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**ROFECOXIB IS A POTENT, METABOLISM-DEPENDENT
INHIBITOR OF CYP1A2 – IMPLICATIONS FOR IN VITRO
PREDICTION OF DRUG INTERACTIONS**

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A list of non-standard abbreviations:

CYP, cytochrome P450

DHHA, dihydrohydroxy acid

GSH, glutathione

K_i , inhibitor concentration that supports half the maximal rate of inactivation

k_{inact} , maximal rate of inactivation

K_{obs} , initial rate of inactivation

SOD, superoxide dismutase

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ABSTRACT:

Rofecoxib was recently found to greatly increase plasma concentrations of the cytochrome P450 1A2 (CYP1A2) substrate drug tizanidine in humans, but there are no published in vitro studies on the CYP1A2 inhibiting effects of rofecoxib. Our objective was to investigate whether rofecoxib is a direct-acting or metabolism-dependent inhibitor of CYP1A2 in vitro. The effect of rofecoxib on the O-deethylation of phenacetin (20 μM) was studied using human liver microsomes. The effect of preincubation time on the inhibitory potential of rofecoxib was also studied, and the inhibitor concentration that supports half the maximal rate of inactivation (K_I) and the maximal rate of inactivation (k_{inact}) were determined. Rofecoxib moderately inhibited phenacetin O-deethylation (IC_{50} 23.0 μM), and a 30-min-preincubation with microsomes and NADPH considerably increased its inhibitory effect (IC_{50} 4.2 μM). Inactivation of CYP1A2 by rofecoxib required NADPH, and was characterized by a K_I of 4.8 μM and a k_{inact} of 0.07 min^{-1} . Trapping agents, glutathione, superoxide dismutase, and mannitol, or dialysis could not reverse the inactivation of CYP1A2 caused by rofecoxib. Fluvoxamine concentration-dependently decreased the rofecoxib-caused inactivation of CYP1A2. In conclusion, rofecoxib is a potent, metabolism-dependent inhibitor of CYP1A2, a CYP-form contributing to rofecoxib metabolism. The results provide a mechanistic explanation for the interactions of rofecoxib with CYP1A2 substrates, and may partially explain its nonlinear pharmacokinetics.

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Rofecoxib is a cyclooxygenase-2-selective nonsteroidal anti-inflammatory drug, which was recently withdrawn, possibly temporarily, from clinical use due to its cardiovascular side effects. Previous studies have shown that rofecoxib moderately increases plasma concentrations and effects of theophylline (Bachmann et al., 2003) and the *R*-isomer of warfarin (Schwartz et al., 2000). Quite recently, rofecoxib in therapeutic doses of 25 mg per day was found to increase more than 10-fold the plasma concentrations of the cytochrome P450 1A2 (CYP1A2) substrate tizanidine in humans (Backman et al., 2006b). The effects on tizanidine pharmacokinetics suggest that rofecoxib can be a relatively potent inhibitor of CYP1A2. However, hitherto, there have been no published in vitro studies on the effect of rofecoxib on CYP1A2 activity nor has the type of CYP1A2 inhibition by rofecoxib been clarified.

Rofecoxib itself is extensively metabolized via complex oxidative, reductive and back reduction pathways (Figure 1) (Merck & Co., 2002; Slaughter et al., 2003). In human liver microsomal incubations, rofecoxib has been found to be metabolized to 5-hydroxyrofecoxib by several CYP enzymes, CYP1A2 and CYP3A4 having the greatest contributions (Slaughter et al., 2003). In liver S9 fractions, the metabolite profile of rofecoxib has been more complex, including the formation of both 5-hydroxyrofecoxib and different 3,4-dihydroxy acid (DHHA) derivatives; the latter are considered to represent the major pathways in vivo (Halpin et al., 2002). Thus, it has been suggested that CYP enzymes play a minor role in its metabolism. However, in our recent study, the plasma concentrations of rofecoxib correlated to the plasma caffeine-paraxanthine ratio in vivo, suggesting a role for CYP1A2 in the metabolism of rofecoxib (Backman et al., 2006b).

Our purpose was to explore in vitro the CYP1A2 inhibitory potency of rofecoxib, and to elucidate the type of possible CYP1A2 inhibition.

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METHODS

Chemicals and microsomes. Rofecoxib (Sequoia Research Products Limited, Pangbourne, UK), phenacetin, acetaminophen, β -NADPH (Sigma Aldrich, St Louis, MO, USA), methanol, acetonitrile and ethyl acetate (Rathburn Chemicals Ltd., Walkerburn, Scotland) were used in this study. Other chemicals were from Merck (Darmstadt, Germany). Pooled human liver microsomes were obtained from BD Biosciences (Woburn, MA, USA). Human liver tissue had been collected in accordance with all pertinent regulations.

Incubation conditions. Phenacetin O-deethylation (acetaminophen formation) was used as an index reaction for CYP1A2 activity (Distlerath et al., 1985; Tassaneeyakul et al., 1993). All incubations were performed in duplicate (all the data were within 12% of their mean) in a shaking water bath at 37°C. The incubations were carried out in 0.1 M sodium phosphate buffer (pH = 7.4), containing 5.0 mM MgCl_2 , 1.0 mM β -NADPH, 20 μM phenacetin and human liver microsomes (0.1 mg ml^{-1} protein) in a total volume of 1000 μl . The incubation mixture components, except β -NADPH, were premixed and the resulting mixture was kept in room temperature for 3 minutes, and incubations were commenced by addition of β -NADPH. Aliquots of 200 μl were removed at 20 min, the reactions were stopped with 100 μl of acetonitrile including the internal standard, hydrochlorothiazide, and cooling on ice. To evaluate the inhibitory effect of rofecoxib on CYP1A2 activity, various concentrations of rofecoxib were added to the incubation mixture prior to addition of β -NADPH. Rofecoxib was dissolved in acetonitrile, and phenacetin in methanol, the final solvent concentration not exceeding 1%.

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To evaluate the potential of metabolism-dependent inhibition by rofecoxib, incubation mixtures were prepared as described earlier, but without the substrate. Preincubations were started by addition of β -NADPH. At 30 min, phenacetin was added to the mixtures and the incubations were allowed to proceed for 20 min. To determine the K_i (the inhibitor concentration needed to cause half-maximal rate of inactivation) and k_{inact} (the maximal rate of inactivation with saturable concentration of the inhibitor), the preincubation mixture was prepared as described above, except using a microsomal protein concentration of 1 mg ml^{-1} . 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 β -NADPH, in a final volume 1000 μl , to determine residual CYP1A2 activity. Thus, rofecoxib was diluted to one tenth of its original concentration, so that in the final incubation mixture the “direct” inhibition of CYP1A2 by the highest rofecoxib concentration would not exceed 25%.

To determine the effect of trapping agents and an alternative competitive inhibitor on CYP1A2 inactivation, the preincubation mixture was prepared as in k_{inact} and K_i determinations. Preincubations were carried out with or 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 an aliquot of 100 μl was transferred to another incubation tube (final volume of 1000 μl), and the residual phenacetin-O-deethylation activity was measured as described above.

To evaluate the effect of dialysis on the inhibitory effect of rofecoxib, human liver microsomes were preincubated with or without 12 μM rofecoxib for 30 min as described earlier (1 mg ml^{-1} of microsomal protein). The samples were immediately

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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 4°C, followed by a 10-fold dilution and measurement of phenacetin O-deethylation activity as described above.

Measurement of drug concentrations. Acetaminophen and its internal standard, hydrochlorothiazide, were extracted with 5.0 mL of ethyl acetate. After centrifugation, the supernatant was evaporated to dryness under nitrogen, and the residue was reconstituted with 100 µL of the mobile phase (ammonium acetate 6 mM, pH 9.0: acetonitrile, 78 : 22). Thereafter, acetaminophen concentration was determined by high-performance liquid chromatography with UV detection (230 nm) (Demotes-Mainard et al., 1984; Pufal et al., 2000). 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 acetaminophen was 10 nM.

The nonspecific binding of 1 µM and 12 µM rofecoxib to the microsomes (1 mg/ml of microsomal protein) was determined with equilibrium dialysis for 18 hours at 37°C. Rofecoxib and its internal standard, etoricoxib were extracted with 5.0 ml of methyl-tert-butylether. After centrifugation, the supernatants were evaporated to dryness under nitrogen, and the residues were reconstituted with 100 µL of the mobile phase (acetonitrile : water, 45 : 55). Rofecoxib concentrations were determined by HPLC with UV detection (270 nm) (Slaughter et al., 2003). The limit of quantification for rofecoxib was 20 nM.

Data analysis. The IC₅₀-values were determined by nonlinear regression analysis with Sigma Plot 8.0 (SPSS Inc., Chicago, IL, USA). For estimation of inactivation

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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 of inactivation of CYP1A2 activity by each inhibitor concentration (K_{obs}) was determined by linear regression analysis of the natural logarithm of percentage of activity remaining versus preincubation time data (Kitz and Wilson, 1962). Thereafter, the K_{obs} values were used to determine the inhibitor concentration needed to cause half-maximal rate of enzyme inactivation (K_I) and the maximal rate of inactivation (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) (Kitz and Wilson, 1962). When extrapolated over 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 . Then, the K_I and k_{inact} were estimated by nonlinear regression using the following equation (Jones et al., 1999):

$$K_{obs} = \frac{k_{inact} \cdot [I]}{K_I + [I]} \quad (1)$$

In vivo simulations. In vitro data describing mechanism-based inhibition (the k_{inact}/K_I ratio) can be used to evaluate the potential clinical impact of the inhibition according to the following equation (Lu et al., 2003):

$$\frac{AUC_{po(i)}}{AUC_{po(c)}} = \frac{1}{\frac{f_m \cdot f_{m,P450}}{(1 + [k_{inact} / K_I \cdot [I] / k_e])} + 1 - f_m \cdot f_{m,P450}} \quad (2)$$

where $AUC_{po(i)}$ is the area under the concentration time curve of the substrate during inactivation of the enzyme caused by the inhibitor, $AUC_{po(c)}$ is the control value, $f_m \cdot f_{m,P450}$ represents the fraction of the substrate dose cleared by the CYP enzyme, and k_e

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is the rate constant for enzyme degradation in the absence of the inhibitor. In a previous study, k_e estimates ranging from 0.0005 to 0.00026 min^{-1} have yielded the most accurate in vivo predictions of CYP1A2 inhibition by the mechanism-based inhibitor zileuton (Lu et al., 2003). In our simulations, a CYP1A2 half-life of 38.6 h (i.e. $k_e = 0.0003 \text{ min}^{-1}$) was assumed, based on a previous estimate of the in vivo CYP1A2 half-life (Faber and Fuhr, 2004; Ghanbari et al., 2006). This value is similar to the in vitro half-life estimate of 36 hours determined for CYP1A2 in liver slices (Renwick et al., 2000). It should be noted that the equation used assumes that the substrate is completely absorbed from the gastrointestinal tract, its hepatic clearance can be described by the well-stirred pharmacokinetic model, the substrate is metabolized in the liver only, its pharmacokinetics are linear, and the inhibitor does not affect its absorption or clearance by other mechanisms.

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RESULTS

Effect of rofecoxib on CYP1A2 activity. Rofecoxib inhibited CYP1A2 activity with an IC_{50} value of 23.0 μ M in human liver microsomes, when no preincubation was carried out (Figure 2). A 30-min-preincubation of rofecoxib in the presence of β -NADPH increased the inhibition of CYP1A2 considerably (Figure 2), suggesting that rofecoxib is a metabolism-dependent inhibitor of CYP1A2. The IC_{50} value of rofecoxib with the preincubation was 4.18 μ M. This preincubation-dependent inactivation of CYP1A2 required β -NADPH (data not shown).

Inactivation of CYP1A2 by rofecoxib. The inhibition of CYP1A2 activity by rofecoxib was preincubation time- and concentration-dependent (Figure 3). The inactivation variables K_I and k_{inact} for CYP1A2 were 4.78 μ M and 0.070 min^{-1} , respectively, when determined with the non-linear regression method (Figure 3). The k_{inact} value implies that 7% of CYP1A2 is inactivated each minute when a saturating concentration of rofecoxib is incubated with human liver microsomes. The partition ratio (number of molecules metabolized via non-inactivating pathways per one inactivating event), estimated as described previously (Kunze and Trager 1993) and assuming a CYP1A2 proportion of about 20% (Klein et al., 2006), was about 3. The nonspecific binding of rofecoxib to microsomes was less than 5% (data not shown).

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 (Table 1). Fluvoxamine concentration-dependently diminished the rofecoxib-induced inactivation of the enzyme (Table 1). For example,

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1.0 μM fluvoxamine decreased the inactivation caused by 12 μM rofecoxib from 63% to 6%.

Prediction of in vivo drug interactions. Dependence of the AUC change of a CYP1A2 substrate on rofecoxib concentration is shown for drugs with different fractions metabolized by CYP1A2 (Figure 4). When clinically relevant total plasma rofecoxib concentration of 1 μM , and the K_I of 4.78 μM and the k_{inact} of 0.070 min^{-1} of rofecoxib were used in simulations, marked (> 3-fold) increases in the AUC of compounds extensively (> 70%) metabolized by CYP1A2 were predicted. The unbound mean peak plasma concentration of rofecoxib, 150 nM after a 25 mg daily dose assuming a 85% protein binding in plasma (Merck & Co., 2002), was predicted to cause an about 8-fold increase in the AUC of a drug, whose $f_m \cdot f_{m,\text{CYP1A2}}$ is 0.98. The corresponding predicted increase in the AUC was 2.6-fold for a drug, whose $f_m \cdot f_{m,\text{CYP1A2}}$ is 0.70, but only 1.54-fold for a drug, whose $f_m \cdot f_{m,\text{CYP1A2}}$ is 0.40.

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DISCUSSION

In previous pharmacokinetic studies, rofecoxib has moderately increased the plasma concentrations of *R*-warfarin and theophylline (Schwartz et al., 2000; Bachmann et al., 2003), and greatly increased the plasma concentrations of tizanidine (Bachman et al., 2006b). In the present in vitro study, we wanted to elucidate the mechanism of these interactions by studying the effect of rofecoxib on CYP1A2 activity (phenacetin-O-deethylation) in human liver microsomes. Our findings are consistent with a potent mechanism-based inhibition of CYP1A2 by rofecoxib, thus, providing a likely mechanistic explanation for the interactions.

Rofecoxib inhibited CYP1A2 with an IC_{50} value of 23.0 μ M without preincubation. However, typical total plasma concentrations of rofecoxib are less than 10% of this IC_{50} -value. Therefore, direct inhibition of CYP1A2 is unlikely to explain the strong interaction with tizanidine in humans (Bachmann, 2006). Preincubation of rofecoxib in the presence of NADPH considerably increased its inhibitory effect. This inactivation of CYP1A2 was dependent on preincubation time, rofecoxib concentration, and NADPH, indicating that the inactivation proceeded via catalytic step(s). The decrease in inactivation observed in the presence of increasing concentrations of the competitive CYP1A2 inhibitor, fluvoxamine, and the lack of effect of glutathione, superoxide dismutase and mannitol, suggest that the inactivating metabolite is formed at the CYP1A2 active site and does not leave the active site before the inactivation takes place. These findings are consistent with criteria for mechanism-based inhibition (Silverman, 1995).

In a further experiment, the K_I and k_{inact} values of rofecoxib for CYP1A2 were 4.8 μ M and 0.070 min^{-1} . Prediction of in vivo interactions on the basis of these values

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(Figure 4), and the free C_{\max} of rofecoxib, 0.15 μM with 25 mg rofecoxib daily (Merck & Co., 2002; Backman et al., 2006b), conformed fairly well, but not exactly, with the findings of the pharmacokinetic studies with tizanidine, theophylline and *R*-warfarin (Schwartz et al., 2000; Bachmann et al., 2003; Backman et al., 2006b). The fraction of the substrate metabolized by CYP1A2 ($f_m \cdot f_{m,\text{CYP1A2}}$) is crucial for in vitro – in vivo extrapolations. The $f_m \cdot f_{m,\text{CYP1A2}}$ values for tizanidine, theophylline, and *R*-warfarin are approximately 0.98, 0.7, and ≤ 0.4 , respectively (Kaminsky and Zhang, 1997; Rasmussen et al., 1997; Yao et al., 2001; Granfors et al., 2004b; Backman et al., 2006a). In the in vivo studies with healthy volunteers, rofecoxib (25 mg daily) has raised the AUC of tizanidine, theophylline and *R*-warfarin 13.6-fold, 1.51-fold, and 1.38-fold, respectively (Schwartz et al., 2000; Bachmann et al., 2003; Backman et al., 2006b). Thus, our predictions seem to slightly underestimate the interaction with tizanidine (8-fold predicted increase in AUC), and overestimate the interactions with theophylline (2.6-fold predicted increase) and *R*-warfarin (1.54-fold predicted increase).

It is possible that the $f_m \cdot f_{m,\text{CYP1A2}}$ values of tizanidine, theophylline, and *R*-warfarin in the healthy volunteer studies differed from our estimates, partially explaining this apparent slight discrepancy in the predictions. However, a more likely explanation is that rofecoxib affected mainly the first-pass metabolism of the high clearance drug tizanidine, while 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 absorption may largely determine the extent of interaction with tizanidine. On the other hand, the mean or trough concentrations of rofecoxib (about 50%-70% lower than the C_{\max}) may be a more important determinant for interactions with drugs, which do not have significant

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first-pass metabolism, like theophylline and warfarin. Moreover, it is obvious that the C_{\max} of rofecoxib in the portal vein is higher than its C_{\max} in peripheral blood, further facilitating the inactivation of CYP1A2 during the absorption of rofecoxib.

The free rofecoxib C_{\max} in portal vein can be estimated from the following equation:

$$C_{\max, \text{portal, free}} = f_u \cdot \left(C_{\max} + \frac{k_a F_a D}{Q_h} \right) \quad (3)$$

where f_u is the unbound fraction (15%), D is the dose (25 mg), F_a is the oral bioavailability (93%), k_a is the absorption rate constant, and Q_h is the hepatic blood flow (1500 ml min^{-1}) (Merck & Co., 2002). As the peak concentration of rofecoxib can occur at 2 hours and its half-life averages 20 hours (Merck & Co., 2002; Backman et al., 2006b), the k_a of rofecoxib can be estimated to approximate 0.035 min^{-1} . Thus, according to equation 3, the $C_{\max, \text{portal, free}}$ of rofecoxib could be about 400 nM, i.e. 2.7 times higher than the free C_{\max} in peripheral blood. This would yield a prediction of a 14.8-fold increase in the AUC of a drug with a $f_m \cdot f_{m, \text{CYP1A2}}$ of 0.98.

The molecular structure of rofecoxib includes a furanone ring, which is the site of its CYP1A2-mediated oxidation to 5-hydroxyrofecoxib (Figure 1), and a possible structure involved in the observed inactivation of CYP1A2. Furan ring, which lacks the ketone group of furanone ring, is one of the most common substructures causing mechanism-based inhibition (Fontana et al., 2005). It has been suggested that the mechanism includes the formation of an epoxide intermediate, which can be nucleophilically attacked by a nitrogen atom of a protein sidechain or a haem, leading to a covalent binding between the inhibitor and the CYP enzyme. However, metabolism of the furan ring of furafylline has been questioned as a basis for CYP1A2 inactivation (Racha et al., 1998). Interestingly, the spontaneous

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formation of a reactive maleic anhydride, which binds covalently to elastin fibers, has been suggested to be involved in the cardiovascular toxicity of rofecoxib (Reddy and Corey, 2005).

CYP1A2 is one the major hepatic drug metabolizing CYP enzymes. It is largely responsible for the metabolism of many important xenobiotics including caffeine, theophylline, clozapine, olanzapine, duloxetine, tacrine, riluzole, lidocaine, zolmitriptan and tizanidine (Berthou et al., 1991; Bertilsson et al., 1994; Ha et al., 1995; Madden et al., 1995; Zhang and Kaminsky, 1995; Ring et al., 1996; Wang et al., 2000; Granfors et al., 2004a), and partially involved in the metabolism of e.g. tricyclic antidepressants and the *R*-isomer of warfarin (Zhang et al., 1995; Kaminsky and Zhang, 1997; Olesen and Linnet, 1997). In addition, CYP1A2 plays a central role in the metabolism of certain endogenous compounds such as melatonin, 17 β -estradiol, and uroporphyrinogen (Aoyama et al., 1990; Lambrecht et al., 1992; Facciola et al., 2001). Some animal data suggest that CYP1A2 can have important housekeeping functions, in addition to its role in drug metabolism (Smith et al., 2003).

Rofecoxib has previously been suggested to have a relatively low propensity to interact with drugs metabolized by CYP enzymes (Merck & Co., 2002; Slaughter et al., 2003). However, the inhibition of CYP1A2 by rofecoxib is clinically relevant as documented by the clinical studies with tizanidine, theophylline and warfarin (Schwartz et al., 2000; Bachmann et al., 2003; Backman et al., 2006b). It is probable that the metabolism of other CYP1A2 substrate drugs also is markedly inhibited by rofecoxib. This is likely to be particularly important when the victim drug has a low non-CYP1A2 mediated clearance and a small therapeutic range, like, for example, clozapine. It is also possible that rofecoxib can affect the CYP1A2 mediated metabolism of dietary and endogenous compounds, such as caffeine and estrogens,

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which might explain some of the adverse effects of rofecoxib. Rofecoxib has inhibited the metabolism of caffeine in healthy volunteers (Backman et al., 2006b), and according to recent findings, intake of caffeine may be associated with an increased risk of myocardial infarction among individuals with “slow caffeine metabolism” (Cornelis et al., 2006).

In our recent *in vivo* study, the AUC of rofecoxib correlated significantly with the caffeine/paraxanthine ratio (Backman et al., 2006b), suggesting that CYP1A2 has a significant role in the metabolism of rofecoxib. However, rofecoxib is mainly reduced to different 3,4-dihydrohydroxy acid (DHHA) derivatives *in vivo* (Figure 1), and CYP enzymes have been suggested to play only a minor role in its metabolism (Merck & Co., 2002; Slaughter et al., 2003). *In vitro*, CYP1A2 and CYP3A4 have catalyzed the 5-hydroxylation of rofecoxib, but the contributions of non-CYP mediated pathways to the total metabolism of rofecoxib have been greater (Slaughter et al., 2003).

The concentrations of rofecoxib used in the above mentioned *in vitro* study (Slaughter et al., 2003) were 10 μM and 60 μM , and the incubation time was 15 minutes. These concentrations are about 100 times higher than the unbound concentrations of rofecoxib in plasma with typical clinical dosing. Such high concentrations can rapidly inactivate CYP1A2 (K_i 4.8 μM , k_{inact} 7% per minute). Therefore, the previous *in vitro* studies may have underestimated the contribution of CYP1A2 to the metabolism of rofecoxib. Another factor potentially leading to underestimation of the role of CYP enzymes is the NADPH-dependent back-reduction of 5-hydroxyrofecoxib to rofecoxib *in vitro* (Slaughter et al., 2003). Accordingly, it is possible that CYP1A2 contributes to the metabolism of rofecoxib *in vivo*. Thus, autoinhibition of the CYP1A2 mediated metabolism of rofecoxib could also explain

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its nonlinear pharmacokinetics in vivo (Merck & Co., 2002; Bachmann et al., 2003). Moreover, autoinhibition can be a pitfall leading to erroneous conclusions when the metabolism of a drug is studied in vitro using concentrations much higher than its free plasma concentrations in humans in vivo.

In conclusion, rofecoxib is a potent, metabolism-dependent inhibitor of CYP1A2. It also seems that the role of CYP1A2 in the metabolism of rofecoxib itself is greater than what has been recognized previously. The results provide a mechanistic explanation for the interactions of rofecoxib with CYP1A2 substrates and possibly for its nonlinear pharmacokinetics.

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FIGURE LEGENDS

Figure 1. Metabolic pathways of rofecoxib in vitro (Slaughter et al., 2003).

Figure 2. Effect of rofecoxib on phenacetin O-deethylation (percent of control activity) with (*solid circles*) or without (*open circles*) 30-min-preincubation. Incubations were conducted in human liver microsomes (0.1 mg/ml of protein) with 20 μ M phenacetin.

Figure 3. Preincubation-time- and concentration-dependent inhibition of phenacetin O-deethylation by rofecoxib (0-48 μ M) in human liver microsomes (1 mg ml⁻¹). Aliquots were removed from the preincubation mixtures at indicated time points and diluted 10-fold for measurement of residual CYP1A2 activity. The rate of inactivation of CYP1A2 activity by each inhibitor concentration (K_{obs}) was determined by linear regression analysis of the natural logarithm of percentage of activity remaining versus preincubation time data (A). The K_I and k_{inact} were calculated from the double-reciprocal plot of the K_{obs} versus inhibitor concentration [I] (B), and by nonlinear regression analysis of the K_{obs} versus rofecoxib concentration data according to the equation 1, as described in Methods (C).

Figure 4. Prediction of the effect of rofecoxib on the pharmacokinetics of CYP1A2 substrates in vivo ($AUC_{po(i)} / AUC_{po(c)}$). The figure illustrates how the effect of rofecoxib on the AUC of the substrate depends both on the concentration of rofecoxib at the enzyme site and on the fraction of the substrate metabolized by CYP1A2 ($f_m \cdot f_{m,CYP1A2}$ varying from 0.4 to 1.0). The simulation was carried out using equation 2, as described in Methods, for rofecoxib concentrations ranging from 0 μ M to 1 μ M (the peak total concentration in plasma during treatment with 25 mg rofecoxib daily).

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Table 1. The effect of trapping agents, the competitive inhibitor fluvoxamine, and dialysis on the inactivation of CYP1A2 by 12 μ M rofecoxib. Each experiment is compared to its own control i.e. the trapping agent, fluvoxamine or dialysis without rofecoxib.

Variable	Percent of control
Control	100 \pm 1.4
Rofecoxib	37.4 \pm 0.4
Rofecoxib with GSH (2 mM)	34.3 \pm 1.3
Rofecoxib with SOD (1000 U/ml)	31.5 \pm 4.5
Rofecoxib with mannitol (1 mM)	36.1 \pm 1.7
Rofecoxib with fluvoxamine	
Fluvoxamine (0.01 μ M)	36.1 \pm 8.3
Fluvoxamine (0.1 μ M)	82.8 \pm 13.9
Fluvoxamine (1.0 μ M)	94.0 \pm 0.5
Rofecoxib with dialysis (3 x 2 h at 4 °C)	36.9 \pm 0.8

Values are presented as mean \pm S.E.M. GSH, glutathione; SOD, superoxide dismutase.

Figure 1.

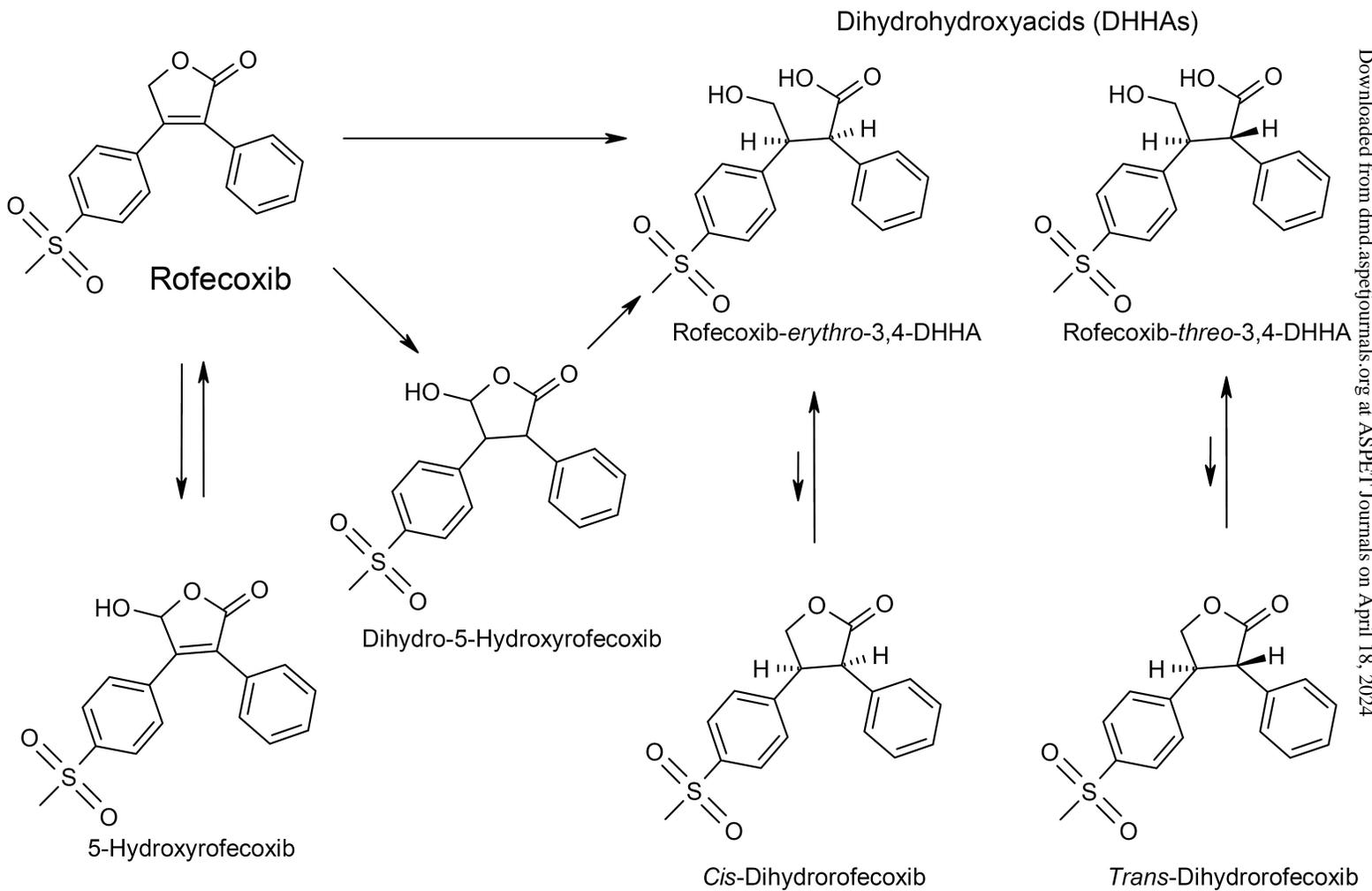


Figure 2.

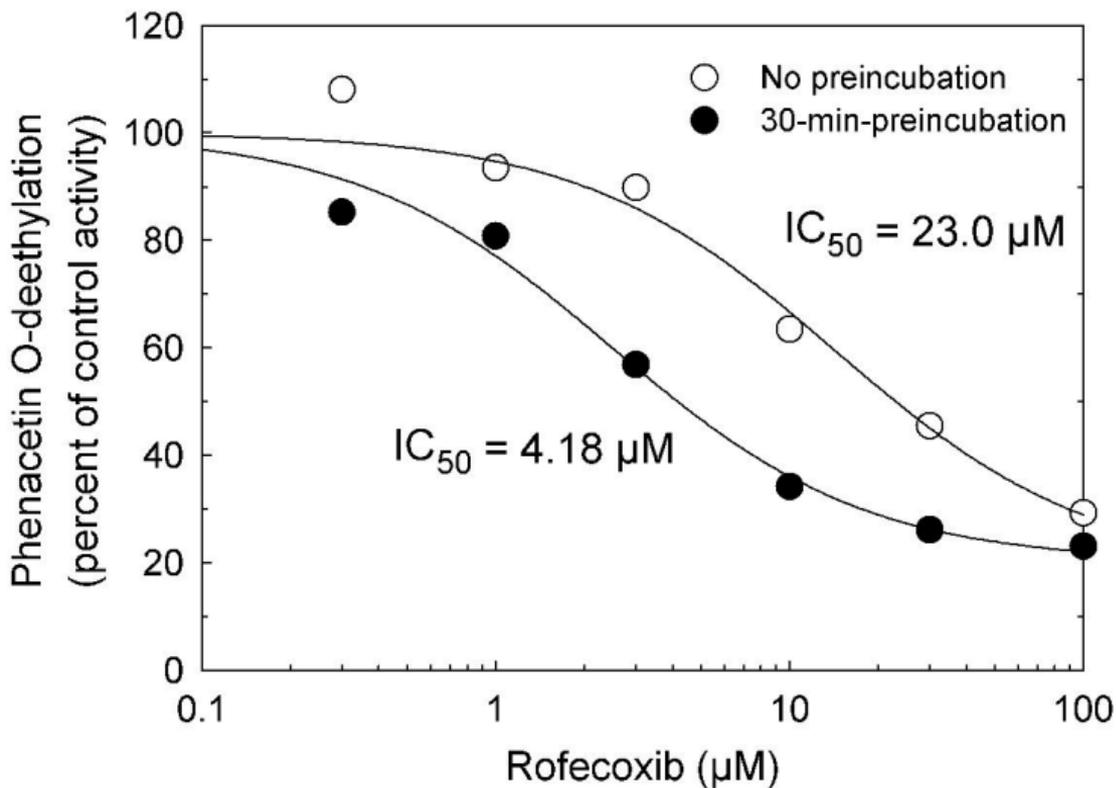


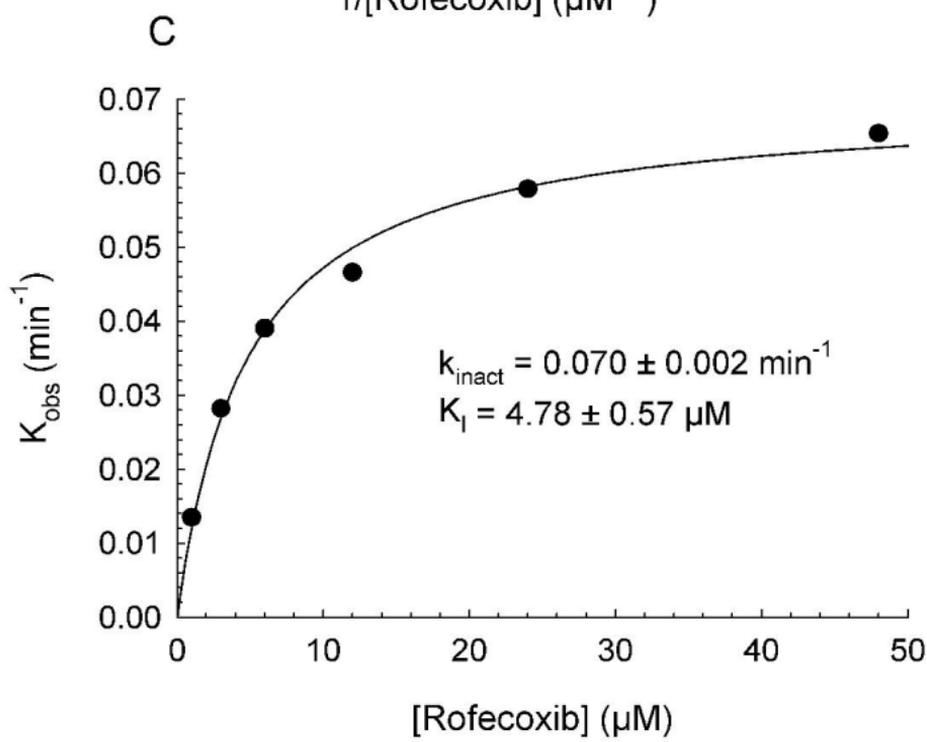
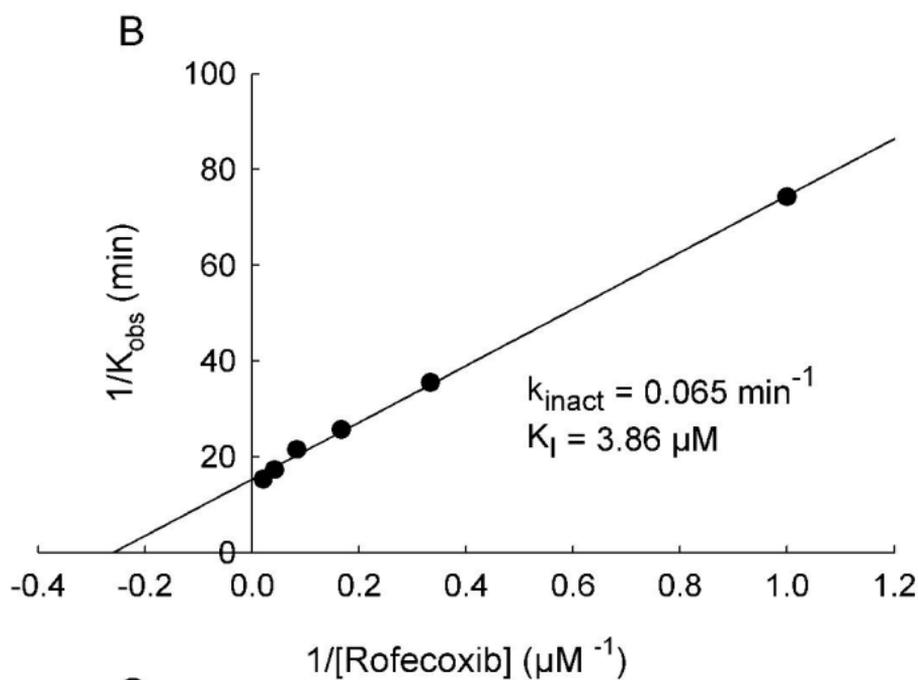
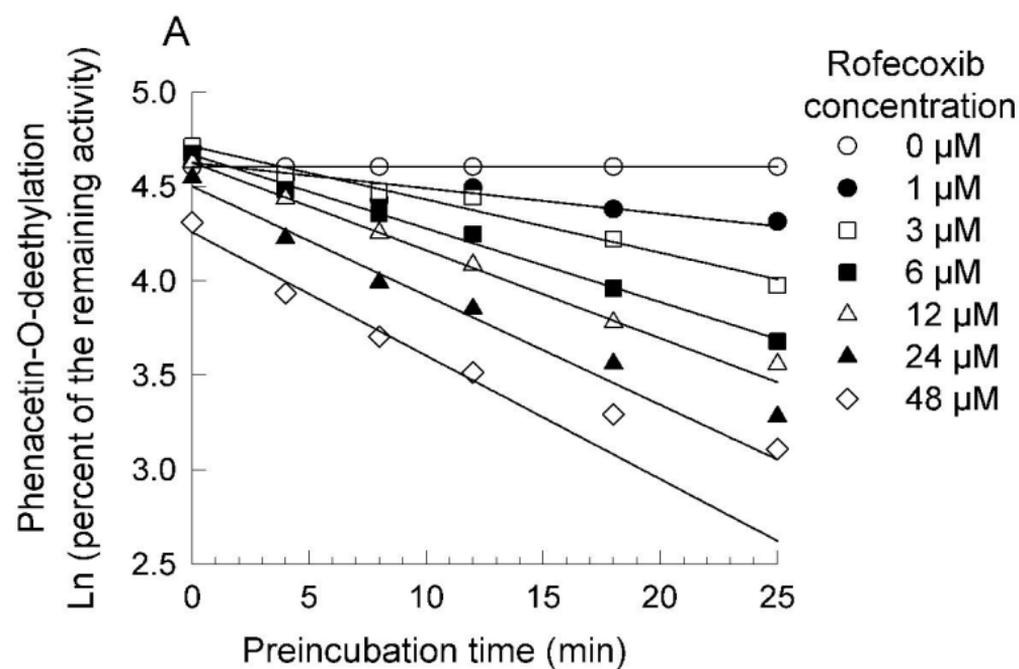
Figure 3.

Figure 4.

