Reevaluation of the Microsomal Metabolism of Montelukast – Major Contribution by CYP2C8 at Clinically Relevant Concentrations

Anne M. Filppula, Jouko Laitila, Pertti J. Neuvonen, and Janne T. Backman

Department of Clinical Pharmacology, University of Helsinki (A.M.F., J.L., P.J.N., J.T.B.) and Helsinki University Central Hospital (P.J.N., J.T.B.), Helsinki, Finland
Montelukast is metabolized by CYP2C8 \textit{in vitro}

Janne T. Backman, MD
Department of Clinical Pharmacology, University of Helsinki
and Helsinki University Central Hospital
P.O. Box 705, FI-00029 HUS, Finland
Tel: +358 9 471 73914
Fax: +358 9 471 74039
E-mail: janne.backman@helsinki.fi

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\textbf{Nonstandard Abbreviations:} AUC, area under the concentration-time curve; CL\textsubscript{B}, \textit{in vivo} blood clearance, CL\textsubscript{h}, hepatic clearance; CL\textsubscript{int}, intrinsic clearance; DDC, diethyldithiocarbamate; HLM, human liver microsomes; MBI, mechanism-based inhibition; m/z, mass-to-charge ratio; P450, cytochrome P450; RAF, relative activity factor.
Abstract

According to published in vitro studies, cytochrome P450 3A4 catalyzes montelukast 21-hydroxylation (M5 formation), whereas CYP2C9 catalyzes 36-hydroxylation (M6), the primary step in the main metabolic pathway of montelukast. However, montelukast is a selective competitive CYP2C8 inhibitor, and our recent in vivo studies suggest that CYP2C8 is involved in its metabolism. We therefore reevaluated the contributions of different P450 enzymes, particularly that of CYP2C8, to the hepatic microsomal metabolism of montelukast using clinically relevant substrate concentrations in vitro. The effects of P450 isoform inhibitors on montelukast metabolism were examined using pooled human liver microsomes, and montelukast oxidations by human recombinant CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5 were investigated. The results verified the central role of CYP3A4 in M5 formation. The CYP2C8 inhibitors gemfibrozil 1-O-β glucuronide and trimethoprim inhibited the depletion of 0.02 µM montelukast and formation of M6 from 0.05 µM montelukast more potently than did the CYP2C9 inhibitor sulfaphenazole. Correspondingly, recombinant CYP2C8 catalyzed montelukast depletion and M6 formation at a 6 times higher intrinsic clearance than did CYP2C9, while other P450 isoforms produced no M6. Based on depletion of 0.02 µM montelukast, CYP2C8 was estimated to account for 72% of the oxidative metabolism of montelukast in vivo, with a 16% contribution for CYP3A4 and 12% for CYP2C9. Moreover, CYP2C8 catalyzed the further metabolism of M6 more actively than did any other P450. In conclusion, CYP2C8 plays a major role in the main metabolic pathway of montelukast at clinically relevant montelukast concentrations in vitro.
Introduction

Montelukast is a potent and selective leukotriene D4 receptor antagonist, which is widely used for the treatment of asthma. The compound is absorbed rapidly after oral administration, has a bioavailability of 60-70%, and is highly bound to plasma proteins (>99%) (Cheng et al., 1996; Singulair Label, http://www.accessdata.fda.gov/drugsatfda_docs/label/2010/020829s055,020830s056,021409s031lbl.pdf). Montelukast undergoes extensive metabolism and the majority of the metabolites are excreted in bile, with less than 0.2% being eliminated in urine (Balani et al., 1997). Primary metabolites include a phenol (M3), diastereomeric 21-hydroxylated metabolites (M5a and M5b), and diastereomeric methylhydroxylated metabolites (M6a and M6b) (Fig. 1). A dicarboxylic acid metabolite (M4a and M4b) generated by further oxidation of M6, is the quantitatively most important metabolite in bile, followed by M3, M6, and M5. In addition, trace amounts of an acyl glucuronide metabolite (M1) and diastereomeric sulfoxides (M2a and M2b) are found in bile (Balani et al., 1997; Singulair Clinical Pharmacology and Biopharmaceutics Review(s), http://www.accessdata.fda.gov/drugsatfda_docs/nda/98/020829s000_Singular_Clin_Pharma rm_Biopharm.pdf).

Early in vitro studies have shown that cytochrome P450 enzymes are exclusively responsible for all montelukast oxidations. According to these studies, CYP3A4 mediated M2 formation with a minor contribution from CYP2A6. CYP3A4 also catalyzed M5 formation, whereas CYP2C9 played an important role in M6 formation (Chiba et al., 1997). The enzymes mediating the formation of M3 and the secondary metabolite M4 were not investigated, and while most other hepatic drug-metabolizing P450 isoforms were studied, the roles of CYP2C8 and CYP3A5 were not evaluated. Furthermore, these studies were carried out at montelukast concentrations of 100-500 µM, i.e., at least 10,000 times higher than the therapeutic unbound montelukast concentrations in plasma.
Although montelukast has been identified as a highly potent and selective competitive inhibitor of CYP2C8 \textit{in vitro} (Walsky et al., 2005a; Walsky et al., 2005b), it does not inhibit CYP2C8 \textit{in vivo} (Jaakkola et al., 2006; Kajosaari et al., 2006; Kim et al., 2007). The lack of \textit{in vivo} inhibitory effect suggests that the concentrations of montelukast in hepatocytes are much lower than its Ki for CYP2C8 (<0.15 µM), consistent with the small unbound fraction of montelukast in plasma. In a recent study, ligand-protein binding interaction between montelukast and CYP2C8 was demonstrated by X-ray crystallography (Schoch et al., 2008). The binding of montelukast positioned its benzyl ring near the heme iron. These studies and the fact that the metabolites M3, M4, and M6 result from the oxidation of the benzyl ring of montelukast, suggested that montelukast can be oxidized by CYP2C8 and prompted us to carry out a series of \textit{in vitro} and \textit{in vivo} investigations on this issue.

In our recently published \textit{in vivo} study, the strong CYP2C8 inhibitor gemfibrozil raised the plasma exposure to montelukast in humans almost 5-fold, consistent with an important role for CYP2C8 in the metabolism of montelukast (Karonen et al., 2010). The present \textit{in vitro} study was conducted to reevaluate the contributions of different P450 enzymes to the hepatic microsomal metabolism of montelukast at clinically relevant concentrations. In particular, we wanted to focus on characterizing the possible role and relative importance of CYP2C8 in the main metabolic pathway of montelukast (M6 formation and its subsequent metabolism to M4).
Methods

Chemicals and Microsomes. Montelukast (sodium) was obtained from Toronto Research Chemicals Inc. (TRC; North York, ON) and Sequoia Research Products Ltd. (Oxford, UK). Montelukast-d6, montelukast sulfoxide (M2, mixture of diastereomers), montelukast 1,2-diol (M6, mixture of diastereomers), and gemfibrozil 1-\(\beta\)-glucuronide were also from TRC. Diethyldithiocarbamate (DDC), furafylline, 8-methoxy-psoralen, omeprazole, quinidine, sulfaphenazole, trimethoprim, troleandomycin, and \(\beta\)-NADPH (reduced form) were purchased from Sigma-Aldrich (St. Louis, MO), ketoconazole from Janssen Biotech (Olen, Belgium), and clopidogrel from Sequoia. Pooled human liver microsomes (HLM), recombinant human P450 isoforms (Supersomes™) containing cytochrome b5 protein (except for CYP1A2 and CYP2D6), and control supersomes were from BD Biosciences (Woburn, MA). Other chemicals were from J. T. Baker (Deventer, Holland) and Merck (Darmstadt, Germany).

Incubation Conditions. Incubations were conducted in a shaking water bath at 37°C in 0.1 M sodium phosphate buffer (pH 7.4) in duplicates or triplicates (all control incubations were made in triplicates). HLM or recombinant P450 isoform, buffer, and 5 mM MgCl\(_2\) were premixed and kept on ice until the start of the experiment. In order to keep the possible nonspecific binding of montelukast equal in the experiments (Walsky et al., 2005b) and to enable precise comparisons of results with different P450 isoforms, an equal protein concentration (0.05 or 0.1 mg/ml) was used in parallel experiments (resulting in variable P450 contents in recombinant isoform studies). With the exception of inhibition studies, experiments were started by premixing montelukast for 3 min with HLM or recombinant P450 isoform buffer mixes, followed by addition of 1 mM \(\beta\)-NADPH to initiate reaction in a final incubation volume of 0.5 or 1 ml. After incubation, reactions were stopped by moving 50 or 100 \(\mu\)l of the incubation mixture to 150 or 300 \(\mu\)l acetonitrile containing 1% acetic acid and internal standard (50 ng/ml montelukast-d6).
Samples were immediately vortexed, put on ice for at least 10 min, and vortexed further
two times before centrifugation at 20 800 g for 10 min.

All stock solutions of montelukast, its metabolites, and inhibitors were prepared in
methanol or acetonitrile. Solutions containing montelukast and/or metabolites were
protected from light and incubations were conducted in amber Eppendorf tubes. All
incubations (including controls) contained the same concentration of organic solvent
(1%). When metabolite formation rate was measured, the incubation time was optimized
within the linear range for metabolite formation, depending on substrate turnover rate in
each specific experiment (<20% turnover of substrate was required). Determination of
the free fraction of montelukast in HLM incubations was attempted using both
equilibrium dialysis and ultrafiltration. However, accurate determination was not
achieved due to technical limitations, possibly because of binding of montelukast to the
ultrafiltration/dialysis apparatus (Walsky et al., 2005b).

**Inhibition Studies.** Competitive inhibitors tested were trimethoprim (100 μM),
sulfaphenazole (10 μM), omeprazole (10 μM), quinidine (10 μM), and ketoconazole (1
μM) as inhibitors of CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, respectively
(Baldwin et al., 1995; Newton et al., 1995; Bourrie et al., 1996; Ko et al., 1997; Eagling
et al., 1998; Wen et al., 2002). Mechanism-based inhibitors (MBIs) examined were
furafylline (20 μM), 8-methoxy-psoralen (0.5 μM), clopidogrel (1 μM), gemfibrozil 1-O-β
glucuronide (60 μM), DDC (100 μM), and troleandomycin (100 μM) as inhibitors of
CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2E1, and CYP3A4, respectively (Newton et
al., 1995; Bourrie et al., 1996; Draper et al., 1997; Koenigs et al., 1997; Eagling et al.,
1998; Richter et al., 2004; Ogilvie et al., 2006). In competitive inhibitor studies,
montelukast and inhibitor were (simultaneously) premixed with HLM (0.1 mg/ml protein
concentration) for 3 min before β-NADPH addition. In MBI studies, except for that with
gemfibrozil 1-O-β glucuronide, the MBI was first premixed with HLM (0.1 mg/ml) for 3
min before addition of β-NADPH. After a 15 min preincubation, including β-NADPH, montelukast was added to start the reaction.

Inhibition by gemfibrozil 1-O-β glucuronide (60 μM) was investigated by premixing it with 2 mg/ml HLM and MgCl₂ in buffer. After addition of β-NADPH, the solution was preincubated for 30 min. An aliquot of 25 μl was then moved to another tube containing β-NADPH and MgCl₂ in buffer, and montelukast was immediately added to start the reaction in a final incubation volume of 0.5 ml. Thus, the protein and inhibitor concentrations had been diluted 20-fold in order to avoid possible competitive inhibition of other P450 enzymes by the inhibitor.

In the first screening experiment, 0.4 µM montelukast was incubated with each inhibitor, or after preincubation, for 15 min and metabolite formation rate was measured. Based on results from this experiment, selected inhibitors (8-methoxy-psoralen, gemfibrozil 1-O-β glucuronide, trimethoprim, sulfaphenazole, ketoconazole, and troleandomycin) were further tested in a montelukast depletion study, where 0.02 μM montelukast was incubated for 90 min. In addition, the effects of gemfibrozil 1-O-β glucuronide, trimethoprim, and sulfaphenazole on M6 formation were tested with a concentration of 0.05 μM montelukast.

**Metabolite Inhibition Studies.** To evaluate the possible inhibition of montelukast metabolism by its sulfoxide metabolite (M2), which was found in a small quantity as an impurity in montelukast sodium, 0.01, 0.1, and 1 μM M2 and 0.1 μM montelukast were incubated with HLM (0.1 mg/ml) for 12 min or with recombinant CYP2C8 or CYP2C9 (0.05 mg/ml protein) for 3 or 6 min, respectively.

**Recombinant P450 Isoform Studies.** Recombinant CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5 were first tested in a screening study, where 1 μM montelukast was incubated with each P450 isoform for 60 min (0.1 mg/ml protein). In a further experiment with selected
isoforms, including CYP2C8, CYP2C9, CYP3A4, and CYP3A5, 0.02 and 0.1 µM montelukast were incubated for 3 (CYP2C8 and CYP3A4) or 12 min (CYP2C9 and CYP3A5), and metabolite formation was measured. In a depletion study, 0.02 µM montelukast was incubated with CYP2A6, CYP2C8, CYP2C9, CYP3A4, and CYP3A5. Additionally, 10 µM montelukast was incubated with CYP2A6 (0.3 mg/ml protein) for 60 min and metabolite formation was monitored, because CYP2A6 was previously reported to catalyze M2 formation (Chiba et al., 1997).

**Enzyme Kinetics of M6 Formation in Recombinant CYP2C8 and CYP2C9.** Various concentrations of montelukast (0.03-30 µM) were incubated with CYP2C8 and CYP2C9 to evaluate the enzyme kinetics of M6 formation. The incubation time was 3 min for CYP2C8 and 10 min for CYP2C9, and the protein concentration was 0.05 mg/ml.

**M6 Metabolism with Recombinant P450 Isoforms.** In a preliminary screening experiment, M4 formation from 1 µM M6 was investigated with different P450 isoforms. In a further experiment, M6 depletion and M4 formation were examined by incubating 0.02 µM M6 with CYP2C8, CYP2C9, and CYP3A4 (0.05 mg/ml protein) for 30 min.

**Measurement of Montelukast and Metabolites.** Montelukast and its oxidative metabolites were quantified by use of SCIEX API 2000 liquid chromatography-tandem mass spectrometry system (Sciex Division of MDS Inc, Toronto, ON). Chromatography was performed on a Symmetry C8 reversed phase column (2.1x150 mm; Waters Corporation, Milford, MA) using gradient elution. The mobile phase consisted of acetonitrile and 1% acetic acid in water. The mobile phase gradient comprised 1 min at 50% acetonitrile, 9 min to 90% acetonitrile, 4 min at 90% acetonitrile, 0.1 min to 50% acetonitrile, and finally 6.9 min at 50% acetonitrile. Aliquots of 10 or 15 µl were injected at a mobile phase flow rate of 200 µl/min. The mass spectrometer was operated in positive multiple reaction monitoring (MRM) mode and ion transitions monitored were $m/z$ (mass-to-charge ratio) 586.2 to $m/z$ 422.3 for montelukast, $m/z$ 602 to $m/z$ 422 for
M2, m/z 616 to m/z 452 for M4, m/z 602 to m/z 147 for M5a/b and M3, m/z 602 to m/z 438 for M6 and M3, and m/z 592.2 to m/z 427 for the internal standard. These transitions represent the product ions of the [M+H]^+ ions.

Retention times and mass spectra of M2a/b and M6a/b were identical to those of the corresponding authentic standards. The M6 standards yielded only one peak, which was presumed to represent both diastereomers of the metabolite. The peaks of M3, M4, and M5 were identified by their ion transitions (Balani et al., 1997). For M5, two peaks with distinct retention times were found, corresponding to its previously described diastereomeric forms (Balani et al., 1997; Chiba et al., 1997), and indicated by the lower case letter a or b according to the retention time (Fig. 2). In agreement with previous studies (Chiba et al., 1997), preliminary experiments indicated that M2 formation is catalyzed by CYP3A4 (and CYP3A5). However, the quantitative importance of M2 is negligible in humans (Balani et al., 1997; Karonen et al., 2010; Singulair Clinical Pharmacology and Biopharmaceutics Review(s), http://www.accessdata.fda.gov/drugsatfda_docs/nda/98/020829s000_Singular_Clin_Pharm_Biopharm.pdf). In addition, due to the montelukast sulfoxide impurity (~1%) present in montelukast sodium, an accurate quantification of metabolite formation was not possible. Consequently, M2 concentrations were not measured in the present study.

The lower limit of quantification was 2 nM for montelukast and 2.5 nM for M6. The between-day coefficient of variation for montelukast was 13.8% at 2 nM, 9.6% at 20 nM and 11.3% at 200 nM, and for M6 19.2% at 2.5 nM, 6.8% at 20 nM and 9.4% at 200 nM. A signal to noise ratio of 10:1 was used as the limit of quantification for the other metabolites. Their quantities are given in arbitrary units relative to the ratio of the peak height of the metabolite to the peak height of the internal standard. The detection response was shown to be linear for montelukast and all its metabolites in the relevant concentration range, using a 25-fold sample dilution series (r^2 > 0.98).
Data Analysis. The mean values of duplicates or triplicates were used in calculations. Error bars have been omitted for clarity in figures. The kinetics of M6 formation by CYP2C8 and CYP2C9 were analyzed using SigmaPlot® software, version 9.01 (Systat Software, Inc, San Jose, CA). Selection of the best-fit enzyme model was based on the Akaike Information Criterion, \( R^2 \), and on the examination of Michaelis-Menten plots. The results were best described by an uncompetitive substrate inhibition model: \( v = \frac{V_{\text{max}} \times S}{(K_m + S + S^2 / K_i)} \), where \( v \) is velocity (pmol/min/pmol), \( V_{\text{max}} \) is the maximal velocity (pmol/min/pmol), \( S \) is substrate concentration (µM), \( K_m \) is the Michaelis-Menten constant (µM), and \( K_i \) is the inhibitory constant (µM). Intrinsic clearance (\( CL_{\text{int, in vitro}} \)) values were calculated according to \( CL_{\text{int, in vitro}} = \frac{V_{\text{max}}}{K_m} \).

Pseudo-first order depletion rate constants (\( k \)) were determined for the depletion of 0.02 µM montelukast as a function of incubation time in HLM and recombinant enzyme incubations using nonlinear regression analysis (SigmaPlot®). Percentage inhibition of montelukast depletion in HLM was calculated by comparing \( k \) values of inhibitor incubations with those of control incubations. Assuming that the concentration of montelukast is much smaller than the \( K_m \) for its metabolism, its intrinsic clearance can also be expressed as \( CL_{\text{int, in vitro}} = \frac{k}{[M]} \), where \([M]\) is the microsomal protein concentration, or P450 concentration in recombinant enzyme incubations (Venkatakrishnan et al., 2003; Emoto and Iwasaki, 2007). To estimate the relative contributions of different P450 enzymes to the metabolism of montelukast, relative activity factor (RAF) values were calculated for CYP2C8, CYP2C9, and CYP3A4, using the equation \( \text{RAF}_i = \frac{\text{rate for a probe substrate in HLM}_i}{\text{rate for a probe substrate with a recombinant isoform}_i} \) where \( i \) denotes a specific P450 isoform (Crespi and Miller, 1999), using activity data provided by BD Biosciences for the recombinant enzyme and HLM batches used. The recombinant P450 \( CL_{\text{int, in vitro}} \) values were then multiplied with the respective RAF. For comparison, the relative contributions of CYP2C8, CYP2C9,
CYP3A4, and CYP3A5 to montelukast metabolism were also estimated using the relative abundance approach. Relative abundance was calculated as (total HLM P450 concentration) / 100 × Ai (Stringer et al., 2009), where the total P450 concentration in the HLM batch used was 410 pmol P450/mg microsomal protein and Ai was the mean percent content of the respective P450 enzyme in human liver microsomal fractions (Rostami-Hodjegan and Tucker, 2007). The recombinant P450 CL\textsubscript{int, in vitro} values were then multiplied with the respective relative abundance. The RAF and relative abundance adjusted CL\textsubscript{int, in vitro} values and the CL\textsubscript{int, in vitro} obtained from the HLM incubations were thereafter converted to CL\textsubscript{int, in vivo} values using the standard in vitro-in vivo scaling parameters of 40 mg microsomal protein/g liver (Houston and Galetin, 2008) and 25.7 g liver/kg body weight (Davies and Morris, 1993). Hepatic blood clearance (CL\textsubscript{h}) values were calculated using the well stirred model:

\[
CL_h = Q_h \cdot f_u \cdot \frac{CL_{int,in vivo}}{Q_h + f_u \cdot CL_{int,in vivo}}
\]

where Q\textsubscript{h} is the hepatic blood flow (20.7 ml/min/kg) and f\textsubscript{u} is the unbound fraction of montelukast in blood (Houston and Galetin, 2008). The total in vivo blood clearance (CL\textsubscript{h}) of montelukast was calculated with the equation CL\textsubscript{h} = CL\textsubscript{P} × C\textsubscript{P} / C\textsubscript{B}, where C\textsubscript{B} and C\textsubscript{P} are the concentrations in blood and plasma, respectively, and CL\textsubscript{P} is the average in vivo plasma clearance, 0.578 ml/min/kg, as reported after intravenous administration of 3, 9, and 18 mg montelukast (Singulair Pharmacology Review(s), http://www.accessdata.fda.gov/drugsatfda_docs/nda/2002/21-409_Singulair_Phamr.pdf). The mean blood/plasma concentration ratio of montelukast is about 0.65 in humans (Singulair Clinical Pharmacology and Biopharmaceutics Review(s), http://www.accessdata.fda.gov/drugsatfda_docs/nda/98/020829s000_Singular_Clin_Pharma_Biopharm.pdf). Thus, montelukast is minimally bound to blood cells and the value of
unbound fraction in blood can be assumed to approximate that in plasma, 0.004

(Singulair Pharmacology Review(s),

Results

Incubation of 1-10 µM montelukast with HLM resulted in NADPH- and time-dependent montelukast depletion and metabolite formation (data not shown). The reactions were linear with protein concentration up to at least 0.1 mg/ml. Addition of albumin to incubations reduced the rate of montelukast metabolism in a concentration-dependent manner (data not shown). Following incubation of 0.02-0.4 µM montelukast with HLM, four primary metabolites (M2, M3, M5a/b, and M6) were found. The secondary metabolite M4 was also detectable with increasing montelukast concentrations and incubation times.

Inhibition Studies. The effects of eleven P450 inhibitors on metabolite formation rate at 0.4 µM montelukast are shown in Fig. 3. The CYP3A4 inhibitors ketoconazole and troleandomycin inhibited M5a and M5b formation almost completely, by >90%. The formation rate of M6 was reduced by about 20-50% by several inhibitors, including the CYP2C8 inhibitors gemfibrozil 1-O-β glucuronide and trimethoprim, the CYP2C9 inhibitor sulfaphenazole, and the CYP2E1 inhibitor DDC. M3 formation was most potently inhibited by gemfibrozil 1-O-β glucuronide, the CYP2A6 inhibitor 8-methoxy-psoralen and DDC. Furafylline, clopidogrel and quinidine did not significantly affect the formation of any of these metabolites, indicating that CYP1A2, CYP2B6 and CYP2D6, respectively, did not play a significant role in montelukast metabolism. At 0.05 µM montelukast, M6 formation was most potently inhibited by gemfibrozil 1-O-β glucuronide (Fig. 3).

Of the six inhibitors selected for depletion studies, 8-methoxy-psoralen, gemfibrozil 1-O-β glucuronide, trimethoprim, ketoconazole, and troleandomycin, inhibited the depletion rate of 0.02 µM montelukast by approximately 40-60%, while sulfaphenazole did not affect the depletion of montelukast at all (data not illustrated).
Product Inhibition by Montelukast Sulfoxide. Simultaneous incubation of montelukast sulfoxide (M2) and 0.1 µM montelukast with HLM did not affect the formation of M5a/b. However, M2 inhibited M6 (and M3; data not shown) formation in a concentration-dependent manner in both HLM and recombinant CYP2C8 (Fig. 4). In CYP2C9, M2 had no effect on montelukast metabolism.

Montelukast Metabolism by Recombinant P450 Isoforms. Among the ten P450 isoforms tested, CYP3A4 caused the greatest depletion of 1 µM montelukast (Fig. 5), a supratherapeutic montelukast concentration compared to its typical free plasma concentrations. Both CYP3A4 and CYP3A5 catalyzed M5a/b formation, whereas M6 was most effectively formed by CYP2C8 and CYP2C9 (Fig. 5 and 6). M3 was formed by several P450 isoforms, including CYP2C8, CYP3A4, CYP2C9, CYP3A5, and CYP2C19 at 1 µM montelukast.

Further investigation on CYP2C8, CYP2C9, CYP3A4, and CYP3A5 indicated that the relative importance of CYP2C8 in M6 (and M3) formation increased as montelukast concentration was reduced from 1 to 0.1 (Fig. 6) or 0.02 µM (data not shown); at 0.1 and 0.02 µM montelukast, M6 formation rate was about 3 times higher in CYP2C8 than in CYP2C9. CYP2C8 also metabolized 0.02 µM montelukast at a higher depletion rate than did CYP3A4 or CYP2C9, while CYP3A5 and CYP2A6 were not able to cause any significant montelukast depletion (Fig. 7). Furthermore, incubation of 10 µM montelukast with CYP2A6 (0.3 mg/ml protein) for 60 min resulted in no significant montelukast metabolite formation (data not shown). Control supersomes did not metabolize montelukast to any appreciable extent.

M6 Metabolism by Recombinant P450 Isoforms. Incubation of 0.02 µM M6 with CYP2C8 caused a rapid depletion of M6 (approximately 90% in 30 min) and formation of M4, while CYP2C9 and CYP3A4 did not form a detectable amount of M4 (Fig. 7). Recombinant CYP1A2, CYP2A6, CYP2B6, CYP2C19, CYP2D6, CYP2E1, and
CYP3A5 were not able to catalyze formation of M4 from 1 µM M6 during a 30 min incubation (data not shown).

**Kinetics of M6 Formation in CYP2C8 and CYP2C9.** Seven-point M6 formation curves (0.03-30 µM montelukast) in recombinant CYP2C8 and CYP2C9 were best described by a substrate inhibition kinetic model (Fig. 8). The estimated $K_m$ was particularly low for CYP2C8, and therefore, the $CL_{int, in\text{\;vitro}}$ ($V_{max}/K_m$) of M6 formation was 6 times higher for CYP2C8 than for CYP2C9.

**Scaling Montelukast Metabolism to In Vivo.** The $in\text{\;vivo}$ $CL_B$ of montelukast, calculated on the basis of its published total plasma clearance and blood to plasma partition ratio was estimated to average 0.89 ml/min/kg. The $CL_{int, in\text{\;vitro}}$ based on depletion of 0.02 µM montelukast in HLM was 0.14 ml/min/mg, yielding a predicted montelukast $in\text{\;vivo}$ $CL_h$ of 0.54 ml/min/kg, which is approximately 60% of the estimated $CL_B$ in humans. The unadjusted $CL_{int, in\text{\;vitro}}$, and the RAF and relative abundance adjusted $CL_{int, in\text{\;vitro}}$, as well as the corresponding predicted $CL_h$ from depletion of 0.02 µM montelukast by recombinant enzymes are presented in Table 1. Using the RAF approach, the contribution of CYP2C8 to the total metabolism of montelukast $in\text{\;vivo}$ was estimated to average 72%, followed by CYP3A4 (16%) and CYP2C9 (12%) (Table 1). The relative abundance approach resulted in a higher predicted significance for CYP3A4 (60%) and lower significance for CYP2C8 (27%), with a 11% contribution for CYP2C9 and 2.3% for CYP3A5. Both approaches slightly overestimated the total $in\text{\;vivo}$ $CL_B$: the sum of $CL_h$ of CYP2C8, CYP2C9, CYP3A4, and CYP3A5 was 1.35-1.43 ml/min/kg.
Discussion

The aim of the present *in vitro* study was to comprehensively reevaluate the contributions of different P450 enzymes to the metabolism of montelukast at clinically relevant substrate concentrations, with a special emphasis on the main metabolic pathway of montelukast (M6 formation). Inhibition studies with HLM and incubations performed with recombinant P450 isoforms verified that M5a/b is mainly formed by CYP3A4, and suggested a minor involvement of CYP3A5 in its formation. However, with regard to M6 formation, the results indicated a major role for CYP2C8, leaving only a small contribution for CYP2C9. This conclusion is based principally on the following findings. The CYP2C8 inhibitors gemfibrozil 1-O-β-glucuronide (Ogilvie et al., 2006) and trimethoprim (Wen et al., 2002) inhibited M6 formation from 0.05 µM montelukast much more potently than did the CYP2C9 inhibitor sulfaphenazole. Recombinant CYP2C8 mediated M6 formation at an about 6 times higher CL<sub>int</sub>, *in vitro* than did CYP2C9. Moreover, CYP2C8 catalyzed the depletion of montelukast (0.02 µM) and mediated the further metabolism of M6 (0.02 µM) more actively than did any of the other P450 forms. Thus, our results substantiate a major contribution for CYP2C8 in the biotransformation of therapeutic montelukast concentrations and importantly complement earlier montelukast information stating that only CYP2A6, CYP2C9, and CYP3A4 participate in montelukast metabolism (Singulair Label, http://www.accessdata.fda.gov/drugsatfda_docs/label/2010/020829s055,020830s056,021409s031lbl.pdf).

In the earlier *in vitro* study (Chiba et al., 1997), the microsomal enzymes mediating M3 and M4 formation were not investigated. Our present findings suggest that M3 is mainly formed by CYP2C8, with a smaller contribution by CYP3A4, CYP2C9, CYP3A5, and CYP2C19, while M4 formation is mediated almost exclusively by CYP2C8. Previously reported CYP2A6-mediated M2 formation could not be corroborated in our
study, despite incubating a 10 µM montelukast concentration with recombinant CYP2A6. Moreover, CYP2A6 did not cause any significant depletion of 0.02 µM montelukast, indicating that any possible contribution of CYP2A6 to the metabolism of montelukast is unlikely to be clinically relevant. A possible explanation for the difference to the previous study is that a very high 500 µM montelukast concentration and 60 min incubation time were used in the previous study, allowing even a low catalytic activity to be detected. It should be noted, however, that such high montelukast concentrations, exceeding over 10 000 times its therapeutic free concentrations in plasma, could not have given clinically relevant estimates of the contributions of different P450 enzymes, because of the saturation of the CYP2C8-mediated metabolism of montelukast. Moreover, the observed modest inhibitory effects of 8-methoxy-psoralen on montelukast metabolism may have been due to inhibition of other enzymes than CYP2A6 (Dierks et al., 2001).

The CL$_{int}$, in vitro values calculated from the formation kinetics of M6 in CYP2C8 and CYP2C9 were almost identical to those based on substrate depletion data, indicating that M6 formation accounts for the majority of montelukast metabolism by these enzymes. The CL$_h$ values calculated using the RAF method and well-stirred model suggested a relative contribution of 72% for CYP2C8 in the total oxidative metabolism of montelukast, while the relative abundance approach predicted a contribution of 27% for CYP2C8. Both approaches resulted in a minor, 11-12% contribution for CYP2C9, whereas the contribution for CYP3A4 differed markedly (16 vs. 60%). The RAF approach accounts for variations in both quantities and activities between HLM and recombinant P450 enzymes, and it has been shown to provide more reliable predictions than does the relative abundance method (Stringer et al., 2009). Also our in vivo results are more consistent with the RAF approach (Karonen et al., 2010). In healthy volunteers, gemfibrozil, a strong in vivo CYP2C8 inhibitor (Backman et al., 2002; Niemi et al., 2003a; Niemi et al., 2003b; Tornio et al., 2008; Backman et al., 2009), increased
the mean area under the plasma concentration-time curve (AUC) of montelukast 4.5-fold, equivalent to an almost 80% reduction in oral clearance, and reduced the AUC of M6 and M4 by 40% and >90%, respectively (Karonen et al., 2010). These findings are almost exactly in accordance with the RAF based >70% contribution for CYP2C8, as well as with our in vitro data predicting a crucial role for CYP2C8 in M6 formation and its further metabolism to M4.

The extended, three-branched active site cavity of CYP2C8 allows it to oxidize substrates of high molecular weight and T or Y shape (Schoch et al., 2004). A recent X-ray crystallography study has demonstrated binding of montelukast into the active site cavity of CYP2C8, positioning the benzyl ring of montelukast near the heme iron (Schoch et al., 2008). The metabolites M3, M4, and M6 result from the oxidation of the benzyl ring or its side chain. Accordingly, our data indicating that these metabolites are mainly formed by CYP2C8, is in agreement with the X-ray crystallographic findings.

There were several methodological challenges in the present study, e.g., due to the protein binding of montelukast in plasma as well as in microsomes. In vivo, only ~0.4% of montelukast is unbound in plasma (Singulair Pharmacology Review(s), http://www.accessdata.fda.gov/drugsatfda_docs/nda/2002/21-409_Singulair_Pharmr.pdf), and use of low montelukast concentrations was therefore critical for the clinical relevance of the present in vitro study. After a 10 mg oral dose of montelukast, its peak concentration approximates 500 ng/ml in humans (Zhao et al., 1997; Karonen et al., 2010), corresponding roughly to a free plasma concentration of 0.003 µM. Using a sensitive LC/MS/MS method for montelukast and metabolite quantification, we were able to incubate substrate concentrations as low as 0.02 µM, which is reasonably close to the unbound plasma concentrations. Our enzyme kinetic results, indicating a very low $K_m$ for CYP2C8-mediated M6 formation (0.050 µM; compared to 0.19 µM for CYP2C9), and the increased inhibition of M6 formation by
gemfibrozil 1-O-β glucuronide when montelukast concentration was decreased from 0.4 to 0.05 µM confirmed the necessity to use low montelukast concentrations for clinically relevant predictions.

Apart from plasma protein binding, montelukast is subject to nonspecific binding as microsomal protein concentrations are increased (Walsky et al., 2005b). Unfortunately, like in a previous study (Walsky et al., 2005b), measurement of the nonspecific binding of montelukast could not be accomplished. However, in our experiments, protein concentration was minimized and standardized (0.05 or 0.1 mg/ml), in order to minimize binding and to allow reliable predictions and comparisons between experiments. In addition, incubation conditions were carefully optimized to obtain initial rate conditions with HLM and each P450 isoform studied, paying special attention to high substrate turn-over conditions (low substrate concentrations). The good accuracy of the predicted in vivo CLh of montelukast indicates that the optimization of conditions was successful. The CLh based on montelukast depletion in HLM was approximately 60% of the in vivo CLB, while the total CLh, calculated with the RAF approach based on depletion in CYP2C8, CYP2C9, and CYP3A4 was roughly 1.5 times higher than CLB.

The major methodological limitations of our study were the lack of reference compounds for montelukast metabolites M3, M4, and M5, and the sulfoxide (M2) impurity of montelukast sodium products. Therefore, M3, M4, and M5 concentrations were quantified as arbitrary units only, and it was not feasible to perform enzyme kinetic experiments, apart from that on M6 formation. Moreover, concomitant incubation of M2 and montelukast with HLM, CYP2C8, and CYP2C9 indicated potent inhibition of CYP2C8 by M2, with 50-70% inhibition of M6 formation already at 0.1 µM M2. Interestingly, M6 formation in CYP2C8 was best described by the substrate inhibition enzyme kinetic model, with a Ki of 11 µM for substrate inhibition. With a 1% M2 impurity, a montelukast concentration of 11 µM would contain a 0.11 µM concentration of M2,
indicating that the impurity can be the sole explanation for the observed substrate inhibition kinetics with CYP2C8. In contrast, since M2 did not markedly inhibit M6 formation in CYP2C9, this impurity does not provide an explanation for the substrate inhibition kinetics of M6 formation in CYP2C9. Nevertheless, it should be noted that the 1% M2 impurity in montelukast sodium is unlikely to compromise our main results and conclusions, because its inhibitory effect is negligible at the ≤1 µM montelukast concentrations used in the present study. Yet, the present findings provide a cautionary example highlighting that even <1% impurities in substrate drug products may seriously interfere with drug metabolism in vitro, especially with high substrate concentrations.

In conclusion, the present in vitro study indicates that CYP2C8 plays a crucial role in the metabolism of montelukast at clinically relevant concentrations, and is the major enzyme mediating the formation of the main in vivo metabolite M6 and its further metabolite M4. These conclusions are in good agreement with our recent clinical data showing that the strong CYP2C8 inhibitor gemfibrozil markedly increases the concentrations of montelukast in humans (Karonen et al., 2010). Furthermore, unlike the product information of montelukast states, the present findings suggest that the contributions of CYP3A4 and CYP2C9 to the total metabolism of montelukast are only small and the contribution of CYP2A6 negligible. Moreover, the present data highlight the importance of using clinically relevant substrate concentrations and carefully validated incubation conditions in in vitro screening of drug metabolism.
Authorship Contributions

Participated in research design: Filppula, Backman, Laitila, and Neuvonen

Conducted experiments: Filppula and Laitila

Performed data analysis: Filppula and Backman

Wrote or contributed to the writing of the manuscript: Filppula, Backman, Laitila, and Neuvonen

Other: Backman and Neuvonen acquired funding for the research
References


Footnotes

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International Union of Basic and Clinical Pharmacology.

Please address correspondence to: Janne T. Backman, Department of Clinical Pharmacology, University of Helsinki and Helsinki University Central Hospital, P.O. Box 705, FI-00029 HUS, Finland. E-mail: janne.backman@helsinki.fi.
Legends for figures

Fig. 1.
The oxidative metabolism of montelukast. The P450 isoforms responsible for the formation of the metabolites M2, M3, M4, M5, and M6 are shown according to Chiba and co-workers (Chiba et al., 1997) and Filppula et al. (present work).

Fig. 2.
Representative chromatograms of montelukast, its oxidative metabolites, and the internal standard. Representative chromatograms from a 30 min incubation of 0.5 µM montelukast with HLM in the presence of NADPH (A), and from a 10 min incubation of 0.02 µM M6 with CYP2C8 and NADPH (B) are shown. M3 was monitored at both m/z 602 to m/z 147 and m/z 602 to m/z 438, and quantification was based on the signal at m/z 602 to m/z 438. For other metabolites, the ion transitions monitored are described in Methods. When exposed to light, a small fraction of montelukast and metabolites were converted to their respective cis isomers (data not shown), as previously documented for montelukast (Ochiai et al., 1998). Therefore, incubations were conducted in amber tubes.

Fig. 3.
The effects of different P450 isoform inhibitors on M3, M5a/b, and M6 formation.
Montelukast (0.4 µM) and inhibitors were incubated with pooled HLM (0.1 mg/ml protein) for 15 min. The inhibitors tested were furafylline 20 µM (FURA), 8-methoxy-psoralen 0.5 µM (8-M-P), clopidogrel 1 µM (CLOP), gemfibrozil 1-O-β glucuronide 60 µM (GEM-G), trimethoprim 100 µM (TRIM), sulfaphenazole 10 µM (SULF), omeprazole 10 µM (OME), quinidine 10 µM (QUIN), diethyldithiocarbamate 100 µM (DDC), ketoconazole 1 µM (KETO), and troleandomycin 100 µM (TAO). A 15 or 30 min preincubation of the inhibitor
with HLM preceded the incubation in experiments with FURA, 8-M-P, CLOP, GEM-G, DDC, and TAO. M3 and M5a/b concentrations formed in 0.05 µM montelukast incubations were below quantification levels.

Fig. 4.

*The inhibitory effect of montelukast sulfoxide (M2) on M6 formation.* Montelukast (0.1 µM) and various concentrations of M2 were incubated with HLM (0.1 mg/ml protein), CYP2C8 (16 pmol/ml; 0.05 mg/ml protein), and CYP2C9 (20 pmol/ml; 0.05 mg/ml protein).

Fig. 5.

*Recombinant P450 isoform screening.* Depletion of 1 µM montelukast and formation of M3, M5a/b, and M6 during a 60 min incubation with CYP1A2 (25 pmol/ml), CYP2A6 (10 pmol/ml), CYP2B6 (35 pmol/ml), CYP2C8 (30 pmol/ml), CYP2C9 (24 pmol/ml), CYP2C19 (25 pmol/ml), CYP2D6 (15 pmol/ml), CYP2E1 (33 pmol/ml), CYP3A4 (20 pmol/ml), and CYP3A5 (20 pmol/ml) are shown. The protein concentration in all incubations was 0.1 mg/ml. Concentrations of M3 and M5a/b are given as arbitrary units, i.e., quantitative comparisons between metabolites are not possible.

Fig. 6.

*Metabolite formation rates during incubation of montelukast with main recombinant P450 isoforms.* Concentrations of 0.1 and 1 µM montelukast were incubated with CYP2C8 (30 pmol/ml), CYP2C9 (24 pmol/ml), CYP3A4 (20 pmol/ml), and CYP3A5 (20 pmol/ml). The protein concentration was 0.1 mg/ml in all incubations. M3 and M5a/b formation velocities are based on arbitrary units, i.e., quantitative comparisons between metabolites are not possible.
Fig. 7.

Metabolism of low montelukast and M6 concentrations by recombinant P450 isoforms.

Depletion of 0.02 µM montelukast by CYP2A6 (10 pmol/ml), CYP2C8 (30 pmol/ml), CYP2C9 (24 pmol/ml), CYP3A4 (20 pmol/ml), and CYP3A5 (20 pmol/ml) was tested during a 30 min incubation (A). Incubations contained 0.1 mg/ml protein.

Depletion of 0.02 µM M6 (B) and formation of M4 (C) by CYP2C8 (16 pmol/ml), CYP2C9 (20 pmol/ml), and CYP3A4 (10 pmol/ml) were tested during a 30 min incubation. The protein concentration in the incubations was 0.05 mg/ml. M4 data is presented in arbitrary units, i.e., quantitative comparisons to montelukast and M6 are not possible.

Fig. 8.

Enzyme kinetics of M6 formation by recombinant CYP2C8 and CYP2C9. Enzyme kinetic results were best described by a substrate inhibition model; $V_{\text{max}}$, $K_m$, and $K_i$ for M6 formation in CYP2C8 (16 pmol/ml) were 0.18 pmol/min/pmol, 0.050 µM, and 11 µM, respectively, and in CYP2C9 (20 pmol/ml) 0.11 pmol/min/pmol, 0.19 µM, and 7.4 µM, respectively. The calculated $CL_{\text{int, in vitro}}$ was 3.6 ml/min/nmol for CYP2C8 and 0.59 ml/min/nmol for CYP2C9. Incubations contained 0.05 mg/ml protein.
Tables

**TABLE 1**

*Intrinsic and hepatic clearance values for recombinant CYP2C8, CYP2C9, CYP3A4, and CYP3A5.* Values are calculated on the basis of the depletion rate of 0.02 µM montelukast with the relative activity and relative abundance approaches. Numbers within brackets indicate estimated values when CYP3A5 is excluded from the calculations.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$\text{CL}_{\text{int, in vitro}}$</th>
<th>Relative activity factor (RAF)</th>
<th>Relative abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{CL}_{\text{int RAf}}$</td>
<td>$\text{CL}_{h}$</td>
<td>% of total $\text{CL}_{h}$</td>
</tr>
<tr>
<td></td>
<td>ml/min/nmol</td>
<td>ml/min/mg</td>
<td>ml/min/kg</td>
</tr>
<tr>
<td>CYP2C8</td>
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<td>0.97</td>
</tr>
<tr>
<td>CYP2C9</td>
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<td>0.040</td>
<td>0.16</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>1.8</td>
<td>0.053</td>
<td>0.22</td>
</tr>
<tr>
<td>CYP3A5</td>
<td>0.16</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
FIG. 7

A) Montelukast (nM) vs. Time (min) for CYP2A6, CYP2C8, CYP2C9, CYP3A4, and CYP3A5.

B) M6 (nM) vs. Time (min) for the same enzymes.

C) M4 (unit) vs. Time (min) for the same enzymes.