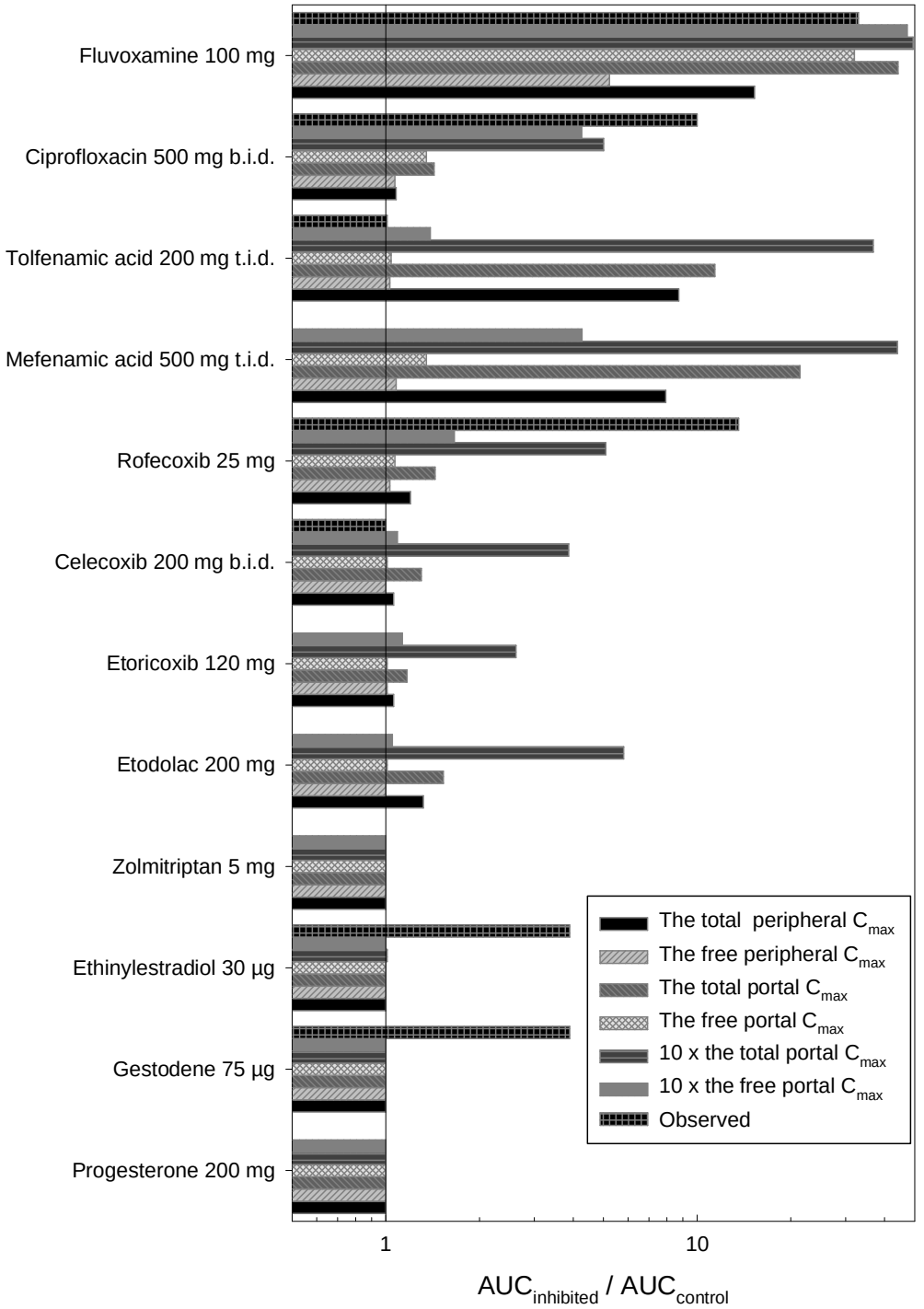
The predicted *in vivo* inhibition values based on the observed *in vitro* inhibition constants, competitive inhibition model and reported *in vivo* plasma inhibitor concentrations are presented in Figure 9. The free portal C_{max} of the inhibitor predicts well the effect of fluvoxamine and the lack of *in vivo* effect of tolfenamic acid and celecoxib (despite their relatively strong *in vitro* effects) on tizanidine pharmacokinetics *in vivo* (Figure 9). However, even if 10 times the total portal inhibitor concentration is used, the simple competitive inhibition model greatly underestimates the effects of ciprofloxacin, rofecoxib and oral contraceptives on tizanidine pharmacokinetics *in vivo* in humans (5-, 5- and 1-fold increase in the AUC, respectively). The free portal C_{max} of mefenamic acid was predicted to increase the AUC of tizanidine by 35%, but other *in vitro* CYP1A2 inhibitors in the present study were predicted not to have any significant effect *in vivo* (Figure 9).

In addition to the prediction based on the free C_{max} of tolfenamic acid and the K_i obtained without albumin, the prediction based on the total C_{max} of tolfenamic acid and the K_i values obtained with increasing albumin concentration also approached the observed effect (6.7-, 2.7-, 1.5- and 1.1-fold increase in the AUC with 0.3, 1, 3 and 10 mg/ml of albumin). When the protein binding of tolfenamic acid was underestimated (95%), the AUC of tizanidine was predicted to increase by 50%-63%.

2.2. Mechanism-based inhibition model (Study II)

When the total plasma rofecoxib concentration (~1 µM) and the *in vitro-in vivo* extrapolation method for a mechanism-based inhibitor were used in the prediction, >3-fold increases in the AUC of compounds >70% metabolized by CYP1A2 were predicted. The free C_{max} of rofecoxib (150 nM) was predicted to cause about an 8-fold increase in the AUC of tizanidine. However, using the estimated free portal C_{max} of rofecoxib (400 nM) in the prediction yielded a 14.8-fold increase in the AUC, correlating well with our finding *in vivo*.

Figure 9. Effects of CYP1A2 inhibitors on the AUC of tizanidine (98% metabolized by CYP1A2), predicted using the competitive inhibition model and various C_{max} values for $[I]_{in\ vivo}$, compared to observed effects.



3. *In vivo* studies

3.1. The effect of rofecoxib, tolfenamic acid and celecoxib

3.1.1 Tizanidine pharmacokinetics (Studies I, III and IV)

Rofecoxib greatly increased the plasma concentrations of tizanidine. The tizanidine C_{max} increased to 6.1-fold (range 4.3-14.70-fold; $P < 0.001$) and the $AUC_{0-\infty}$ increased to 13.6-fold (range 7.4-22.5-fold; $P < 0.001$) the placebo values. The $t_{½}$ was also increased from 1.6 ± 0.2 hours to 3.0 ± 0.6 hours ($P < 0.001$) by rofecoxib. However, tolfenamic acid and celecoxib had no significant effect on the $AUC_{0-\infty}$, C_{max}, t_{max} or $t_{½}$ of tizanidine (Figure 10).

Rofecoxib considerably prolonged the t_{max} of the tizanidine metabolite M-3, and of the secondary metabolite M-4. Also, the $t_{½}$ values of M-3 and M-4 increased 4.4-fold and 1.6-fold respectively. In addition, rofecoxib increased the $AUC_{0-\infty}$ of M-3 ($P = 0.01$), and decreased the C_{max} of M-4 ($P < 0.001$). The tizanidine/M-3 and tizanidine/M-4 ratios in plasma were increased over 10-fold by rofecoxib ($P < 0.001$). Tolfenamic acid had no significant effect on the $AUC_{0-\infty}$, C_{max}, t_{max} or $t_{½}$ of M-3. The $AUC_{0-\infty}$ of M-4 was slightly decreased by tolfenamic acid. Celecoxib did not affect the AUCs of the metabolites M-3, M-4 and M-5. The C_{max} of M-3 slightly increased during the celecoxib phase, and that of the minor metabolite M-10 decreased.

Rofecoxib increased the urinary excretion of tizanidine 12-fold ($P = 0.04$) and that of M-10 2-fold ($P < 0.001$), but decreased the excretion of M-4 by 48% ($P = 0.002$). Rofecoxib increased several-fold the tizanidine to metabolite (M-3, M-4, M-5, M-9, M-10) ratios in urine.

To sum up, rofecoxib increased the plasma concentrations of tizanidine and decreased those of the metabolites, whereas tolfenamic acid and celecoxib did not affect tizanidine concentrations.

3.1.2. Caffeine test (Studies I, III and IV)

Rofecoxib increased the caffeine/paraxanthine ratio 2.4-fold ($P = 0.008$) (Figure 10). There was a good correlation between the effect of rofecoxib on the CYP1A2 probes tizanidine and caffeine. The AUC of tizanidine, the tizanidine/M-3 AUC ratio and the tizanidine/M-4 AUC ratio correlated well with the caffeine/paraxanthine ratio ($P < 0.02$). During the placebo phase, the tizanidine/metabolite (M-4, M-5 and M-10) Ae ratios correlated significantly with the caffeine/paraxanthine ratio ($P < 0.02$).

Moreover, the changes in tizanidine/metabolite (M-3, M-4, M-5 and M-9) Ae ratios caused by rofecoxib correlated with the changes in the caffeine/paraxanthine ratio caused by rofecoxib ($P < 0.05$).

Tolfenamic acid had no significant effect on the caffeine/paraxanthine ratio, which was 1.07 ± 0.47 (range 0.57-2.19) during the placebo phase and 1.22 ± 0.64 (range 0.45-2.53) during the tolfenamic acid phase ($P = 0.52$). The caffeine/paraxanthine ratio was not significantly altered by celecoxib but there was a slight decreasing trend (1.65 ± 0.85 and 1.30 ± 0.40 during placebo and celecoxib phases, respectively, $P = 0.10$).

To summarize, rofecoxib decreased paraxanthine formation from caffeine, but tolfenamic acid and celecoxib did not have a significant effect. In addition, there was a correlation between the changes in tizanidine metabolism and the changes in caffeine metabolism caused by rofecoxib.

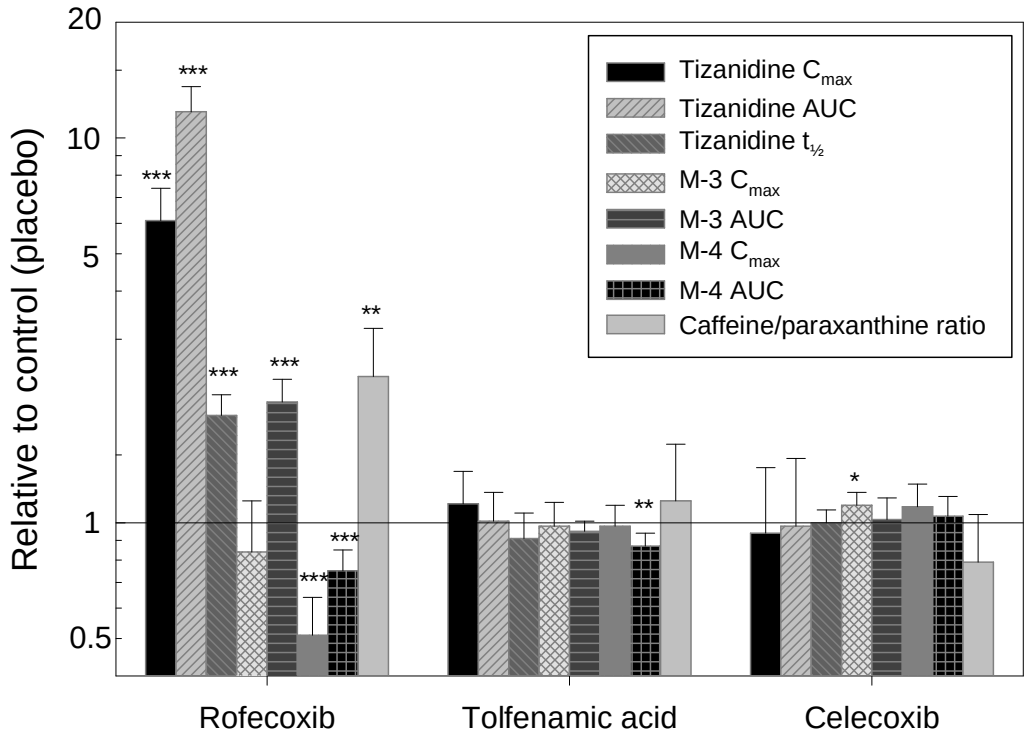


Figure 10. The effects (mean difference with symmetric 95% CI) of rofecoxib (25 mg daily), tolfenamic acid (200 mg t.i.d.) and celecoxib (200 mg b.i.d.) on the pharmacokinetics of tizanidine (single oral dose of 4 mg during rofecoxib and tolfenamic acid and 2 mg during celecoxib treatments) and its metabolites M-3 and M-4, and the caffeine/paraxanthine ratio. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.1.3. Tizanidine pharmacodynamics (Studies I, III and IV)

Tizanidine caused much stronger pharmacodynamic responses (blood pressure, heart rate, subjective drowsiness and drug effect and DSST) during the rofecoxib phase than during the placebo phase (Figure 11). There was no significant difference between the phases in the heart rate-corrected QT-interval.
Neither tolfenamic acid nor celecoxib significantly affected the pharmacodynamics of tizanidine (Figure 11).

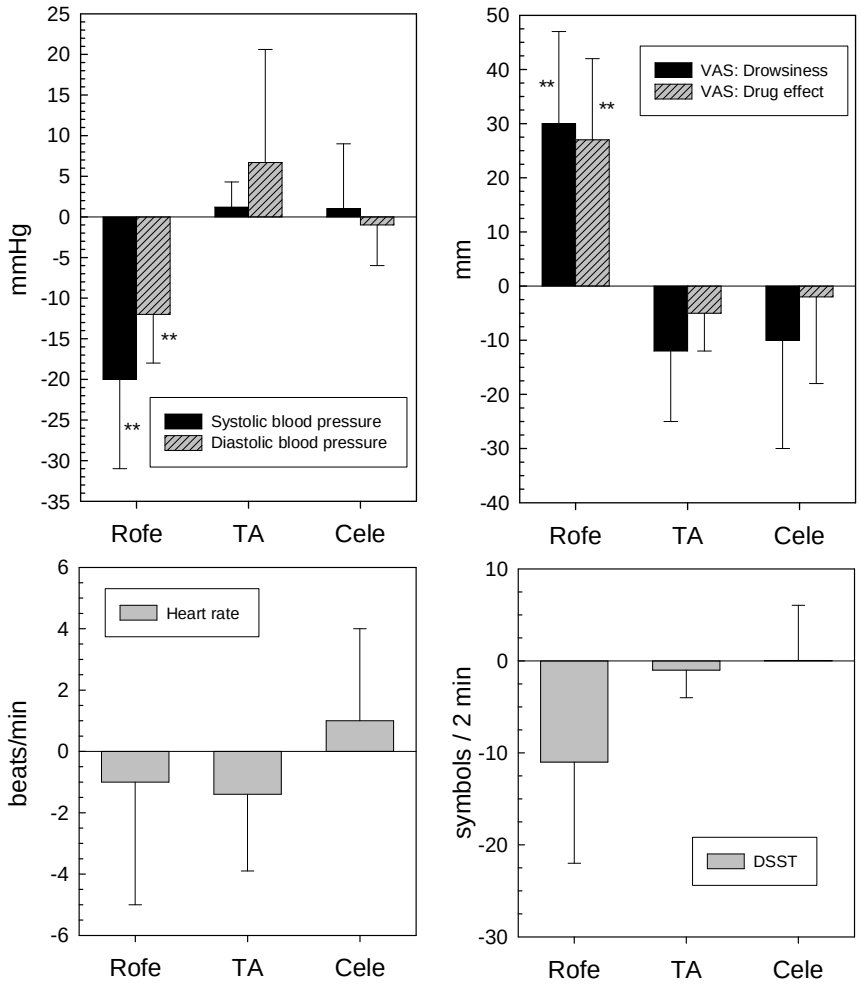


Figure 11. The mean difference (with 95% CI) between phases (inhibitor-placebo) in maximum responses of the pharmacodynamic variables of tizanidine - blood pressure, heart rate, subjective drowsiness and drug effect on the visual analog scale (VAS), and digit symbol substitution test (DSST). Rofe, rofecoxib; TA, tolfenamic acid; Cele, celecoxib. ** $P < 0.01$

3.2. Rofecoxib, tolfenamic acid and celecoxib concentrations (Studies I, III and IV)

The mean C_{max} of rofecoxib was 0.30 ± 0.07 µg/ml (corresponding to 0.95 ± 0.22 µM). The trough concentration of rofecoxib on day 4, the AUC_{0-25} of rofecoxib, and the $AUC_{0-\infty}$ of rofecoxib correlated significantly with the placebo phase caffeine/paraxanthine ratio ($P < 0.05$). The $AUC_{0-\infty}$ of rofecoxib also correlated with the placebo phase $AUC_{0-\infty}$ of tizanidine ($P = 0.037$).

The mean C_{max} of tolfenamic acid in plasma was 3.4 ± 2.1 µg/ml (corresponding to 13.0 ± 7.9 µM). The mean AUC_{0-8} of tolfenamic acid was 10.8 ± 3.7 µg·h/ml, and its $t_{½}$ was 2.2 ± 0.8 h.

The mean C_{max} of celecoxib was 610 ± 230 ng/ml (corresponding to 1.60 ± 0.60 µM), and its mean AUC_{0-12} was 6.9 ± 2.1 µg·h/ml. There was a 4.6-fold variation in the C_{max} and a 2.9-fold variation in the AUC_{0-12} of celecoxib between the subjects.

DISCUSSION

1. Methodological considerations

1.1. *In vitro* studies

The aim of the *in vitro* studies was to elucidate the mechanisms behind observed *in vivo* interactions, and in addition, to study the CYP1A2 inhibitory potency of certain drugs for which such data was not previously available. *In vitro* studies can be performed using HLMs or rhCYP enzymes. In the present study, only HLMs were used. Pooled HLMs contain all CYP enzymes in the same proportions as they are found in the average population, so the effect of inter-individual variation is minimized.[128] To study inhibition of a specific CYP enzyme in HLMs, a marker substrate, preferably exclusively metabolized by the CYP, is needed. HLMs are good for studying metabolism-dependent inhibition especially if there is an inhibitory metabolite which is formed by a different CYP than the one it inhibits.[128]

Phenacetin O-deethylation to paracetamol is a well established marker for *in vitro* CYP1A2 activity.[41,128] The microsomal protein concentration, incubation time and phenacetin concentration were chosen from a range where phenacetin depletion is less than 20% during the incubation and paracetamol formation is linear.[128] A low microsomal protein concentration was also chosen to minimize nonspecific binding and depletion of the inhibitor caused by metabolism. The appropriate substrate concentrations for K_i determinations were chosen according to the guidelines ¼ K_m, ½ K_m, K_m and 2 K_m.[128] The inhibitor concentrations used for the IC_{50} and K_i determinations were also chosen based on the screening tests. In the preincubation studies, the incubation time was shorter than during the regular incubations to minimize direct inhibition; at the same time, phenacetin concentration had to be increased for analytical purposes, but without compromising the above mentioned principles. When mechanism-based inhibition of CYP1A2 caused by rofecoxib was further studied, a 10-fold dilution step was included to minimize the effect of direct inhibition.[128,194] This experimental design requires a high protein concentration during the preincubation, which might lead to increased nonspecific microsomal binding and underestimation of the inactivation potency of the inhibitor.[194] However, in the present study, the nonspecific binding of rofecoxib was less than 5% (Study II).

Organic solvents are known to inhibit CYP enzymes.[27,86] However, they are often needed in *in vitro* studies due to the poor solubility of many drugs. The solvents used in this study were methanol and acetonitrile, which at a concentration of 1% have only minimal effects on CYP1A2. In addition, control samples contained equal amounts of the solvents to allow observation of any possible effect of the

inhibitor on enzyme activity. Also, the ionic strength (0.1 M sodium phosphate) and the pH (7.4) were chosen to mimic hepatic cytoplasmic conditions, as changes in the pH may influence, for example, the protein binding of the compounds.

The present study could have been improved by determining the inhibitor concentrations in all incubations. Some compounds had some solubility problems and other might have experienced nonspecific microsomal binding, and thus, the *in vitro* inhibitory potency would be underestimated.

1.2. *In vitro-in vivo* predictions

The *in vitro-in vivo* predictions were made with different inhibitor concentrations, as a universal consensus has not been reached on which inhibitor concentration to be used as $[I]_{in\ vivo}$ would best estimate the situation *in vivo*.[24,93,94,167] As the $[I]_{in\ vivo}$ can not be measured, the predictions are based on values obtained from peripheral plasma samples or calculation of the portal concentration based on the peripheral concentration. General recommendations are difficult to make as different compounds behave differently due to their diverse chemical properties. For example, some compounds (e.g. basic or lipid-soluble) seem to be concentrated in the hepatocytes due to passive diffusion or active transport, while others may be actively transported out of the hepatocytes.[150] Consideration of protein binding of the inhibitor is usually recommended.[7,94,143,209] However, in some cases predictions made with the total concentration more accurately correlate with the *in vivo* results, and have been recommended to avoid underprediction of *in vivo* interactions.[23,93] In the present study, when using the free portal C_{max} values, the predictions were rather accurate for the effects of fluvoxamine, tolfenamic acid and celecoxib on the AUC of tizanidine, but the effects of ciprofloxacin and oral contraceptives were underestimated even when 10-fold the portal C_{max} value was used.
One potential source of error in *in vitro-in vivo* predictions is simply the conditions of *in vitro* data determination.[24] Using a high protein concentration in the incubation might increase the nonspecific binding of the inhibitor, and thus lead to underestimation of the inhibitory effect. In the present study, this issue was minimized by using a low microsomal protein concentration. An additional unsolved issue arises when mechanism-based inhibition is involved, as the k_{deg} of the enzyme can not be measured.[142,194] Previous studies have estimated the *in vivo* k_{deg} for CYP1A2 to be 0.0003 min^{-1} (half-life of CYP1A2 38.6 h),[47,67] which is in agreement with the estimated *in vitro* half-life in liver slices (36 h).[162]

As some *in vivo* interactions are still grossly underestimated based on *in vitro* results,[143] it might be wise to study the effects of moderate *in vitro* CYP inhibitors

in vivo as well using a sensitive and specific probe substrate regardless of any negative prediction.

1.3. *In vivo* studies

In vivo studies were carried out in a randomized, placebo controlled, cross-over design with a 2-4 week wash-out period. Inter-individual variation (caused by, for example, genetic differences or body weight) does not need to be considered in this design because subjects serve as their own controls, and 10 to 12 subjects are sufficient to shown an increase of 30% in the AUC with a $P < 0.05$. Thus, the number of subjects exposed to the drugs can be minimized. Balanced randomisation reduces period effects, and a sufficient wash-out period minimizes the carry-over effect. Tizanidine and caffeine were used as probe substrates for CYP1A2 activity due to their specificity.

Prior to ingestion of the substrate drug, the subjects fasted overnight, which was necessary to ensure similar conditions for absorption of the drugs during both phases. The premedication periods were chosen so that steady state would be reached during that time. The timing of the ingestion of the inhibitor (at 8.00 h) and the substrate (at 9.00 h) was chosen to ensure the absorption of the inhibitor before and during the absorption of the substrate.

Tobacco smoke can induce CYP1A2, and therefore, smokers were excluded from the study. Also, the intake of other drugs including oral contraceptives, alcohol, additional caffeine, grapefruit juice, and charcoal-broiled food was limited during the studies to exclude their effect on CYP1A2 activity.

Clinically relevant doses for all the drugs were used in the studies. However, in Study IV the tizanidine dose was lower (2 mg instead of 4 mg) due to the requirements of the Ethics Committee. This compromised the measurement of tizanidine and metabolite concentrations during the absorption and elimination phases. However, had there been a significant inhibition of tizanidine metabolism, it would have been, without a doubt, detected even with this lower dose.

CYP1A2 is crucial to the elimination of tizanidine, the major metabolites of which are M-3 and M-4. For tizanidine metabolites, reference compounds were not available and the concentrations were measured in arbitrary units only. However, with a dilution series, their analytics could be validated, and the measurement of arbitrary units should not compromise the evaluation of the effect of the inhibitors on the formation of these metabolites.

To evaluate the clinical relevance of tizanidine interactions, pharmacodynamic effects were also measured during the study days. The subjects were trained to perform three widely-used psychomotor tests, because training significantly enhances performance. Also the measurement of blood pressure and heart rate was always done twice in a sitting position.

Inhibition of CYP enzymes is thought to occur rather rapidly. Thus, simultaneous ingestion of inhibitor with a substrate drug may lead to inhibition. However, induction takes more time. Intestinal CYPs and transporters are considered to be induced within a week but for hepatic enzymes it seems to take longer.[58,119] Thus, a longer celecoxib pre-treatment might have revealed actual CYP1A2 induction which was only implied in Study IV. It is also possible that drugs that appear inhibitors in simultaneous single exposure may induce drug metabolism in long term treatment.

2. The effect of rofecoxib on CYP1A2 activity *in vitro* and *in vivo*

Rofecoxib, at a usual daily dose of 25 mg, impaired caffeine metabolism and increased the $AUC_{0-\infty}$ of tizanidine on average nearly 14-fold, indicating potent inhibition of CYP1A2. The concentration-dependent[71] and potentially hazardous adverse effects of tizanidine (e.g. hypotension, bradycardia, somnolence) were increased by rofecoxib. Rofecoxib affected both the presystemic and systemic metabolism of tizanidine as indicated by the increases in C_{max}, $t_{½}$ and AUC. The observed effects were markedly stronger than the previously detected effects of rofecoxib on theophylline and *R*-warfarin metabolism.[6,172]

Rofecoxib caused a several-fold increase in the tizanidine/metabolite ratios, and a 2.4-fold increase in the caffeine/paraxanthine ratio. These two ratios correlate with one another, suggesting that CYP1A2 is involved in the formation of all tizanidine metabolites. Moreover, the increase in the caffeine/paraxanthine ratio correlated with the increases in the plasma concentration and urinary excretion of tizanidine, and the tizanidine/metabolite ratios. Thus, rofecoxib appears to be a potent inhibitor of CYP1A2 *in vivo*, and it inhibits the CYP1A2-mediated metabolic pathways of tizanidine.

Rofecoxib seems to decrease the formation of all tizanidine metabolites except M-10. Urinary excretion of M-10 was increased during the rofecoxib phase, and there was no correlation between the increase in the tizanidine/M-10 ratio and the caffeine/paraxanthine ratio, suggesting that, in addition to CYP1A2, other enzymes are also involved in the formation of M-10.

In vitro, rofecoxib inhibited CYP1A2, with an IC_{50} value of 23.0 µM without preincubation. However, the total C_{max} of rofecoxib is less than 10% of this IC_{50} value, and direct inhibition is not sufficient to explain the strong interaction with tizanidine in humans. The inhibitory effect of rofecoxib was considerably increased by preincubation. The dependence of inactivation of CYP1A2 on the preincubation time, rofecoxib concentration, and NADPH indicated that the inactivation proceeded by a series of catalytic step(s). The inactivation decreased in the presence of increasing concentrations of fluvoxamine (a competitive CYP1A2 inhibitor), and was not altered in the presence of glutathione, superoxide dismutase, and mannitol, suggesting that the inactivating metabolite is formed at the CYP1A2 active site and stays there until the inactivation occurs. The above findings indicate that rofecoxib is a mechanism-based inhibitor of CYP1A2.[176]

The K_I and k_{inact} values of rofecoxib for CYP1A2 were 4.8 µM and 0.070 min^{-1}. According to these values and the C_{max} of rofecoxib (0.15 µM with 25 mg of rofecoxib daily), increases in the AUCs of tizanidine, theophylline, and *R*-warfarin

($f_{m,CYP1A2}$ 0.98, 0.7, and 0.4, respectively) of 8-fold, 2.6-fold and 1.5-fold could be predicted, whereas the observed increases were 13.6-fold, 1.51-fold, and 1.38-fold, respectively.[6,172] Thus, the prediction slightly underestimates the interaction with tizanidine, and overestimates the interactions with theophylline and *R*-warfarin. A likely explanation is that rofecoxib mainly affected the first-pass metabolism of the high-clearance drug tizanidine, whereas it affected the metabolism of the low-clearance drugs theophylline and *R*-warfarin exclusively during their elimination phase. Thus, the plasma concentration of rofecoxib at the time of tizanidine's first-pass largely determines their interaction, whereas the mean or trough concentrations of rofecoxib seem to be more important for interactions with drugs with insignificant first-pass metabolism. Using the portal C_{max} of rofecoxib, which is approximately 400 nM, would lead to the prediction of an increase in the AUC of tizanidine of 14.8-fold, correlating very well with the finding *in vivo*.

The furanone ring that is a part of the molecular structure of rofecoxib is similar to a furan ring, which is one of the most common substructures causing mechanism-based inhibition of CYPs.[56] Covalent bonding between the inhibitor and the P450 enzyme is suggested to occur via the formation of an epoxide intermediate, which can then be nucleophilically attacked by a nitrogen atom of a protein side chain or a heme. However, metabolism of the furan ring of furafylline has been questioned as a basis for CYP1A2 inactivation.[156]

The metabolism of rofecoxib itself is extensive and complex, including oxidative, reductive and back reduction pathways, and CYP enzymes are considered to have only a minor role in it.[134,177] However, in the present study, the AUC of rofecoxib correlated well with the caffeine/paraxanthine ratio, suggesting that CYP1A2 has a significant role in the elimination of rofecoxib. *In vitro*, CYP1A2 and CYP3A4 have been found to catalyze the 5-hydroxylation of rofecoxib[177] when rofecoxib concentrations of 10 µM and 60 µM were used. As such high concentrations can rapidly inactivate CYP1A2 (K_I 4.8 µM, k_{inact} 7% per minute), the role of CYP1A2 in the metabolism of rofecoxib may have been underestimated. In addition, the NADPH-dependent back-reduction of 5-hydroxyrofecoxib to rofecoxib may have caused the role of CYP enzymes in the metabolism of rofecoxib to be underestimated.[177] Accordingly, it is possible that CYP1A2 contributes to the metabolism of rofecoxib *in vivo*, and the autoinhibition of the CYP1A2-mediated metabolism of rofecoxib could also explain its nonlinear pharmacokinetics *in vivo*.[6,134]

Taken together, it can be concluded that rofecoxib is a potent mechanism-based inhibitor of CYP1A2, and it also considerably impairs the metabolism of the CYP1A2 substrates tizanidine and caffeine *in vivo*. In addition, CYP1A2 seems to play a role in the metabolism of rofecoxib itself.

3. The effect of tolfenamic acid on CYP1A2 activity *in vitro* and *in vivo*

When studied without albumin, tolfenamic acid potently inhibited phenacetin O-deethylation *in vitro* (IC_{50} 1.8 µM), which was almost as strong as the inhibition caused by fluvoxamine (K_i 0.12–0.24 µM) and considerably stronger than that by ciprofloxacin (K_i 180 µM). The total C_{max} of tolfenamic acid *in vivo* yields the prediction of a significant interaction with tizanidine (9.3-fold increase in the AUC). However, taking the protein binding of tolfenamic acid (99.7%) into account,[151] the free C_{max} of tolfenamic acid (0.04 µM) predicts only a 1.03-fold increase in the AUC. This fits very well with our observation that tolfenamic acid did not cause a significant change in plasma tizanidine concentration or the caffeine test in humans.

The *in vitro* CYP1A2 inhibitory effect of tolfenamic acid was markedly reduced by the addition of albumin to the incubation medium. Only 42% inhibition was observed even at the highest, clinically irrelevant, tolfenamic acid concentration (100 µM) when an albumin concentration of 10 mg/ml (one-fourth of the physiological concentration) was used. Accordingly, the prediction with the K_i estimate obtained with 10 mg/ml albumin and the total C_{max} of tolfenamic acid produced only an 11% increase in the AUC of tizanidine.

The degree of protein binding is often not known exactly. Assuming a 5% free fraction for tolfenamic acid (a 15-fold overestimate) would predict a 50–63% increase in the AUC of tizanidine. Addition of albumin to microsomal incubations has been suggested to improve predictions of drug clearance.[15,124,199] However, a low protein concentration is generally preferred to minimize protein binding of probe substrates and inhibitors.[15] The present findings suggest that in order to more reliably evaluate *in vivo* inhibition potency, it would be reasonable to perform *in vitro* studies also by adding plasma proteins (albumin or orosomucoid) to the incubation medium when it is not possible to define the exact degree of protein binding of the drug or new chemical entity. This method should perform well when the inhibitor is highly bound to plasma proteins and the probe substrate, such as phenacetin, is largely unbound.

In summary, tolfenamic acid has been found to be a potent inhitor of CYP1A2 *in vitro* without albumin. Due to the low free tolfenamic acid concentration *in vivo* caused by high plasma protein binding, it does not inhibit the metabolism of the CYP1A2 substrates tizanidine and caffeine *in vivo*. Addition of albumin to the incubation medium considerably diminishes the *in vitro* inhibitory effect of tolfenamic acid, and can be considered a useful tool in simulating protein binding *in vivo*.

4. The effect of celecoxib on CYP1A2 activity *in vitro* and *in vivo*

Celecoxib was a moderately potent inhibitor of the CYP1A2 mediated O-deethylation of phenacetin *in vitro* in HLMs (K_i 25.4 µM). However, it did not affect the metabolism of caffeine or tizanidine *in vivo* in humans, unlike the other COX-2 selective inhibitor rofecoxib. When the CYP1A2-mediated drug interaction potential was evaluated, the *in vitro-in vivo* predictions were good when based on the free peripheral or portal C_{max} of celecoxib, whereas predictions made using the total C_{max} tended to overestimate the effect of celecoxib on the tizanidine AUC *in vivo*.

Tizanidine has been found to be a useful probe substrate for CYP1A2 due to its selectivity and sensitivity to the effects of CYP1A2 inhibitors and inducers.[10,69-72] For example, as was shown by the present thesis, rofecoxib increased the AUC of tizanidine 13.6-fold, even though it was first considered to be only a moderately potent inhibitor of CYP1A2, because the AUC of theophylline was raised only by 1.5-fold. To avoid false negative conclusions, it is important to use selective and sensitive *in vivo* probe substrates when estimating the potential for *in vivo* drug-drug interactions.[15,148] It can be concluded that celecoxib (400 mg/day) does not have a clinically relevant CYP1A2 inhibition potency as it did not impair the metabolism of tizanidine or caffeine.

A weak induction of CYP1A2 by celecoxib was suggested by the slight increase in the C_{max} of M-3, and the slight trend towards a decrease in the caffeine-paraxanthine ratio during the celecoxib phase. Formation of the minor metabolite M-10 decreased during the celecoxib phase. As it is thought to be formed partly by CYPs other than CYP1A2 (Study I),[8,70] it is possible that celecoxib inhibits this other CYP enzyme that is responsible for M-10 formation.

As rofecoxib was found to be a potent mechanism-based inhibitor of CYP1A2, and it also inhibited CYP1A2 *in vivo*, it was of interest to find out whether celecoxib would have these same properties. While the chemical structures of the COX-2 inhibitors are much alike, celecoxib includes a pyrazole ring instead of the furanone ring, which is a possible substructure involved in the mechanism-based inhibition of CYP1A2 by rofecoxib.[56] Thus, this difference in the structures of these two molecules may explain their different effects on CYP1A2.

In summary, celecoxib is a moderately potent inhibitor of CYP1A2 *in vitro*, but it is unlikely to cause clinically significant inhibition of CYP1A2 *in vivo*, as it does not inhibit the metabolism of the selective CYP1A2 substrates tizanidine and caffeine *in vivo*.

5. Inhibition of CYP1A2 *in vitro*

The model CYP1A2 inhibitor fluvoxamine is known to be a potent inhibitor of CYP1A2,[96] and in previous studies, its *in vitro* K_i values have ranged from 0.035 to 0.24 µM.[22,143] However, the *in vivo* K_i with theophylline as a CYP1A2 substrate has been found to be lower than the *in vitro* value.[208] In Study V, the incubations carried out with a low microsomal protein concentration (0.1 mg/ml) resulted in a K_i of 0.011 µM, which is in agreement with the *in vivo* finding. Compared to its *in vivo* effects, ciprofloxacin has been found to be a surprisingly weak inhibitor of CYP1A2 *in vitro*, with K_i values around 200 µM.[62,80] In the present study, the K_i for ciprofloxacin was 145 µM, which is still quite high compared to its free plasma concentration. Ciprofloxacin did not exhibit a clear time-dependent inhibition of CYP1A2, although the data hints at the possibility. Further studies are needed to elucidate this matter.

In vivo in humans, the metabolism of the CYP1A2 substrates theophylline, caffeine and tizanidine has been found to be impaired by oral contraceptives containing ethinylestradiol and gestodene.[2,70,166] *In vitro*, ethinylestradiol has been found to moderately inhibit CYP2C8, CYP2C19 and CYP3A4, and it has been reported to be a mechanism-based inhibitor of CYP2B1, CYP2B6 and CYP3A4.[210] Gestodene, on the other hand, is a mechanism-based inhibitor of CYP3A4, but no effect on CYP1A2 has been observed even at high concentrations.[74] In the present study, ethinylestradiol potently inhibited phenacetin-O-deethylation, while gestodene had only a weak effect. No sign of mechanism-based inhibition of CYP1A2 by either of the compounds was observed. A moderate inhibition of CYP1A2 by another progestogen, desogestrel, was found in the present study. The inhibition of CYP1A2 by desogestrel was further increased by a 30-min preincubation, which indicates metabolism-dependent, or even mechanism-based, inhibition of CYP1A2. Etonogestrel, the primary metabolite of desogestrel,[65] did not, however, inhibit CYP1A2 *in vitro* in the present study. Progesterone showed some weak inhibition of CYP1A2 which was increased by preincubation.

As muscle relaxants are often used in combination with pain killers, the effect of several NSAIDs, in addition to those mentioned above, and zolmitriptan on CYP1A2 activity were investigated. Tolfenamic acid and mefenamic acid have been in clinical use for decades. However, their effects on CYP1A2 were not known prior to this study. They both proved to be relatively strong inhibitors of CYP1A2 *in vitro* with K_i values of 1.39 µM and 3.47 µM, respectively. Celecoxib was known to inhibit CYP2D6 *in vitro* with a K_i of 4.2 µM.[52] In the present study, the K_i for celecoxib with CYP1A2 was about six times higher than that reported for CYP2D6. Etoricoxib was known to have IC_{50} values of > 100 µM for CYP1A2, 2C9, 2C19, 2D6, 2E1 and 3A *in vitro*.[104] In the present study, etoricoxib inhibited

CYP1A2 with an IC_{50} of 240 µM, which is in good agreement with previous findings. In contrast to rofecoxib, the inhibitory potencies of celecoxib and etoricoxib were not affected by preincubation. The primary metabolism of zolmitriptan to N-desmethyl-zolmitriptan is mainly CYP1A2-mediated, which makes it a candidate inhibitor of the enzyme.[204] In fact, moderate inhibition of CYP1A2 by zolmitriptan, with a K_i of 60.1 µM, was discovered.

Taken together, potent inhibition of CYP1A2 by fluvoxamine, tolfenamic acid, mefenamic acid and rofecoxib was observed *in vitro*. Ethinylestradiol, celecoxib, desogestrel and zolmitriptan showed moderately potent inhibition of CYP1A2. Ciprofloxacin, etodolac, etoricoxib and gestodene had only a weak CYP1A2 inhibitory effect, and the other tested drugs inhibited CYP1A2 less than 35% at 100 µM.

6. *In vitro-in vivo* predictability and predictions

One of the aims of this work was to evaluate the accuracy of *in vitro-in vivo* predictions. This was done by comparing the *in vivo* effects of fluvoxamine, ciprofloxacin, oral contraceptives containing ethinylestradiol and gestodene, rofecoxib, tolfenamic acid and celecoxib on tizanidine pharmacokinetics with the predictions based on *in vitro* data from the present study. The *in vivo* effects of the above mentioned compounds (Studies I, III, IV)[70-72] are comparable as their effects on tizanidine pharmacokinetics have been studied under equivalent conditions.

The drastic increase in the AUC of tizanidine caused by fluvoxamine was well predicted when the estimated free portal C_{max} of fluvoxamine was used (32- and 33-fold increases predicted and observed, respectively).[71] The use of the free peripheral C_{max} of fluvoxamine resulted in underestimation of the AUC increase. Thus, the extent of this interaction seems to be determined mainly by the hepatic concentration of fluvoxamine during the first-pass of tizanidine.

The effects of ciprofloxacin and oral contraceptives on tizanidine pharmacokinetics *in vivo* are greatly underestimated based on *in vitro* data and any inhibitor concentration used *in vivo*. Ignoring metabolism-dependent inhibition is known to greatly underestimate *in vivo* effects.[194] Another explanation has been proposed to be accumulation of the inhibitor in the hepatocytes, either due to passive diffusion or active transport, leading to an increased inhibitor concentration at the enzyme active site. It is possible that fluoroquinolones become concentrated in the hepatocytes due to their lipophilic and basic properties.[73] Enoxacin has also been found to moderately inhibit CYP1A2 *in vivo* despite its weak inhibitory potency *in vitro*.[143] In the present study, the slight increase in the inhibitory potency of ciprofloxacin caused by preincubation could be due to either mechanism-based inhibition or formation of inhibitory metabolites. The former has been described for ciprofloxacin in studies with dog microsomes.[160] In addition, the M1 metabolite of ciprofloxacin has been found to be a somewhat stronger inhibitor of CYP1A2 than the parent compound *in vitro*.[61] As about 90% of ethinylestradiol ends up in the liver, at least in rodents, the concentration of ethinylestradiol might also be relatively high in the hepatocytes.[84,210] However, the extent of the *in vivo* interaction between ethinylestradiol and CYP1A2 substrates was underestimated, even when 10-fold the estimated portal C_{max} was used. Ethinylestradiol did not show indications of time-dependent inhibition of CYP1A2. However, conjugates may also inhibit CYP enzymes, as shown by a glucuronide of gemfibrozil.[145] Glucuronidation and sulfation are important routes for the elimination of ethinylestradiol,[210] but the effects of these ethinylestradiol conjugates on CYP1A2 seem to be unknown.

Tolfenamic acid and celecoxib were found to be potent and moderately potent inhibitors of CYP1A2 *in vitro*, respectively. However, neither one of them inhibited the metabolism of tizanidine or caffeine in humans (Studies III and IV). A low free inhibitor concentration caused by high plasma protein binding explains well the lack of *in vivo* inhibition despite the *in vitro* effects.[151] Accordingly, the predictions based on the free portal C_{max} values of tolfenamic acid and celecoxib correlated well with the lack of *in vivo* interactions, whereas the total portal C_{max} values overestimated the effects.

Mefenamic acid was discovered to be a rather potent inhibitor of CYP1A2 *in vitro*. However, mefenamic acid is also highly bound to plasma proteins (about 99%).[44] When the free portal C_{max} was used, mefenamic acid was predicted to slightly increase the AUC of tizanidine. Thus, despite the high protein binding of mefenamic acid, its *in vivo* effect on CYP1A2 might be somewhat stronger than that of tolfenamic acid. Etoricoxib had only a weak effect on CYP1A2, but it was recently found to inhibit *R*-warfarin metabolism, which is partly mediated by CYP1A2.[171] The role of CYP1A2 inhibition in this interaction could be elucidated by using a specific CYP1A2 substrate like tizanidine. According to our predictions, clinically significant inhibition of CYP1A2 seems likely only if etoricoxib is concentrated in the hepatocytes. Zolmitriptan was discovered to moderately inhibit CYP1A2, but it was predicted not to cause a significant interaction with CYP1A2 substrates *in vivo*.

Desogestrel was found to be a moderately potent CYP1A2 inhibitor, and its effect was increased by preincubation. However, desogestrel is rapidly and practically completely metabolized to etonogestrel *in vivo*,[65] which in the present study did not inhibit CYP1A2 *in vitro*. Thus, it seems unlikely that desogestrel would have clinically significant interactions with CYP1A2 substrates *in vivo*. Progesterone showed some time-dependent inhibition of CYP1A2. Although progesterone was predicted not to significantly affect CYP1A2 *in vivo*, the clinical relevance of this finding might be worth investigating further.

As quantitative prediction of *in vivo* metabolic drug-drug interactions from *in vitro* data has proved to be challenging, different approaches to predicting *in vivo* interactions have been suggested in order to reduce the number of studies needed to understand the *in vivo* interaction potential based on *in vitro* data. The rank order approach proposes that the rank order of *in vitro* and *in vivo* inhibitory potencies are the same.[144] Thus, if a drug does not cause at least a 100% increase in the AUC of a substrate metabolized by the CYP most potently inhibited *in vitro*, it is unlikely to cause clinically significant interactions with substrates of the CYP enzymes less potently inhibited. However, the probe substrates need to be sensitive (and selective), and metabolism-dependent inhibition needs to be excluded. For example,

when theophylline was used as a CYP1A2 substrate, the *in vivo* order of potency of fluvoxamine was CYP1A2 < CYP2C19 (3.3-fold and 9.9-fold increase in the AUC of theophylline and mephenytoin, respectively).[144] However, fluvoxamine inhibited CYP1A2 most potently *in vivo* when the more sensitive CYP1A2 substrates caffeine (undetectable paraxanthine concentrations in urine)[96] and tizanidine (33-fold increase in its AUC)[71] were used. In the present study, the applicability of the rank order approach was evaluated in the case of celecoxib in regard to CYP2D6 and CYP1A2. Although metoprolol is not an optimal CYP2D6 substrate (only 80% metabolized by CYP2D6)[57], the rank order approach performed well in this case.

To summarize, the *in vivo* effect of fluvoxamine and the lack of effects of tolfenamic acid and celecoxib could be well predicted when using the free inhibitor concentration and the competitive inhibition model. However, the effects of ciprofloxacin and oral contraceptive steroids are underestimated even when using the total inhibitor concentration. The mechanism of inhibition and possible accumulation of the inhibitor in the hepatocytes were considered, but no satisfying explanation for the lack of correlation was found. Besides mefenamic acid, other drugs for which *in vivo* data is lacking are not predicted to interact with CYP1A2 substrates *in vivo*. However, as the *in vitro-in vivo* correlations are not always accurate, one should not overlook moderate *in vitro* inhibition.

7. Clinical implications

CYP1A2 is one of the major hepatic drug-metabolizing enzymes. It is largely responsible for the metabolism of many important xenobiotics including caffeine, theophylline, clozapine, olanzapine, duloxetine, tacrine, riluzole, lidocaine, zolmitriptan, and tizanidine.[12,13,69,76,129,165,198,211] In addition, CYP1A2 plays a central role in the metabolism of certain endogenous compounds such as melatonin, 17-β-estradiol, and uroporphyrinogen.[5,49,117] Thus, inhibition of CYP1A2 may affect the pharmacokinetics of many compounds.

Symptomatic hypotension, bradycardia and somnolence have been observed in case reports (one with a fatal outcome) during co-administration of rofecoxib and tizanidine,[53,107] demonstrating the potential hazards of this interaction to patients. Similar exaggerated pharmacodynamic effects of tizanidine were also seen in our healthy volunteers after ingestion of tizanidine during the rofecoxib phase.

Rofecoxib has previously been suggested to have a relatively low propensity to interact with drugs metabolized by CYP enzymes.[134,177] However, the inhibition of CYP1A2 by rofecoxib is clinically relevant, as documented by clinical studies with tizanidine (Study I), theophylline, and warfarin.[6,172] It is likely that the metabolism of other CYP1A2 substrate drugs is also markedly inhibited by rofecoxib. This inhibition is likely to be particularly important when the victim drug has a low non-CYP1A2-mediated clearance and a narrow therapeutic range, like, for example, clozapine. However, due to the possibly temporary withdrawal of rofecoxib from the market, these combinations are not relevant to clinical practice.

Urine volume during the first 12 h after tizanidine ingestion was markedly lower during the rofecoxib phase than during the placebo phase (Study I). Thus, concomitant administration of rofecoxib and tizanidine seems to adversely affect renal function, and may increase the risk of fluid accumulation and edema. This renal effect was probably mainly due to the hypotension caused by the high plasma concentrations of tizanidine. However, rofecoxib may also decrease renal function and cause fluid retention, which may have contributed to the decreased urine excretion.[90,203]

Rofecoxib inhibited the metabolism of caffeine in healthy volunteers (Study I), and according to recent findings, caffeine intake may be associated with an increased risk of myocardial infarction among individuals with impaired caffeine metabolism.[30,31] Thus, the inhibition of CYP1A2-mediated caffeine metabolism by rofecoxib may contribute to its increased cardiovascular risk. Celecoxib seems to have a lower cardiovascular risk than rofecoxib, but the risk can not be excluded with daily doses of celecoxib higher than 200 mg.[17] At least in theory, the lack of

CYP1A2 inhibition could partly explain the lower cardiovascular risk of celecoxib compared to rofecoxib. However, there are also other significant differences between these two coxibs, including their effect on the vascular endothelium.[146]

The present study suggests that the role of CYP1A2 in the metabolism of rofecoxib is greater than was previously thought. Thus, rofecoxib itself might be susceptible to the effects of CYP1A2 inhibitors such as oral contraceptive steroids, ciprofloxacin or fluvoxamine, and their co-administration might lead to an accumulation of rofecoxib during daily administration, and thereby increase the risk of adverse events.

As was demonstrated by tolfenamic acid and celecoxib, plasma protein binding of the inhibitor should be considered when making *in vitro-in vivo* extrapolations. As in many cases the exact fraction bound is unknown, the use of albumin in the *in vitro* incubations could have predictive value. In the case of tolfenamic acid, addition of only one-fourth of the physiological albumin concentration nearly abolished inhibition of CYP1A2. Thus, an interaction between tolfenamic acid and CYP1A2 substrates is highly unlikely *in vivo*, even if the plasma protein concentration were slightly decreased, e.g., by hepatic or renal disease.

In order to get better pain relief, centrally acting muscle relaxants are often prescribed together with NSAIDs. Combinations of tizanidine and a long-acting NSAID have been proposed to be useful e.g. for the treatment of analgesic rebound headache.[178] Because increased tizanidine plasma concentrations can lead to severe adverse effects, it is important that care is taken when tizanidine is used in combination with any potent CYP1A2 inhibitor.[71,72] The present study demonstrates that the concomitant use of celecoxib and tolfenamic acid with tizanidine does not lead to increased tizanidine plasma concentrations, making their co-administration preferable to the tizanidine and rofecoxib combination. Of the other drugs studied here and for which *in vivo* data is lacking, only mefenamic acid seems to pose a potential risk.

CONCLUSIONS

The following conclusions can be made based on the studies in this thesis:

1 Rofecoxib is a potent inhibitor of CYP1A2 *in vivo*, and it greatly increases the plasma concentrations and adverse effects of tizanidine. Therefore, coadministration of rofecoxib and tizanidine is best avoided. It seems that CYP1A2 plays a significant role in the elimination of rofecoxib, and the findings here raise concerns about the potential risks related to the interactions of rofecoxib with both CYP1A2 substrate and inhibitor drugs. However, at the moment, these findings are not relevant to clinical practice, because rofecoxib has been withdrawn from the market, at least temporarily.

2 Rofecoxib is a potent, metabolism-dependent inhibitor of CYP1A2 *in vitro*. The results provide a mechanistic explanation for the interactions of rofecoxib with CYP1A2 substrates and, possibly, for its nonlinear pharmacokinetics.

3 Tolfenamic acid is an inhibitor of CYP1A2 *in vitro* but does not affect the pharmacokinetics of tizanidine or caffeine *in vivo* in humans. It is important to consider protein binding when interpreting *in vitro* data. If the exact degree of protein binding is unknown, an alternative is to test the effect of albumin on the inhibitory effect *in vitro*.

4 Despite moderate CYP1A2 inhibition *in vitro*, in humans, celecoxib does not inhibit the metabolism of tizanidine or the formation of paraxanthine from caffeine, which are highly selective markers for CYP1A2 activity. Thus, it is also unlikely to cause clinically significant increases in the concentrations of other CYP1A2 substrates.

5 Except for rofecoxib, and possibly mefenamic acid, the *in vitro* data does not support the likelyhood of significant CYP1A2 inhibition *in vivo* by the other tested NSAIDs.

6 In most cases, it seems to be preferable to use the free plasma concentration of the inhibitor when estimating *in vivo* drug-drug interactions based on *in vitro* data. However, the mechanism of inhibition and accumulation of the inhibitor in the hepatocytes might interfere with the predictions.

7 The *in vivo* effects of ciprofloxacin and oral contraceptive steroids are underestimated based on *in vitro* data, and further studies are warranted to clarify their mechanisms of CYP1A2 inhibition in humans.

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Marjo Karjalainen

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