HEPATIC MICROSOMAL METABOLISM OF MONTELUKAST, A POTENT LEUKOTRIENE D\textsubscript{4} RECEPTOR ANTAGONIST, IN HUMANS

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ABSTRACT:
Montelukast (L-706,631, MK-0476, SINGULAIR), a potent and selective leukotriene D\textsubscript{4} (CysLT\textsubscript{1}) receptor antagonist, is currently under development for the treatment of asthma. In vitro studies were conducted using human liver microsomes to evaluate: 1) the difference in the metabolic kinetics of montelukast between adult and pediatric subjects; 2) the relative contribution of flavin-containing monoxygenase and cytochrome P450 (P450) to the sulfoxidation; and 3) the P450 isoforms responsible for montelukast oxidation. No statistically significant difference was observed in the in vitro kinetics for acetyl glucuronidation and oxidative metabolism between the two age groups. Results from studies on heat inactivation of flavin-containing monooxygenase and immunoenzyme inhibition by an anti-rat NADPH P450 reductase antibody on montelukast oxidation indicated that all oxidative metabolism of montelukast—including diastereomeric sulfoxidations, as well as 21- and methyl-hydroxylations—are catalyzed exclusively by P450. Five in vitro approaches have been used to identify the P450 isoforms responsible for the human liver microsomal oxidation of montelukast. The experimental results consistently indicated that CYP3A4 catalyzes sulfoxidation and 21-hydroxylation, whereas CYP2C9 selectively mediates methyl-hydroxylation.

Montelukast (L-706,631, MK-0476, SINGULAIR), (R)-1-[(1-(3-(2-(7-chloro-2-quinolinyl)-ethyl)phenyl)-3-(2-hydroxy-2-propyl)phenyl)propyl]thiomethyl] cyclopropane acetic acid—a potent and selective leukotriene D\textsubscript{4} (CysLT\textsubscript{1}) receptor antagonist (1, 2)—is currently under development for the treatment of asthma. Montelukast undergoes extensive metabolism in humans, with only a small fraction of the dose being excreted as the parent drug in bile. 1 The major metabolites identified in plasma and bile samples from experimental animals and humans are: 1) acetyl glucuronide (M1); 2) diastereomeric sulfoxides (M2a and M2b, each configuration yet to be identified); 3) diastereomeric 21-hydroxylated metabolites (M5a and M5b for S and R isomers, respectively); and 4) diastereomeric methyl-hydroxylated metabolites (M6a and M6b; corresponding configuration yet to be identified). 2 In the present studies, we focused on the metabolic pathways that form these primary metabolites (fig. 1) found in the in vitro studies. In addition, because patients of all ages may receive asthma treatment, the in vitro montelukast metabolism in both adult and pediatric subjects were examined. This study was designed to evaluate: 1) the difference in the in vitro metabolic kinetics of montelukast between adult and pediatric subjects; 2) the relative contribution of FMO and P450 to the sulfoxidation; and 3) the P450 isoforms responsible for montelukast oxidation by using a combination of five in vitro approaches (3).

Materials and Methods

Chemicals. [14\textsuperscript{C}]Montelukast was synthesized at Merck Research Laboratories (Rahway, NJ). The carbon-14 label was incorporated at the isopropyl positions (fig. 1) with a specific activity of 21.22 mCi/mg. Nonradiolabeled compounds—montelukast; I-761,114 (M2a, sulfoxide form a); I-761,159 (M2b, sulfoxide form b); I-772,146 (M5a, 21-hydroxylated metabolite); I-772,145 (M5b, 21R-hydroxylated metabolite); I-775,066 (M6a, methyl-hydroxylated metabolite form a); and I-775,065 (M6b, methyl-hydroxylated metabolite form b)—were synthesized at Merck Frost (4) (Montreal, Quebec, Canada). Diastereomeric configurations of M2 and M6 were not yet determined.

Marker substrates and metabolites were obtained from the following sources: testosterone, methimazole, phenacetin, acetaminophen, and coumarin were from Sigma Chemical Co. (St. Louis, MO); hydroyxylated metabolites of testosterone were from Steraloids, Inc. (Wilton, NH); tolbutamide and methyl-hydroxylated tolbutamide were from Research Biochemical International (Natick, MA); and 7-hydroxylated coumarin, S-mephenytoin, and its 4’- hydroxylated metabolite were from Gentest Corp. (Woburn, MA).

Chemical inhibitors were obtained from the following sources: ketoconazole was from Research Diagnostics, Inc. (Flanders, NJ); and L-754,394 was synthesized at Merck Research Laboratories (West Point, PA). L-754,394 is a potent and selective inhibitor of CYP3A4 (5); sulfaphenazole and furafylline were from Gentest Corp.; troleandomycin, quinidine, and diethyldithiocarbamate were from Sigma Chemical Co. All other reagents were of analytical grade.

Human Liver Microsomes, Antibodies, and Microsomal Fractions Specifically Expressing Human P450 Isomers. Microsomal fractions of human liver were provided by Keystone Skin Bank (Exton, PA). Subject codes A, B, C, D, E, F, G, H, I, J, K, and L used in the manuscript correspond to the original microsomes codes HHH-050, 057, 059, 065, 079, 095, 096, 099, 106, 123, 124, and 127, respectively. Microsomes were used as they were supplied.

Goat polyclonal anti-rat NADPH P450 reductase antibody was obtained from Gentest Corp. Rabbit polyclonal antibody prepared against rat CYP3A1 and phenytoin control IgG were obtained from Gentest Corp. (Phoenix, AZ). The rabbit antibody prepared against human CYP2C9 was kindly provided by Dr. Jerome Lasker (Mount Sinai Medical Center, NY). A mouse monoclonal antibody for human CYP2A6 was obtained from Gentest Corp.

Microsomal fractions, prepared from AHH-1 TK+/- cell lines transfected...
Montelukast Metabolism Studies.

Montelukast metabolism was measured in a reaction mixture consisting of an NADPH- or UDPGA-fortified liver microsomes (or NADPH-fortified human recombinant P450s) according to the method outlined herein.

Oxidative Metabolism of Montelukast. The incubation mixture (final volume of 125 µl in 0.15 M Tris-HCl buffer, pH 7.4) consisted of an NADPH-generating system (10 mM G6P, 2 IU/ml G6P dehydrogenase, 10 mM MgCl₂), 1 mM EDTA, 2 mg/ml of liver microsomes, and various concentrations of montelukast. After a 5-min preincubation at 37°C, the reaction was initiated by the addition of 5 µl of 50 mM NADPH. The incubation was conducted at 37°C for 30 min, and was terminated by adding 125 µl of ice-cold acetonitrile containing 10 µM of α-naphthoflavone (as an internal standard). The resulting suspension was mixed vigorously and centrifuged at 14,000g for 4 min to precipitate protein. An aliquot (100 µl) of the supernatant was directly injected onto the HPLC (system A) described herein.

Acyl Glucuronidation of Montelukast. The incubation mixture (final volume of 300 µl in 0.15 M Tris-HCl buffer, pH 7.4) consisted of 20 mM UDPGA, 10 mM MgCl₂, and 2 mg/ml of liver microsomes. After a 5-min preincubation at 37°C, the reaction was initiated by the addition of various concentrations of 14C-montelukast. The incubation was conducted at 37°C for 30 min, and the reaction was terminated by adding 300 µl of ice-cold acetonitrile. The resulting suspension was mixed vigorously and centrifuged at 14,000g for 4 min to precipitate protein. An aliquot (200 µl) of the supernatant was directly injected onto the HPLC (system B) described herein.

Identification Studies on Monooxygenase. FMO activity was selectively inactivated by a preincubation of human liver microsomal suspension (0.25 mg) at 45°C for a designated time period. After inactivation, the microsomal suspension was used to measure montelukast metabolism and marker activities. Montelukast metabolism was determined according to the method described previously. Methimazole S-oxygenase, testosterone 2β/6β-hydroxylase, and tolbutamide methyl hydroxylase activities were determined according to the method described herein.

For the anti-rat NADPH P450 reductase antibody study, the human liver
were generated by using equations and kinetic parameters listed in Table I.

For mechanism-based P450 inhibition studies with furafylline, troleandomycin, L-754,394 and diethyldithiocarbamate, the microsomal suspension was preincubated at 37°C for 30 min. The same assay method as described in the previous section was used to measure the montelukast metabolism.

Immunochemical inhibition studies were performed with anti-rat CYP3A1 antibody, anti-human CYP2C9 antibody, or anti-human CYP2A6 antibody. The human liver microsomal suspension (0.13 mg) was preincubated with the designated amount of antibody for 30 min at room temperature. After preincubation, the reaction mixture containing an NADPH-generating system (same composition as described previously) and montelukast (final concentration = 500 μM) was added to the microsomes and incubated for 5 min at 37°C. The reaction was initiated by the addition of 5 μl of NADPH and conducted for 60 min at 37°C. The same assay as described in the previous section was used to measure the montelukast metabolism.

Recombinant P450 Studies. Recombinant P450 isoforms (0.5 mg/incubation) were incubated with an NADPH-generating system (the same composition as described in the previous section) and 500 μM montelukast for 60 min at 37°C. The same assay method as described in the previous section was used.

Competitive Interaction Studies. P450 isoform-selective marker metabolism (testosterone 2β-6β-hydroxylation, phenacetin O-deethylation, coumarin 7-hydroxylation, tolbutamide methyl hydroxylation, S-mephenytoin 4′-hydroxylation, and debrisoquine 4-hydroxylase activities) was measured in a reaction mixture (final volume = 150 μl in 0.15 M Tris-HCl buffer, pH 7.4) consisting of an NADPH-generating system (20 mM G6P, 4 IU/ml G6P dehydrogenase, 10 mM MgCl2), 1 mM EDTA, 1 mg/ml of liver microsomes, marker substrate (final concentration specified herein), and various concentrations of montelukast. For the studies on the effect of montelukast on marker activities, the following marker substrate concentration was used: 5 μM of phenacetin; 2 μM of coumarin; 10 μM of tolbutamide; 200 μM of 5-methoxyveratrine; 50 μM of debrisoquine; and 20 μM of testosterone. For an interaction study between montelukast metabolism and tolbutamide methyl-hydroxylation or testosterone 6β-hydroxylation, various combinations of montelukast and tolbutamide or testosterone were used. After 5-min preincubination at 37°C, the reaction was initiated by the addition of 5 μl of 50 mM NADPH and terminated by adding ethyl acetate after 20 min. The assay was conducted according to the method described herein.

Correlation Studies. Human liver microsomes (0.125 mg) obtained from 12 individual subjects were incubated with 500 μM of montelukast, testoterone, nifedipine, tolbutamide, or coumarin to measure montelukast metabolism and P450 marker activities. The same reaction mixtures and incubation conditions as described in the previous section were used for the montelukast metabolism. For the marker metabolism, the reaction mixture (final volume of 250 μl in 0.15 M Tris-HCl buffer, pH 7.4) consisted of an NADPH-generating system (10 mM G6P, 4 IU/ml G6P dehydrogenase, 10 mM MgCl2), 1 mM EDTA, 1 mg/ml of liver microsomes, 500 μM marker substrate. After 5-min preincubation at 37°C, the reaction was initiated by the addition of 5 μl of 50 mM NADPH. The incubation was conducted at 37°C for 20 min. The assay method for each metabolism was described herein.

HPLC Assay for Montelukast and Metabolites (System A). Montelukast and its acyl glucuronide conjugate were measured by an HPLC system consisting of pump/autoinjector (Spectra Physics P4000/AS3000, Fremont, CA) and spectrofluorometer (Spectra Physics FL2000). An assay was performed on an Inertsil ODS-2 column (5 μm, 4.6 × 250 mm, GL Science, Tokyo, Japan). The HPLC method involved the following gradient system using 1 mM ammonium composition as described previously) and montelukast (final concentration = 50 μM) NADPH. The incubation was performed at 37°C for 30 min. The same HPLC assay as described herein (system A) was used to measure the montelukast metabolism.

For mechanism-based P450 inhibition studies with furafylline, troleandomycin, L-754,394 and diethyldithiocarbamate, the microsomal suspension (0.125 mg) was preincubated with an NADPH-generating system (same composition as noted previously), 5 μl of 50 mM NADPH, and various concentrations of inhibitor for 30 min at 37°C. After preincubation, 12.5 μl of 1 mM montelukast (final concentration = 100 μM) was added to start montelukast metabolism. The reaction was conducted for 30 min at 37°C. The same procedure as described was used to measure the montelukast metabolism.

Data represent the average of three determinations with SD. Simulation curves were generated by using equations and kinetic parameters listed in Table I. Microsomal suspension (0.13 mg) was preincubated with a designated amount of antibody for 30 min at room temperature. After preincubation, the microsomal suspension was used for the montelukast metabolism study according to the method described previously, and for the testosterone and tolbutamide metabolism studies as described herein.

Identification Studies on P450 Isoforms. To determine which P450 isoforms are responsible for montelukast metabolism, we conducted the five in vitro approaches adopted previously (3), namely: 1) chemical inhibition; 2) immunochemical inhibition; 3) metabolism by recombinant P450 isoforms; 4) competitive effect on marker activities; and 5) a correlation analysis.

Chemical and Immunochemical Inhibition Studies. For the competitive P450 isoform-selective inhibition studies with coumarin, sulfaphenazole, S- mephenytoin, quinidine, and ketoconazole, the incubation mixture (final volume = 100 μl in 0.15 M Tris-HCl buffer, pH 7.4) consisted of an NADPH-generating system (10 mM G6P, 2 IU/ml G6P dehydrogenase, 10 mM MgCl2), 1 mM EDTA, 2 mg/ml of liver microsomes, 100 μM montelukast, and various concentrations of inhibitor. After 5 min preincubation at 37°C, the reaction was initiated by the addition of 5 μl of 50 mM NADPH. The incubation was performed at 37°C for 30 min. The same HPLC assay as described herein (system A) was used to measure the montelukast metabolism.

For mechanism-based P450 inhibition studies with furafylline, troleandomycin, L-754,394 and diethyldithiocarbamate, the microsomal suspension (0.125 mg) was preincubated with an NADPH-generating system (same composition as noted previously), 5 μl of 50 mM NADPH, and various concentrations of inhibitor for 30 min at 37°C. After preincubation, 12.5 μl of 1 mM montelukast (final concentration = 100 μM) was added to start montelukast metabolism. The reaction was conducted for 30 min at 37°C. The same procedure as described was used to measure the montelukast metabolism.
of 20 mm protein. M/cm was used to calculate nmoles of product formed/minute/milligram of mixtures with and without methimazole. The extinction coefficient 28,200 of increase in the difference in absorbance at 412 nm between identical assay min at 37°C. Enzyme reaction was initiated by the addition of 0.2 mM After the addition of 0.02 mM dithiothreitol, samples were preincubated for 5

\[ \text{Methyl-Hydroxylation} \]

<table>
<thead>
<tr>
<th>Species</th>
<th>Acyl Glucuronidation</th>
<th>Sulfoxidation</th>
<th>21-Hydroxylation</th>
<th>Methyl-Hydroxylation</th>
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<tr>
<td></td>
<td>( K_{M} )</td>
<td>( V_{\text{max}} )</td>
<td>( k )</td>
<td>( K_{M} )</td>
</tr>
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<td>Adult</td>
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<td></td>
<td>±7</td>
<td>±4.6</td>
<td>±48</td>
<td>±0.040</td>
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</table>

Separate incubations were conducted to measure acyl glucuronidation \((M_1)\) formation and oxidations \((M_2, M_5, \text{and } M_6)\) formations (see Materials and Methods). Data represent the average \( ± \) SD of results obtained from three individual subjects. Acyl glucuronidation and oxidations of montelukast analyzed by the following eqs. 1 and 2, respectively.

\[ \nu = \frac{V_{\text{max}} \cdot S}{K_{M} + S + k \cdot S} \]  

\[ \nu = \frac{V_{\text{max}} \cdot S}{K_{M} + S} \]

where \( \nu \), \( S \), and \( k \) represent metabolic velocity, montelukast concentration, and linear constant to analyze second component observed in the higher substrate concentration, respectively. No statistically significant difference \((p > 0.05)\) was observed in parameters between adult and pediatric subjects.

acetate as mobile phase A and acetonitrile as mobile phase B. Mobile phase A was adjusted to pH 3.5 with glacial acetic acid. The flow rate was set at 1.1 ml/min. From 0 to 4 min, the ratio of mobile phase A to B remained at 55:45. After the addition of 0.02 mM dithiothreitol, samples were preincubated for 5 min at 37°C. Enzyme reaction was initiated by the addition of 0.2 mM NADPH, and 0.5 mg/ml of liver microsomes in 0.15 M Tris-HCl \((pH\ 7.4)\). The resulting mixture was vortex-mixed, and the ethyl acetate layer was separated by centrifugation, followed by evaporation to dryness under nitrogen. The residue was reconstituted in 150 μl of 20% methanol in water. An HPLC assay was performed on a Supelco LC 18 column \((5 \, \text{mm}, 4.6 \, \text{mm})\) with a Spectra-Physics HPLC system. The HPLC method involved the isocratic elution for 15 min with 45:55 (v/v) mixture of water (mobile phase A) and methanol (mobile phase B) at a flow rate of 1.0 ml/min. Chromatograms were monitored at 254 nm.

**Phenacetin O-deethylase Assay (CYP1A2).** Reaction was terminated by the addition of ice-cold ethyl ether \((3 \, \text{ml})\). After 20 μl of 50 μM 3-aceamidophenol was added to each sample as an internal standard, the resulting mixture was vortex-mixed, and the ethyl ether layer was separated by centrifugation, followed by evaporation to dryness under nitrogen. The residue was reconstituted in 150 μl of 20% methanol in water. An HPLC assay was performed on a Supelco LC 18 column \((5 \, \text{mm}, 4.6 \, \text{mm})\) with a Spectra-Physics HPLC system. The HPLC method involved the isocratic elution for 6 min with 87:13 (v/v) mixture of water (mobile phase A) and methanol (mobile phase B) at a flow rate of 1.0 ml/min. Both mobile phases were adjusted to pH 3.5 with H3 PO4. A linear gradient was then run until 11 min, when the proportion of mobile phase A was decreased from 87% to 50%. Mobile phase A remained at 50% for 2 min and was reverted back to 87% at 18 min. Chromatograms were monitored at 254 nm.

**Tolbutamide Methyl Hydroxylase Assay (CYP2A6 Marker).** Reaction was terminated by the addition of ice-cold ethyl acetate \((2.5 \, \text{ml})\). After 50 μl of 50 μM p-nitrophenol was added to each sample as an internal standard, the resulting mixture was vortex-mixed, and the ethyl acetate layer was separated by centrifugation, followed by an evaporation to dryness under nitrogen. The residue was reconstituted in 150 μl of 20% methanol in water. An HPLC assay was performed on a Supelco LC 18 column \((5 \, \text{mm}, 4.6 \, \text{mm})\) with a Spectra-Physics HPLC system. The HPLC method involved the isocratic elution for 15 min with 68:32 (v/v) mixture of 0.05 M KH2PO4 (mobile phase A) and methanol (mobile phase B) at a flow rate of 1.5 ml/min. Chromatograms were monitored at 315 nm.

**Nifedipine N-oxidase Assay (CYP3A4 Marker).** Reaction was terminated by the addition of ice-cold ethyl acetate \((2.5 \, \text{ml})\). After 50 μl of 50 μM 6β-hydroxytestosterone was added to each sample as an internal standard, the resulting mixture was vortex-mixed, and the ethyl acetate layer was separated by centrifugation, followed by an evaporation to dryness under nitrogen. The residue was reconstituted in 150 μl of 20% methanol in water. An HPLC assay was performed on a Supelco LC 18 column \((5 \, \text{mm}, 4.6 \, \text{mm})\) with a Spectra-Physics HPLC system. The HPLC method involved the isocratic elution for 10 min with 90:10 (v/v) mixture of 30% methanol in water (mobile phase A) and 10% acetonitrile in methanol (mobile phase B) at a flow rate of 1.0 ml/min. Chromatograms were monitored at 244 nm.
Bisubstrate and NADPH to measure montelukast metabolism (A). The reaction, at 37°C, was initiated by the addition of substrate and NADPH to measure montelukast metabolism (A) and P450/FMO marker activities (B).

The metabolic velocity for montelukast oxidation and acyl glucuronidation was analyzed by a nonlinear least squares program (MULTI) (7). The following equation was used to analyze the relation between metabolic velocity and substrate concentration:

\[ v = \frac{V_{\text{max}} \cdot S}{K_m + S} \cdot k \cdot S, \]

where \( v \), \( K_m \), \( V_{\text{max}} \), and \( S \) represent metabolic velocity, Michaelis-Menten constant, maximum velocity of metabolism, and montelukast concentration, respectively. The linear constant, \( k \), was used to describe the second component observed in the higher substrate concentration range in the acyl glucuronidation kinetics.

Results

Biotransformation of Montelukast in Human Liver Microsomes. All primary metabolites, M1, M2a, M2b, M5a, M5b, and M6a/b (fig. 1) were formed in UDPGA- or NADPH-fortified liver microsomes obtained from adult and pediatric subjects (fig. 2). There was no significant difference in the metabolic profiles for acyl glucuronidation nor in the oxidative metabolism of montelukast between adult and pediatric subjects.

Kinetic Studies. Formation of acyl glucuronide was substantially greater than those of oxidations over the concentration range examined in both adult (fig. 3A) and pediatric (fig. 3B) human liver microsomes. The enzyme kinetic parameters of acyl glucuronidation are listed in table 1. Although the formation of acyl glucuronide was somewhat higher in liver microsomes from pediatric subjects than those from adult subjects, the kinetic parameters did not show statistically significant differences between these two age groups (table 1).

Kinetic parameters also were determined for the oxidative metabolism of montelukast using liver microsomes from pediatric and adult subjects, and are presented in table 1. The \( K_m \) values for sulfoxidations (M2a/b) and 21-hydroxylations (M5a/b) were >3-fold larger than those for methyl-hydroxylations (M6a/b) (table 1). The \( V_{\text{max}} \) of R-isomer of 21-hydroxylation seemed to be greater than that of S-isomer (M5b > M5a), whereas sulfoxidation rates showed little difference between stereoisomers. There was no statistically significant difference in kinetic parameters between adult and pediatric subjects (table 1).

FMO vs. P450 for Montelukast Oxidative Metabolism. To determine whether the FMO system was involved in the sulfoxidation of montelukast (M2), the FMO activity in human liver microsomes was purposely inactivated by a preincubation of the microsomes without NADPH at 45°C as a function of time up to 10 min. Heat preincubation resulted in a dramatic decrease of FMO activity in a time-dependent manner, as evidenced by the decreased activity of methimazole S-oxidation (fig. 4B), whereas it had little effect on the sulfoxidation and hydroxylation reactions of montelukast, as well as P450 marker activities (fig. 4). On the other hand, an anti-rat NADPH P450 reductase antibody strongly (>80%) inhibited sulfoxidation and hydroxylation of montelukast in a concentration-dependent manner (data not shown). Collectively, these results suggest that all oxidative primary metabolism of montelukast is catalyzed exclusively by P450.

Identification of P450 Isoforms Responsible for Montelukast Oxidation. Troleandomycin, 1-t,754,394, and ketoconazole—CYP3A4-selective chemical inhibitors—completely inhibited M5a and M5b formations in a concentration-dependent manner, whereas troleandomycin and 1-t,754,394 had little effect on M6a/b formation (fig. 5, A–C). Sulfoxidations of montelukast (M2a and M2b) also were strongly inhibited by CYP3A inhibitors. In contrast, sulfaphenazole, a CYP2C9-selective chemical inhibitor, showed a marked inhibitory effect on M6a/b formation (fig. 5D). Coumarin, S-mephenyton, furafylline, quinidine, and diethyldithiocarbamate—selective inhibi-
tors of CYP2A6, CYP2C19, CYP1A2, CYP2D6, and CYP2E1, respectively—had little effect on the metabolism of montelukast (fig. 5, E–I).

An anti-rat CYP3A1 antibody strongly inhibited formations of M5a/b, but to a lesser extent, M2a/b, in a concentration-dependent manner without affecting M6a/b formations (fig. 6). In contrast, an anti-human CYP2C9 antibody completely inhibited M6a/b formations, whereas it had little effect on other metabolism (fig. 6). In addition, an anti-human CYP2A6 antibody significantly inhibited formations of M2a/b (to 60% control activity) (data not shown).

Human recombinant CYP3A4 showed a great capability to form M2a/b and M5a/b metabolites, whereas it did not catalyze M6a/b formation (fig. 7). In contrast, a recombinant CYP2C9 formed M6a/b metabolites, but it had little catalytic activity to form M2 and M5 metabolites. Recombinant CYP2A6 also showed some catalytic activities to form M2.

Montelukast strongly inhibited the methyl-hydroxylase activity of tolbutamide and, to a much lesser extent, 6β-hydroxylase of testosterone and 4'-hydroxylase activities of S-mephenytoin, in a concentration-dependent manner (fig. 8). The interaction between montelukast metabolism and tolbutamide methyl hydroxylation was found to be competitive (fig. 9A) with a $K_i$ value of $\sim 15 \mu$M (fig. 9B). Similarly, the interaction was competitive between montelukast metabolism and testosterone 6β-hydroxylation, with a $K_i$ value of $\sim 200 \mu$M (data not shown).

The oxidative metabolism of montelukast and P450 markers was determined in 12 different liver microsomes (fig. 10). There was considerable interindividual variation (>15-fold) in M2 and M5 formations. Similarly, significant interindividual variation was observed in testosterone 6β-hydroxylase and coumarin 7-hydroxylase activities. The extent of variation of M6a/b formation among subjects B–L was much smaller (<3-fold) than that of M2 or M5. Similarly, tolbutamide methyl-hydroxylation showed a smaller variation (<2-fold) than testosterone and coumarin metabolism. The formation rates of M5a and M5b correlated well with CYP3A4-selective marker (testosterone 6β-hydroxylase and nifedipine N-oxidase) activities (table 2). Significant correlations also were observed between M2a/b and CYP3A4 marker activities, with a smaller coefficient value ($r^2 <$

![Fig. 5. Effect of various chemical inhibitors on montelukast oxidative metabolism in human liver microsomes.](image-url)

Effects of (A) troleandomycin, (B) L-754,394, (C) ketoconazole, (D) sulfaphenazole, (E) coumarin, (F) S-mephenytoin, (G) furafylline, (H) quinidine, and (I) diethylthiocarbamate were determined on montelukast metabolism.
0.84) than that for M5 ($r^2 > 0.9$). In contrast, M6a/b formation did not show a significant correlation with these CYP3A marker activities. Tolbutamide methyl-hydroxylation and M6a/b formation showed the highest correlation. The lack of statistical significance is likely due to the low extent of variation (≈2-fold) among subjects that tends to hinder the statistical analysis. No discernible correlation was observed between coumarin 7-hydroxylase activity and montelukast metabolism.

**Discussion**

Comparative studies with human liver microsomes indicated that there is no significant difference in montelukast metabolism between pediatric (ages 6–11) and adult (ages 50–65) subjects. It has been cited in the literature that the human liver matures rapidly, with metabolic capacities similar to, or in excess of, adult values by 6 months of age for the oxidation by P450 and conjugation metabolism by uridine diphosphate glucuronosyltransferase (8–11). This may partially explain why the *in vitro* kinetics of montelukast metabolism, which involve both acyl glucuronidation and P450-catalyzed oxidation, were age-independent.

P450 isoform-selective marker activities were measured in a human liver microsomal reaction mixture with different concentrations of montelukast. Substrate concentrations of marker were: 5 μM for phenacetin O-deethylase; 2 μM for coumarin 7-hydroxylase; 10 μM for tolbutamide methyl-hydroxylation; 200 μM for S-mephenytoin 4′-hydroxylase; 50 μM for debrisoquine 4-hydroxylase; and 20 μM for testosterone 6β-hydroxylase metabolism.

Among the metabolic pathways for montelukast, P450 was found to catalyze exclusively all montelukast oxidations. It has been shown that sulfur-containing functional groups are generally subject to P450- and/or FMO-catalyzed oxidations (12, 13). Therefore, as a compound that contains such a group, the contribution of FMO was investigated.
in the sulfoxidation of montelukast. In these studies, we chose methimazole $S$-oxygenase activity as a marker for FMO, because it has been demonstrated that this reaction is catalyzed by FMO with no apparent contribution from P450s at low concentrations (12–16). The marked thermal lability of the hepatic FMO in conditions above 35°C in the absence of NADPH (15, 17) was used to define the contribution of FMO in the sulfoxidation pathways (17–20). Preincubation of human liver microsomes at 45°C for 5–10 min in the absence of NADPH selectively inactivated FMO-catalyzed methimazole $S$-oxygenation, whereas it had little effect on the P450-catalyzed marker metabolism, as well as any oxidative metabolism of montelukast (fig. 4). These results were further supported by the complete inhibition of montelukast oxidation with an anti-rat NADPH P450 reductase antibody. Although sulfur-containing drugs are generally excellent substrates for FMO, carboxylic acids containing a sulfide group on the terminal carbon have not shown detectable substrate activities (12, 13). The presence of a carboxylic acid on the same side chain structure as the sulfide may similarly prevent montelukast from being a favorable substrate for FMO in human liver microsomes.

Studies to identify the P450 isoforms responsible for montelukast oxidative metabolism consistently indicated that CYP3A4

![Fig. 9. Competitive inhibition of montelukast on tolbutamide methyl-hydroxylation.](image)

Lineweaver-Burk plots (A) of tolbutamide methyl-hydroxylase activity in the presence of different concentrations of montelukast showed that the interaction was competitive. The inhibition constant of montelukast was graphically estimated to be 15 μM by a Dixon plot (B).

![Fig. 10. P450 isoform selective markers (testosterone 6β-hydroxylation, tolbutamide methyl-hydroxylation, and coumarin 7-hydroxylation) and montelukast metabolism in 12 human liver microsomal preparations.](image)

Montelukast and marker activities were measured according to the procedure described in Materials and Methods.
TABLE 2
Correlation between montelukast metabolism and marker enzyme activities in human liver microsomal preparations

<table>
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<tr>
<th>Metabolite</th>
<th>CYP3A4</th>
<th>CYP2C9</th>
<th>CYP2A6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>THO&lt;sup&gt;a&lt;/sup&gt;</td>
<td>M60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>TMB&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>M2a</td>
<td>0.805&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.825&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.0876</td>
</tr>
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<td>0.612&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.636&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.0997</td>
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<tr>
<td>M6a/b</td>
<td>0.483</td>
<td>0.501</td>
<td>0.533</td>
</tr>
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</table>

*a Human liver microsomes obtained from 12 individual donors were used.
*b Marker activities: THO, testosterone 6β-hydroxylation; M60, nifedipine N-oxidation; TMB, tolbutamide methyl-hydroxylation; CTH, coumarin 7-hydroxylation.

Significant correlation was obtained (<i>p</i> < 0.001).
Significant correlation was obtained (<i>p</i> < 0.005).

was a P450 isoform mediating both 21-hydroxylation and sulfoxidation, whereas CYP2C9 selectively catalyzed methyl-hydroxylation. Many pharmaceuticals that contain a carboxylic acid structure and exhibit a pK<sub>a</sub> value below 7 (such as ibuprofen, naproxen, and diclofenac) have been known to undergo CYP2C9-catalyzed metabolism (21). It has been suggested that hydrogen bonding and the presence of a negative charge on the substrate together play an important role in its recognition by P450. Flavonoids and benzoic acid derivatives, which are known to undergo CYP2C9-catalyzed metabolism (21), may be essential for the CYP2C9-selective methyl-hydroxylation metabolism of montelukast.

In summary, no statistically significant difference was observed in the <i>in vitro</i> kinetic parameters for the hepatic microsomal acyl glucuronidation and oxidation of montelukast between adult and pediatric humans. P450 plays an exclusive role in catalyzing the oxidative metabolism of montelukast, and the contribution of FMO was not demonstrated. A regioselective hydroxylation by CYP3A4 and CYP2C9 was observed; CYP3A4 selectively catalyzed 21-hydroxylation, whereas CYP2C9 mediated methyl-hydroxylation. Kinetic studies revealed that acyl glucuronidation and methyl-hydroxylation had smaller <i>K<sub>M</sub></i> values (<100 μM) than both sulfoxidation and 21-hydroxylation. This suggests that acyl glucuronidation and CYP2C9-catalyzed methyl-hydroxylation may play important roles in the metabolism of montelukast.

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References
