METABOLISM OF ROFECOXIB IN VITRO USING HUMAN LIVER SUBCELLULAR FRACTIONS

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ABSTRACT:

The metabolism of rofecoxib, a potent and selective inhibitor of cyclooxygenase-2, was examined in vitro using human liver subcellular fractions. The biotransformation of rofecoxib was highly dependent on the subcellular fraction and the redox system used. In liver microsomal incubations, NADPH-dependent oxidation of rofecoxib to 5-hydroxyrofecoxib predominated, whereas NADPH-dependent reduction of rofecoxib to the 3,4-dihydrohydroxy acid metabolites predominated in cytosolic incubations. In incubations with S9 fractions, metabolites resulting from both oxidative and reductive pathways were observed. In contrast to microsomes, the oxidation of rofecoxib to 5-hydroxyrofecoxib by S9 fractions followed two pathways, one NADPH-dependent and one NAD+-dependent (non-cytochrome P450), with the latter accounting for about 40% of total activity. The 5-hydroxyrofecoxib thus formed was found to undergo NADPH-dependent reduction ("back reduction") to rofecoxib in incubations with liver cytosolic fractions. In incubations with dialyzed liver cytosol, net hydration of rofecoxib to form 3,4-dihydro-5-hydroxyrofecoxib was observed, whereas the 3,4-dihydrohydroxy acid derivatives were formed when NADPH was present. Although 3,4-dihydro-5-hydroxyrofecoxib could be reduced to the 3,4-dihydrohydroxy acid by cytosol in the presence of NADPH, the former species does not appear to serve as an intermediate in the overall reductive pathway of rofecoxib metabolism. In incubations of greater than 2 h with S9 fractions, net reductive metabolism predominated over oxidative metabolism. These in vitro results are consistent with previous findings on the metabolism of rofecoxib in vivo in human and provide a valuable insight into mechanistic aspects of the complex metabolism of this drug.

Rofecoxib (VIOXX, a registered trademark of Merck and Co., Inc.) is a potent and selective inhibitor of cyclooxygenase-2 (COX-2), which is indicated for the treatment of osteoarthritis, rheumatoid arthritis, and pain. It has been shown to cause significantly fewer gastrointestinal side effects than naproxen, a nonselective COX inhibitor (Bombardier et al., 2000), consistent with the hypothesis that COX-2 selective inhibitors exhibit a gastrointestinal safety advantage over nonselective COX inhibitors (Donnelly and Hawkey, 1997; Jouzeau et al., 1997).

In previous reports from this laboratory, the absorption, distribution, metabolism, and excretion of rofecoxib were examined in the rat, dog, and human (Halpin et al., 2000, 2002). Metabolism studies in laboratory animals and humans indicated that rofecoxib undergoes complex metabolism involving both oxidative and reductive reactions. Although the metabolic pathways of rofecoxib were qualitatively similar between the rat and human, oxidation to 5-hydroxyrofecoxib (5-hydroxyR) predominated in rats (Halpin et al., 2000), whereas reduction of rofecoxib to the corresponding isomeric 3,4-dihydrohydroxy acid (DHHHA) derivatives was the major pathway in humans (Halpin et al., 2002). Furthermore, 5-hydroxyR, the primary oxidative metabolite, was found to undergo reversible metabolism to rofecoxib in rats (Baillie et al., 2001) and to a lesser extent in humans (Halpin et al., 2002).

To better define the intermediates and likely biochemical mechanisms of the processes contributing to the biotransformation of rofecoxib, the present in vitro studies were performed using human liver subcellular fractions. The results clearly demonstrate that the three metabolic pathways observed in vivo (oxidative, reductive, and back reduction) could be replicated in vitro using appropriate liver subcellular fractions and redox cofactors.

Materials and Methods

Chemicals. Rofecoxib was synthesized by the department of Process Research, whereas [furanone-4-14C]rofecoxib was prepared by the Labeled Compound Synthesis Group (Merck and Co., Inc., Rahway, NJ). The chemical and radiochemical purities of the latter were at least 99% (as determined by HPLC analysis), and the specific activity of the stock material was 12.62 mCi/mmol (40.4 μCi/mg). Reference standards of rofecoxib metabolites (5-hydroxyrofecoxib, cis- and trans-3,4-dihydrorofecoxib, and rofecoxib-erythro- and rofecoxib-threo-3,4-dihydrohydroxy acid) were synthesized by the Department of Medicinal Chemistry (Merck Frosst Canada, Kirkland, QC, Canada; Prasit et al., 1999; Nicoll-Griffith et al., 2000). Rofecoxib and all biological samples...
containing rofecoxib were handled under yellow light due to the sensitivity of rofecoxib to natural light. All other chemicals and solvents were obtained from commercial sources and were of the highest quality available.

Liver Subcellular Fractions. Liver microsomal, S9, and cytosolic fractions were prepared using differential centrifugation of liver homogenates from nine male human donors (IIMM, Exton, PA) and were stored at −70°C. The protein concentrations were measured according to the biuret method (Gornall et al., 1949), with bovine serum albumin as a standard. Subcellular fractions were stored individually by donor and pooled as required. A portion of the cytosoïd from five of the nine human donors was dialyzed at 4°C against a mixture of three changes of 0.05 M Tris-HCl, pH 7.5 (500 ml) containing 1.15% (w/v) KC1 to remove endogenous redox cofactors. Micosomes containing human recombinant P450 cDNA-expressed P450 isoforms were from baculovirus-transfected SF21 cell expression systems developed in house and were prepared using methods similar to those above.

Incubations. Incubations with rofecoxib or 5-hydroxyR were carried out under a variety of conditions using human liver subcellular fractions. The different aspects of rofecoxib metabolism examined in vitro included oxidative metabolism using microsomal and S9 fractions, reductive metabolism using S9 and cytosolic fractions, and back reduction of 5-hydroxyR using S9 and cytosolic fractions. Unless otherwise indicated, all incubations (0.5 ml) were performed at 37°C in 100 mM potassium phosphate buffer, pH 7.4, containing 3 mM MgCl2, either in the presence or absence of added redox cofactor. The substrate and protein concentrations, as well as the duration of incubation, were as described in text or individual figures. Microsomal incubations were conducted in the presence of a glucose 6-phosphate-based NADPH-regenerating system (1 mM NADPH, 10 mM glucose 6-phosphate, 2 units/ml glucose-6-phosphate dehydrogenase from torula yeast) and focused on the involvement of P-450-mediated metabolism. Cytosolic incubations were conducted in the presence of a malic acid-based NADPH-regenerating system (1 mM NADPH and 10 mM l-mallic acid, presence of endogenous malic enzyme activity assumed), but focused on NADPH-dependent reductase activities. Incubations with S9 fractions were carried out largely to study the interplay between microsomal and cytosolic enzymes and were conducted using either the malic acid-based NADPH-regenerating system, an NAD+ -regenerating system (1 mM NAD+ and 10 mM ATP, presence of endogenous phosphoglycerokinase, glyceraldehyde-3-phosphate dehydrogenase, and 3-phosphoglycerate activities assumed) or a combined system (1 mM NADPH, 1 mM NAD+, and 10 mM l-mallic acid, endogenous malic enzyme and lactic dehydrogenase activities assumed). In early experiments, incubations were terminated by the addition of 1.5 ml of a cold 2:1 mixture of acetonitrile and methanol containing 1.5% (v/v) acetic acid. However, it was found that the DHHA metabolites cyclized slowly in 500 μl -malic acid, endogenous malic enzyme and lactic dehydrogenase activities assumed). In early experiments, incubations were terminated by the addition of 1.5 ml of a cold 2:1 mixture of acetonitrile and methanol containing 1.5% (v/v) acetic acid. However, it was found that the DHHA metabolites cyclized slowly during the course of a chromatographic run. In all cases, an aliquot (1.0 or 2.0 ml) of diluted, acidified supernatant was subjected to HPLC analysis. Chro-

Precipitated protein was removed by centrifugation and supernatants were therefore, acetic acid subsequently was omitted from the terminating solution. In early experiments, incubations were terminated by the addition of 30 ml of solvent (DH-5-hydroxyR) for structure elucidation. At the end of a 16-h incubation period, the reaction was terminated as described above with 30 ml of solvent mixture. After centrifugation to remove the protein precipitate, the supernatant was dried under vacuum and reconstituted in the initial mobile phase prior to chromatography.

HPLC Analysis. Initially, aliquots of supernatants were adjusted to 5% (v/v) total organic solvent (acetoneitrile plus methanol) with 0.1% aqueous TFA. The propensity of the DHHA metabolites to cyclize under acidic conditions was recognized. TFA was not added to HPLC vials until immediately before injection of the contents. In these cases, aliquots of supernatants were diluted with water to 5.5% (v/v) total organic solvent; 1% aqueous TFA (0.1 volume) was added immediately before injection to match the acidity of the HPLC mobile phase. Under these conditions, very little DHR formation was observed in incubations with hepatic subcellular fractions, and control experiments indicated that the DHHA metabolites did not cyclize to any appreciable extent during the course of a chromatographic run. In all cases, an aliquot (1.0 or 2.0 ml) of diluted, acidified supernatant was subjected to HPLC analysis. Chromatography was performed on a Zorbax RX-C18 column (4.6 × 250 mm, 5-μm particle size) preceded by a Zorbax RX-C18 Reliance guard cartridge (4.6 × 12.5 mm) containing the same stationary phase at the same particle size. The LC system was interfaced to a Waters 990 PDA UV detector (Waters, Milford, MA). Mobile phase reservoirs contained 0.1% aqueous TFA and acetonitrile, respectively, and the flow rate was 1 ml/min. The initial mobile phase was 5% acetonitrile. Analytes were eluted by means of a 30-min linear acetonitrile gradient (5–45%), followed by a 5-min wash with 90% acetonitrile and a 10-min column re-equilibration. Although the linear gradient was initi-

metabolite purification. The dried residue from the supernatant of each 10-ml incubation was reconstituted in 2.1 ml of the initial mobile phase minus TFA [5% (v/v) acetonitrile in aqueous 0.1% TFA], of which 2.0 ml was introduced into the HPLC system as a single injection. Chromatography was performed on an Econosil C18 column (10 × 250 mm, 10-μm particle size). The guard column, PDA UV detector, and mobile phase reservoirs were as described above. The flow rate was 3 ml/min. Analytes were eluted by means of a 55-min linear acetonitrile gradient (5–60%), followed by a 5-min wash with 90% acetonitrile and a 10-min column re-equilibration in 5% acetonitrile. Metabolites were collected from the HPLC peak using an ISCO JOX-200 fraction collector (ISCO, Lincoln, NE) in peak detection mode triggered by UV absorbance at 235 nm. Appropriate fractions were dried with the aid of a SpeedVac evaporator (Thermo Savant, Holbrook, NY). Prior to NMR analysis, analytes were repurified using the above methodology.

LC/MS-MS Analysis. LC/MS-MS studies were conducted in the negative ion mode using a Finnigan TSQ7000 mass spectrometer interfaced to a Hewlett-Packard (Palo Alto, CA) 1090 HPLC system through an electrospray ionization source. Chromatography was performed on a Kromasil C18 column (2.0 × 250 mm, 5-μm particle size). Mobile phase reservoirs contained 0.1% aqueous 4-methylmorpholine and acetonitrile, respectively, and the flow rate was 0.2 ml/min. The initial mobile phase was 5% acetonitrile. Analytes were eluted by varying the acetonitrile composition in a stepwise fashion: a 3 min isocratic phase, a 5% acetonitrile phase followed by a 5 to 25% gradient over 9 min, a 1 min linear gradient at 25%, a 19-min 25 to 60% gradient, a 1 min isocratic phase at 60%, a 5 min 60 to 90% gradient, a 5-min isocratic phase at 90%, a 2 min gradient back to 5% and finally a 10-min column re-equilibration at 5%.

1H NMR Analysis. 1H NMR spectra of purified metabolites were taken in the deuterated solvents indicated, using a Varian (Palo Alto, CA) Unity 400 NMR spectrometer equipped with a Nalorac 3 mm probe.

Determination of Kinetic Parameters. All reported parameters are apparent values. Kinetic parameters were determined with the assistance of curve fitting options in KaleidaGraph (Synergy Software Technologies, Inc., Essex Junction, VT). Apparent Vmax values were determined by fitting enzyme activity (V) versus substrate concentration ([S]) data to the Michaelis-Menten equation (V = Vmax[S]/(Km + [S])) and are expressed ± standard error, where n is the number of data points to which the curve was fit. Apparent Km values were calculated in two ways: by fitting the rearranged Michaelis-Menten equation v = (Vmax/Km)[S]/(1 + [S]/Km) to V versus [S] data, where the ratio Vmax/Km is treated as a single curve-fitting parameter, or the approximation v = (Vmax/Km)[S]/(S) to the linear portions of the activity versus [S] profiles.

Results

The metabolism of rofecoxib was investigated using human liver microsomal, S9, or cytosolic fractions under a variety of redox conditions (Table 1) to establish the likely origin and interrelationships of the observed products.

Oxidative Metabolism by Microsomal Preparations. Under con-
Reactions and kinetics of rofecoxib and 5-hydroxyrofecoxib metabolism by human liver S9, cytosol, and dialyzed cytosol

<table>
<thead>
<tr>
<th>Subcellular Fraction</th>
<th>Cofactors</th>
<th>Metabolites</th>
<th>$K_{m, app}$</th>
<th>$V_{max, app}$</th>
<th>Apparent $V_{max}/K_{m}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$\mu M$ $\pm$ S.E.</td>
<td>pmol $\cdot$ mg protein$^{-1}$ min$^{-1}$</td>
<td>nL $\cdot$ mg protein$^{-1}$ min$^{-1}$</td>
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<tr>
<td>Oxidative metabolism of rofecoxib</td>
<td>Microsomes</td>
<td>NADPH-egs</td>
<td>5-HydroxyR</td>
<td>Not determined</td>
<td>218 $\pm$ 3</td>
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<tr>
<td></td>
<td>S9</td>
<td>NADPH-egs</td>
<td>5-HydroxyR</td>
<td></td>
<td>81 $\pm$ 1</td>
</tr>
<tr>
<td></td>
<td>S9</td>
<td>NAD$^+$, ATP</td>
<td>5-HydroxyR</td>
<td></td>
<td></td>
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<tr>
<td>Formation of dihydro-5-hydroxyrofecoxib from rofecoxib</td>
<td>Dialyzed cytosol</td>
<td>None</td>
<td>DH-5-hydroxyR</td>
<td>224 $\pm$ 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytosol</td>
<td>None</td>
<td>DH-5-hydroxyR</td>
<td>364 $\pm$ 8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S9</td>
<td>None</td>
<td>DH-5-hydroxyR</td>
<td>181 $\pm$ 3</td>
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<td>Reductive metabolism of rofecoxib</td>
<td>Cytosol</td>
<td>NADPH, malic acid</td>
<td>erythro-DHHA</td>
<td>90 $\pm$ 21</td>
<td>5.6 $\pm$ 0.9</td>
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<tr>
<td></td>
<td>S9</td>
<td>NADPH, malic acid</td>
<td>threo-DHHA</td>
<td>214 $\pm$ 40</td>
<td>33.1 $\pm$ 5.2</td>
</tr>
<tr>
<td>Back reduction of 5-hydroxyrofecoxib to rofecoxib</td>
<td>Cytosol</td>
<td>NADPH, malic acid</td>
<td>Rofecoxib</td>
<td>164 $\pm$ 21</td>
<td>35.4 $\pm$ 3.7</td>
</tr>
<tr>
<td></td>
<td>S9</td>
<td>NADPH, malic acid</td>
<td>Rofecoxib</td>
<td>32 $\pm$ 2</td>
<td></td>
</tr>
<tr>
<td>Comprehensive time course of VIOXX in vitro metabolism</td>
<td>S9</td>
<td>NADPH, NAD$^+$, malic acid</td>
<td>All (5-OH-rofecoxib; dihydro-5-OH-R; erythro- and threo-DHHA)</td>
<td>Used for time course only</td>
<td></td>
</tr>
</tbody>
</table>

* Independent values for $K_{m, app}$ and $V_{max, app}$ were determined by nonlinear regression of data to the Michaelis-Menten equation.  
 b,c Apparent $V_{max}/K_{m}$ values were determined by linear regression data to $v = (V_{max}/K_{m})[S]$ (b) or nonlinear regression to $v = (V_{max}/K_{m})(1 + [S]/K_{m})$ (c) (see Materials and Methods). Results are expressed as $\pm$ standard error from curve fit.  
 d NADPH-egs, NADPH-regenerating system (see Materials and Methods).

Ditions favoring oxidative metabolism, rofecoxib was converted almost exclusively to a single product, identified previously by UV, MS, and NMR analysis as 5-hydroxyR (Halpin et al., 2000). Several human recombinant cDNA-derived P450 isoforms, most notably CYP1A2 and 3A4, were found capable of catalyzing that transformation (Fig. 1). Inhibition of the microsomal oxidation of rofecoxib to 5-hydroxyR by 1 mM ketonazole and 10 mM fluvoxamine (60% and 30%, respectively, in the presence of 10 mM rofecoxib) indicated substantial, but not exclusive roles for CYP3A4/5 and 1A2. Minimal inhibition by quinidine and sulfaphenazole under the same conditions supported reaction represents at least a two-step process, with one step catalyzed by a microsomal enzyme and the other by a cytosolic enzyme. In one comparative experiment using S9, the apparent rate of this non-P450-dependent oxidase activity was about 60% of that of the NADPH-dependent P450 activity.

**Reductive Metabolism by Human Liver Cytosolic Fractions.** Incubations of rofecoxib (100 μM) with cytosolic fractions in the presence of an NADPH-regenerating system gave metabolic profiles which were characterized mainly by the formation of reduced species, namely, the isomeric DHHA species and trans-DHR (data not shown).

The biotransformation of rofecoxib to the above reduced metabolites by cytosol was dependent upon the presence of NADPH and functional enzymes (boiled preparations were inactive). Incubation of rofecoxib with dialyzed cytosol (lacking NADPH) produced one major and one minor metabolite (Fig. 3A), which were identified as isomers of DH-5-hydroxyR (see below). When NADPH was added to the incubation mixture, the DH-5-hydroxy metabolites were not detected, but instead, the isomeric DHHA analogs were formed (Fig. 3B). When the major isomer of DH-5-hydroxyR (denoted DH-5-hydroxyRα) was isolated and then incubated with cytosol fortified with NADPH and malic acid, erythro- and threo-DHHA metabolites were produced in a time-dependent manner (Fig. 4). When 5-hydroxyR was incubated with liver cytosolic fractions in the presence or absence of NADPH, DH-5-hydroxyR was not formed (data not shown).

**Identification of 3,4-Dihydro-5-hydroxyrofecoxib (DH-5-HydroxyR).** As noted above, incubations of rofecoxib with dialyzed cytosol produced one major and one minor metabolite (Fig. 3A),
Semipreparative scale incubations generated a quantity of the major metabolite (DH-5-hydroxyR) which, after further HPLC purification, was sufficient for structure elucidation. This metabolite was characterized as DH-5-hydroxyR by the combined application of UV, LC/MS-MS, and NMR spectroscopy. Since the proposed structure contains three chiral centers, a total of eight stereoisomeric forms (enantiomeric pairs of four diastereomers) are possible.

The UV spectra of both the major and minor isomers were closely related to those of DHHA and DHR, but were different from those of rofecoxib and 5-hydroxyR (spectra not shown). The most distinctive difference was a loss of absorbance above \( \lambda \approx 250 \) nm, consistent with a loss of conjugation between the two aromatic rings due to saturation of the furanone 3,4-double bond.

The LC/MS-MS analysis of both isomers afforded a strong negative ion spectrum.
ion signal at \( m/z \) 331 in the Q1 profile (Fig. 5, A and C), which was consistent with the molecular anion \((M - H)^{-}\) of the proposed structure. The ion at \( m/z \) 349 resulted from addition of the elements of \( H_2O \) to the \( m/z \) 331 species, either in the form of a water cluster ion or the molecular species generated by hydration of the aldehyde tautomer that would form upon ring opening of the cyclic hemiacetal in the proposed structure. A fragmentation scheme to rationalize the spectra obtained upon CID of \( m/z \) 331 (Fig. 5, B and D) is shown in Fig. 6. Although the mass spectral data are consistent with the proposed structure, NMR data were required to show that the oxygen atom derived from the net hydration process was present at the 5- rather than the 4-position of the furanone ring.

The major metabolite (DH-5-hydroxyR\(_2\)) was analyzed by \(^1^H\) NMR in acetone-\(d_6 \) (Fig. 7). The most informative spectrum, which was taken at 0°C, revealed four chemical entities (labeled a–d), each of which had a series of three coupled methine protons, consistent with hydroxylation at the 5-, but not at the 3- or 4-positions, of the furanone ring. The most abundant of these forms is labeled a. The doublets between 5.5 and 6.2 ppm were assigned to the methine proton at position 5 (E, hemiacetal proton, if the ring is closed); consistent with an anticipated larger downfield shift than for F or G, those between 4.4 and 4.8 ppm were assigned to the methine proton at position 3 (G), and the double doublets between 3.8 and 4.1 ppm were assigned to the methine proton at position 4 (F). For two of the compounds (a and d), the methine proton bands broaden as the temperature is increased, indicating that the coupling interactions for those protons are unstable. At 50°C, the methine proton signals from a and d coalesce completely (data not shown), indicating that a and d interconvert rapidly with one another at that temperature. The exchanging pair, a and d, constituted about 75% of the total isolate. The postulated structures for DH-5-hydroxyR metabolites suggest a reasonable mechanism for an interconversion, namely, rapid opening and closing of the hemiacetal functionality. The methine protons of b and c do not show temperature-dependent peak broadening. The \( jj \) coupling constants for the EF couplings of a and d (7.3 and 4.5 Hz, respectively) are less than those for b and c (9.3 and 9.4 Hz, respectively), indicating that the dihedral angles between protons E and F are smaller in a and d than they are in b and c. The EF coupling constants for b and c are nearly identical. These data are consistent with the relative orientations of the E and F protons on a and d being more constrained than they are for b and c. Thus, a and d may be in a cyclic hemiacetal form, whereas b and c may be in ring-open form, but perhaps not as free aldehydes since downfield shifts of the E proton signals for b and c are much less than would be expected for free aldehydes. Conversely, since the \(^1^H\) NMR signals for b and c are thermostable, it appears unlikely that those compounds are cyclic hemiacetals, although their structures remain unknown. The larger EF coupling constant for a than for d is consistent with respective \( trans \) and \( cis \) configurations between protons E and F for those species. All four of the FG coupling constants are large (12.6 Hz for a, 12.9 Hz for b–d). For a and d, the putative cyclic hemiacetals, large FG coupling constants are consistent with a \( trans \) configuration between protons F and G. For b and c, which might be ring-opened forms, the large FG couplings merely indicate that the dihedral angles between those protons are large. In summary, the LC/MS-MS and \(^1^H\) NMR data collectively indicate that a and d are 3,4-\( trans \)-dihydro analogs of

![Fig. 5. Mass spectra of purified 3,4-dihydro-5-hydroxyrofecoxib isomers obtained under LC/MS conditions. A and B, conventional spectra; C and D, product ion spectra taken after CID of \( m/z \) 331.](image-url)
5-hydroxyR and that a, the more abundant species, also exhibits 4,5-trans stereochemistry, whereas d is the corresponding 4,5-cis isomer.

**Reductive Metabolism of 5-Hydroxyrofecoxib (5-HydroxyR).** 5-HydroxyR was found to undergo reversible metabolism to rofecoxib in vivo in rats (Baillie et al., 2001) and to a lesser extent in the dog and human (Halpin et al., 2002). In light of this observation, incubations of 5-hydroxyR were performed with human liver subcellular fractions to further characterize metabolism of 5-hydroxyR at the biochemical level. Initially, 5-hydroxyR (10 μM) was incubated with microsomal or cytosolic fractions in the presence of an NADPH-regenerating system for 4 h. The resulting HPLC profile revealed the formation of only a single product, which was identified as rofecoxib (data not shown). There was no evidence for the formation of either DH-5-hydroxyR or the DHHA metabolites. The biotransformation of 5-hydroxyR to rofecoxib was catalyzed primarily by cytosolic enzymes (4 pmol/mg protein/min) rather than by microsomal enzymes (0.16 pmol/mg protein/min). No activity was observed when incubations were carried out in the absence of NADPH or with boiled cytosol. Dicoumarol (100 μM), a quinone reductase inhibitor, reduced this activity by about 65%, whereas quercitrin, an inhibitor of carbonyl reductase, was less effective (about 25% suppression). Ketoconazole, SKF-525A (proadifen), and 4-methylpyrazole, all at 100 μM, had no effect on the cytosolic reductase activity.

**Kinetics of Rofecoxib Metabolism.** Kinetic aspects of rofecoxib metabolism were examined in detail using human liver preparations. Each metabolic activity (oxidation, reduction, and the back reduction of 5-hydroxyR to rofecoxib) was examined in three ways: 1) using a subcellular fraction and choice of redox cofactor that would best support the activity of interest; 2) using S9 and the same choice of redox cofactor as in strategy 1, so that the kinetic properties of different pathways could be compared using the same fraction; and 3) using S9 and a combination of redox cofactors that would allow all of the activities to be present simultaneously. In initial experiments (data not shown), each metabolic activity was examined as a function of incubation time and protein concentration at a substrate concentration

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**Fig. 6. Proposed negative ion CID MS-MS fragmentation scheme for isomers of 3,4-dihydro-5-hydroxyrofecoxib.**

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of 100 μM. From these data, optimal incubation times and protein concentrations for each reaction were selected (Table 1) for the determination of kinetic parameters. Solubility limits precluded using rofecoxib concentrations in excess of 100 μM in incubations with subcellular fractions. A more conservative limit of 60 μM was chosen for the activity versus substrate concentration studies. Activity versus substrate concentration profiles are shown in Fig. 8 for the oxidation, reduction, back reduction, and net hydration pathways using strategy 1. Kinetic parameters calculated from activity versus [S] for strategies 1 and 2 are shown in Table 1. Only in the case of the reduction of rofecoxib by cytosol was there enough curvature in the activity versus [S] curves (Fig. 8) to calculate reliable independent apparent values for $K_m$ and $V_{max}$ (nonlinear regression of Michaelis-Menten equation to data). The smallest apparent $K_m$ value was 90 μM for the formation of erythro-DHHA by cytosol. It is presumed that apparent $K_m$ values for all other metabolic activities would be > 90 μM. Thus, it would be expected that clinically relevant concentrations of rofecoxib would be $<< K_m$ in all cases, such that rates of metabolism would be governed by $V_{max}/K_m$. For all activities studied, it was possible to estimate apparent $V_{max}/K_m$ values from the linear portions of the activity versus [S] curves (Fig. 8). In S9, where all activities were expressed, the intrinsic rate of oxidation of rofecoxib to 5-hydroxyR appeared to be substantially greater than that for the reduction of rofecoxib to the DHHA metabolites. The apparent $V_{max}/K_m$ for the two oxidative pathways combined was 298 nmol·mg protein$^{-1}$ min$^{-1}$, whereas that for the formation of erythro- and threo-DHHA was 80 nmol·mg protein$^{-1}$ min$^{-1}$. However, the intrinsic rate of back reduction of 5-hydroxyR to rofecoxib in S9 was even greater (apparent $V_{max}/K_m$ = 388 nmol·mg protein$^{-1}$ min$^{-1}$) than the intrinsic rate of oxidation of rofecoxib. These data would predict that, under conditions where all activities are expressed simultaneously in S9, 5-hydroxyR would be the most abundant metabolite at early time points, only to be overtaken by the DHHA metabolites at later times, due to the impact of the back reduction pathway. This pattern is exactly what was observed experimentally. Figure 9 shows a time course for the metabolism of rofecoxib by S9 in the presence of NAD$^+$. NADPH, and malic acid, conditions under which all metabolic activities are operating. Under the conditions used, <5% of the initial rofecoxib substrate was consumed during the course of the incubations. Under these conditions, 5-hydroxyR and DH-5-hydroxyR would be expected to approach steady-state concentrations, whereas erythro- and threo-DHHA accumulation should be near linear with time. Accordingly, for Fig. 9, the equation $[M] = k_1[S] + t$, which describes the linear accumulation of metabolite ([M]) after a lag time ($t_0$), was fit to the erythro- and threo-DHHA data, whereas $[M] = k_2[S]/k_3(1 - e^{-k_3t})$, which describes the development of a steady state, where $k_2$ and $k_3$ are, respectively, rate constants for the formation and elimination of a metabolite approaching steady state, was fit to the 5-hydroxyR and DH-5-hydroxyR data. In each case, the correlation between experimental and theoretical was high. The in vitro metabolism pathways of rofecoxib are shown in Fig. 10.

**Lactone Ring Opening of cis- and trans-DHR.** Figure 11 shows time course data for the hydrolysis of cis- and trans-DHR to the DHHA metabolites by cytosol in the absence of added cofactor. The HPLC conditions used did not separate erythro- and threo-DHHA. Although both DHR isomers underwent facile hydrolysis, that of trans-DHR was substantially more rapid than that of cis-DHR. In incubations with S9 in the absence of added cofactor, approximately 84% of each DHR isomer was transformed to the DHHA in 4 h. When boiled S9 was used, these values decreased to 5%. These data indicate that the biotransformation is efficient, enzymatic, and cofactor independent, and that the ring-opened DHHA species are favored thermodynamically over the cyclic DHR substrates under the conditions used.

**Discussion**

Metabolism of rofecoxib by human liver subcellular fractions was found to proceed via a number of pathways which were both oxidative and reductive in nature. One product, 5-hydroxyR, was found to undergo cytosolic reduction back to rofecoxib. A “non-redox” process, by which the elements of water were added to rofecoxib, yielded at least two 3,4-dihydro analogs of 5-hydroxyR. Detailed examination of the rofecoxib 5-hydroxylation pathway revealed that two mechanisms contributed to the formation of the product, one NADPH-dependent in microsomes and the other NAD$^+$-dependent in S9 fractions. The observed reductive pathway (leading to DHHA isomers) was found to be an NADPH-dependent cytosolic process, which may involve multiple enzyme-catalyzed steps. The DH-5-hydroxyR species were formed in incubations of rofecoxib with dialyzed hepatic cytosol in the absence of added cofactor. Although DH-5-hydroxyR may not be an obligatory intermediate in the reduction of rofecoxib to the terminal DHHA metabolites, it was nevertheless reduced rapidly to those metabolites when incubated with NADPH-fortified human liver cytosol.

Available data suggest that the NADPH-dependent formation of 5-hydroxyR in liver microsomes and S9 fractions is mediated by cytochrome P450 enzymes (particularly CYP3A4/5 and 1A2). Several examples can be found in published literature of inferred or demonstrated hydroxylation of the carbon α- to an ester linkage, a number of which lead to ester cleavage (Guengerich et al., 1988; Funaki et al., 1989; Scherling et al., 1992; Dunkov et al., 1997), due to the collapse of the resulting hemiacetal functionality. In cases where the ester is cyclic, i.e., in the form of a lactone (Chauret et al., 1995; Holmes et al., 1995; Delorme et al., 1996; Jacobs and Metzler, 1999), the product of α-carbon hydroxylation is stable and can be observed directly. Presumably, the lactone ring exists in equilibrium with the corresponding open-chain aldehyde in such cases, but the cyclic tautomer is favored due to a combination of steric and thermodynamic factors. In one case involving the hydroxylation of a noncyclic ester, the hydroxy ester functionality remained intact (Gelderblom et al., 1999).

In the specific example of 5-hydroxyR, steric constraints imposed by the furanonyl 3,4-double bond would limit movement of the carboxyl group during hydroxylation, thereby preventing the formation of the corresponding open-chain aldehyde. This suggests that the cyclic lactone is the major metabolite formed in cases where hydroxylation occurs at the carbon α- to an ester linkage.
and aldehyde carbonyl groups away from one another, increasing the probability of recyclization to the point that the open form, though it might exist as a transient species, is not actually observed.

The formation of 5-hydroxyR by the NADH-dependent pathway observed in S9 is more intriguing. The requirement for both microsomal and cytosolic contributions suggests that at least two enzymatic steps are involved in this process, one microsomal and one cytosolic. Lactone cleavage through esterase activity would yield a hydroxy acid highly constrained by the retention of the rofecoxib furanonyl 3,4-double bond (Scheme 1A). NADH-dependent oxidation of the exposed hydroxyl group would result in an aldehyde acid that could close spontaneously to form 5-hydroxyR as described above. Conceptually, this mechanism would be a reversal of that proposed for the back reduction of 5-hydroxyR to rofecoxib (Scheme 1B; Baillie et al., 2001). It seems reasonable that oxidation of rofecoxib by this pathway would be NADH-dependent, whereas the back reduction would be NADPH-dependent, since liver cytoplasmic pools of NAD exist primarily in the oxidized form (NAD\(^+\)) and NADP pools exist primarily in the reduced form (NADPH). Additional evidence for the reversible opening of the furanone rings of rofecoxib and 5-hydroxyR derives from in vivo studies in which rats were dosed with [1,2-\(^{18}\)O]rofecoxib or [5-\(^{18}\)O]5-hydroxyR (Baillie et al., 2001), and the fate of the \(^{18}\)O atoms was followed by LC/MS analysis. These studies provided strong evidence that the furanone ring undergoes reversible opening in vivo through both hydrolytic and redox processes.

Hydrolytic opening of the furanone ring also presents an attractive mechanism for the reduction of rofecoxib to the DHHA metabolites (Scheme 2). According to this mechanism, the initial \(\gamma\)-hydroxy-\(\alpha,\beta\)-enoic acid product of hydrolysis rearranges to form a saturated \(\gamma\)-aldehyde acid that is reduced directly to the isomeric DHHAs. An example of the isomerization of a \(\gamma\)-hydroxy-\(\alpha,\beta\)-unsaturated ketone to a saturated 1,4-diketone, analogous to the rearrangement proposed above, may be found in a report on the in vitro metabolism of norethindrone (Cook et al., 1974) by beagle dog liver homogenate. In those studies, it was shown that 6\(\beta\)-hydroxynorethindrone, a \(\gamma\)-hydroxy-\(\alpha,\beta\)-unsaturated ketone, isomerizes to its 5\(\alpha\)-H diketone derivative, a saturated 1,4-diketone, either spontaneously under acidic conditions or in the presence of liver homogenate at physiological pH without added cofactor. As discussed above, hydrolysis of the furanone ring of rofecoxib, an event that may initiate both the reduction

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**Fig. 8.** Metabolism of rofecoxib and 5-hydroxyrofecoxib by human liver S9 or cytosol as a function of substrate concentration.

Data points are in duplicate for each time point and all data points are shown. Apparent single point for a given time is due to close overlay between duplicates.
of rofecoxib to the DHHAs and the NAD$^+$-dependent oxidation of rofecoxib to 5-hydroxyR, likely would be an enzyme-catalyzed process. Similarly, the work on norethindrone suggests that the isomerization of the γ-hydroxy-α,β-enoic acid hydrolysis product of rofecoxib to the saturated γ-aldehyde acid also would be enzymatic. Thus, the reductive step in the biotransformation of rofecoxib to the DHHAs may be preceded by at least two other enzyme-catalyzed steps. According to this proposed mechanism for the reduction of rofecoxib to the DHHAs, the DH-5-hydroxyR species do not represent obligatory intermediates. Instead, they are formed reversibly as side products, resulting from the spontaneous cyclization of the aldehyde acid. The observation that reduction of the DH-5-hydroxyR species to the DHHAs is much faster than the reduction of rofecoxib to the DHHAs suggests that the reductive step is not rate-limiting in the overall process.

An alternative mechanism for the reduction of rofecoxib to the DHHAs involves direct reduction of the furanone 3,4-double bond to form dihydrolactone analogs of rofecoxib (DHRs), followed by lactone hydrolysis (Scheme 3). Incubations of synthetic DHR species with liver cytosol showed near complete hydrolysis (>80%) to the

![Fig. 9. Metabolism of rofecoxib in human liver S9 fortified with NADPH, NAD$^+$, and malic acid.](image1)

A, threo-DHHA; B, erythro-DHHA; C, 5-hydroxyR; D, DH-5-hydroxyR. A single data point was collected for each metabolite for each time point. The equation $[M] = [S] + k_{i}t$ was fit to threo- and erythro-DHHA traces and $[M] = k_{i}[S]e^{(1 - e^{-kt})}$ was fit to the 5-hydroxyR and DH-5-hydroxyR data (see Materials and Methods). Correlation coefficients ($R^2$) for the curve fits were, respectively, 0.99, 0.99, and 0.97 for A, B, C, and D. Less than 5% of substrate was consumed during the course of this experiment.

![Fig. 10. Biotransformation pathways of rofecoxib in vitro.](image2)

![Fig. 11. Hydrolysis of cis- and trans-DHR to the DHHA metabolites by human liver cytosol.](image3)

Incubations (100 μM substrate, 5 mg of human liver cytosolic protein/ml, and no added cofactors) were conducted at 37°C. *, HPLC conditions used to analyze these samples did not separate erythro-DHHA and threo-DHHA.
DHHA over a period of 4 h. This observation indicates that, under the incubation conditions used (aqueous, neutral pH), hydrolysis to yield the DHHAs was kinetically highly favored over re-condensation to yield the DHRs. Thus, if this direct reduction/hydrolytic mechanism for the reduction of rofecoxib to the DHHAs were operative under the incubation conditions used for these studies, it would likely go undetected. The artifactual condensations of the DHHA metabolites to the DHR species mentioned under Materials and Methods occurred under acidic conditions (often in the presence of organic solvent), where the equilibrium may be shifted in favor of the DHR species. Literature examples of the direct reduction of carbon-carbon double bonds in small heterocyclic rings include various pyrimidines (Wasternack, 1980; Porter et al., 1991; Lu et al., 1992), tetramethrin isomers (Tomigahara et al., 1994), and the tobacco alkaloid \( \text{H}9252 \)-nicotyrine (Liu et al., 2000). Butyrolactone hydrolysis has been observed in numerous instances (Sadee et al., 1972; Clark and Kalman, 1974; Peters et al., 1978; Reuning et al., 1985; Sai and Gorrod, 1995; Verite et al., 1996; Cook et al., 2000). Canrenone, a spiranolactone, was shown to interconvert rapidly with its ring-opened hydroxy acid counterpart (Sadee et al., 1972), and both the opening and closing of the lactone ring appeared to be enzymatic. Analogous to rofecoxib, the furanone ring of digoxin (Heinz and Flasch, 1978) and the pyranone ring of 5-methoxypsoralen (John et al., 1992) have been found to undergo reduction and hydrolytic ring opening, but it is not known whether there is an obligatory order to the two events.

The present in vitro kinetic studies on the metabolism of rofecoxib in human liver subcellular fractions provided valuable insight into likely mechanisms by which rofecoxib undergoes metabolism in human subjects in vivo. Although net reductive metabolism of rofecoxib predominates over oxidative metabolism in liver S9 (as it does in vivo) after 24 h, initial rate kinetic data on the individual pathways in appropriate subcellular fractions favored the opposite. The ultimate net predominance of reductive over oxidative metabolism of rofecoxib in human liver S9 appeared to result from limitations on the amount of 5-hydroxyR that can accumulate due to the facile reduction of this metabolite back to rofecoxib.

In conclusion, the present detailed in vitro studies on the metabolism of rofecoxib by human hepatic subcellular fractions have provided insights into the complexity of the corresponding pathways by which this drug undergoes biotransformations in vivo in humans.
Oxidative (NADPH- and NAD+ -dependent), reductive, back reductive, and non-redox reactions all appear to contribute to the metabolic fate of rofecoxib in such a way that the profile of biotransformation products is dominated by compounds in which the five-membered lactone ring system has undergone change. In this respect, the results of these in vitro experiments are consistent with the findings of previous in vivo studies which highlighted the role of the lactone moiety in dictating the metabolic fate of rofecoxib in preclinical species and humans (Baillie et al., 2001; Halpin et al., 2002; Halpin et al., 2000; Porras et al., 2003).

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