STUDIES ON THE METABOLISM OF THE NOVEL, SELECTIVE CYCLOOXYGENASE-2 INHIBITOR INDOMETHACIN PHENETHYLAMIDE IN RAT, MOUSE, AND HUMAN LIVER MICROSONES: IDENTIFICATION OF ACTIVE METABOLITES

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ABSTRACT:
The metabolism of 2-[1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl]-N-phényethylacetamide (indomethacin phenethylamide, LM-4108), a highly selective cyclooxygenase-2 inhibitor, was studied in rat, mouse, and human liver microsomes. The primary site of oxidation in all species examined was on the methylene carbons of the phenethyl side chain to form the 1'- and 2'-hydroxy and 2'-oxo metabolites as determined by electrospray ionization liquid chromatography-tandem mass spectrometry. Half-lives for the disappearance of 10 μM LM-4108 in rat, human, and mouse liver microsomes (0.15 pmol P450/ml) were 11 min, 21 min, and 51 min, respectively. Indomethacin formation was not observed in incubations with rat, mouse, or human liver microsomes. Both the 2'-hydroxy-LM-4108 and 2'-oxo-LM-4108 metabolites were synthesized and found to be equipotent to the parent compound with regard to COX-2 inhibitory potency and selectivity [2'-hydroxy-LM-4108: IC50(COX-2) = 0.06 μM, IC50(COX-1) >66 μM; 2'-oxo-LM-4108: IC50(COX-2) = 0.05 μM, IC50(COX-1) >66 μM]. The formation of the metabolites was strongly inhibited by specific CYP3A4 inhibitors ketoconazole and troleandomycin but not by other isofrom-selective inhibitors. These findings were confirmed by demonstrating that cloned, expressed CYP3A4 catalyzed side chain oxidation. O-Demethylation was a minor oxidative pathway in contrast to the metabolism of indomethacin and was catalyzed by CYP2D6. Upon intravenous administration of LM-4108 to Sprague-Dawley rats, oxidative metabolism on the phenethyl side chain constituted the rate-limiting steps in its clearance. The active metabolites, 2'-oxo- and 2'-hydroxy-LM-4108, as well as 1'-hydroxy-LM-4108, were all observed in rat plasma and thus may contribute to COX-2 inhibition in vivo. The glucuronides of 2'-hydroxy-LM-4108 and O-desmethyl-2'-hydroxy-LM-4108 were also identified in rat bile.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used agents in the treatment of pain and inflammation.

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1 Abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; LM-4108, 2-[1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl]-N-phényethylacetamide or indomethacin phenethylamide; 1'-hydroxy-LM-4108, 1'-hydroxy-indomethacin-phenethylamide; 2'-hydroxy-LM-4108, 2'-hydroxy-indomethacin-phenethylamide; 2'-oxo-LM-4108, 2'-oxo-indomethacin-phenethylamide; LM-4106, 1'-methyl-indomethacin-phenethylamide; LM-5105, indomethacin-[p-chloro]-phenethylamide; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; ESI, electrospray ionization; CID, collision-induced dissociation; P450, cytochrome P450; TLC, thin-layer chromatography; LC/MS/MS, liquid chromatography/tandem mass spectrometry; Rt, retention time.

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inhibition of COX-1 and COX-2. The COX-2 hypothesis has been validated in animal models of inflammation and in human clinical trials with inhibitors such as celecoxib (McKenna et al., 2002), rofecoxib (Weaver, 2001), valdecoxib (Ormorod et al., 2002), etoricoxib (Gottfriedi et al., 2002), and lumiracoxib (Sorbera et al., 2002), which are selective COX-2 inhibitors.

We recently reported a biochemically based strategy for the facile conversion of carboxylic acid-containing NSAIDs such as indomethacin (Kalgutkar et al., 2000a,b; Kozak et al., 2002) and meclofenamic acid (Kalgutkar et al., 2000a, 2002) to selective COX-2 inhibitors. Derivatization of these compounds to esters or amides produces molecules capable of binding tightly to COX-2 but not COX-1. A variety of different amide or ester analogs of indomethacin have been synthesized, and many of these compounds were found to be highly selective and potent COX-2 inhibitors. 2-[1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1-

Fig. 1. Structures of indomethacin, indomethacin phenethylamide (LM-4108), and two internal standards (LM-4106 and LM-5105).

Materials, Methods

Materials. LM-4108 was synthesized by carbodiimide coupling of phenethylamine to indomethacin as described previously (Kalgutkar et al., 2000b). Furafylline, indomethacin, ketocazone, quercetin, quinidine, sulfaphenazole, ticlodipine, and treoleandomycin were purchased from Sigma-Aldrich (St. Louis, MO). O-Desmethyl indomethacin was a gift from Merck Research Laboratories (West Point, PA). Melting points were determined with a Gal- lenkamp melting point apparatus (Sanyo Gallenkamp, Loughborough, UK) and are uncorrected. Chemical yields are nonoptimized examples of a synthetic procedure. All chemicals were purchased from Aldrich Chemical Co. (Mil- waukee, WI). Methylene chloride was purchased as “anhydrous” from Aldrich and was used as received. All other solvents were HPLC grade. Silica gel (Fisher Scientific Co., Fair Lawn, NJ; 60–100 mesh) was used for column chromatography. 1H NMR spectra in CDCl3 or DMSO-d6 were recorded on a Bruker WP-360 or AM-400 spectrometer (Bruker BioSpin Corporation, Bil- lerica, MA); chemical shifts are expressed in ppm (δ) relative to tetramethyl- silane as internal standard. Spin multiplicities are given as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), bt (broad triplet), q (quartet), and m (multiplet). Coupling constants (J) are given in hertz (Hz). Positive-ion electrospray ionization (ESI) and collision-induced dissociation (CID) mass spectra were obtained on a Fison Finnigan TSQ 7000 mass spectrometer (Thermo Finnigan, MAT GmbH, Bremen, Germany). CID fragmentations were consis- tent with assigned structures. The purity of products was judged to be at least 99% on the basis of their chromatographic homogeneity. Human liver micro- somes (pooled from 15 donors) and mouse liver microsomes were purchased from Intravitus Technologies Inc. (Baltimore, MD). Uninduced and phenobar- bital-induced rat liver microsomes (80 mg/kg sodium phenobarbital i.p. for 4 days) were prepared by differential centrifugation (Guingenberger, 1994). Microsomes were stored at −80°C in glycerol/10 mM Tris-acetate buffer, pH 7.7 (2.8) containing 1 mM EDTA. Protein content was determined by a bicine- ninic acid protein assay with bovine serum albumin as the protein standard (Pierce Chemical, Rockford, IL). Total cytochrome P450 content was deter- mined from the dithionate-reduced CO-binding spectrum (Hewlett-Packard 8452A diode array spectrophotometer) after solubilization of the microsomes (1 mg protein/ml) in 1% sodium cholate, 1% Emulgen 911 (Omurra and Sato, 1986).

(2S)-2-[1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl]-N-(2′- hydroxy-2′-phenyl-ethyl)-acetamide (2′-hydroxy-LM-4108). To a solution of indomethacin (1.0 g, 2.8 mmol) in 25 ml of anhydrous methylene chloride was added triethylamine (780 μl, 5.6 mmol) and bis(2-oxo-3-oxazolidi- nyl)phosphinic chloride (710 mg, 2.8 mg mol). After stirring at room tempera- ture for 10 min. (2S)-2-amino-1-phenylethanol (765 mg, 5.6 mmol) was added. The solution was stirred at room temperature for an additional 6 h and then diluted with methylene chloride. The organic solution was washed with satu- rated sodium bicarbonate, saturated ammonium chloride, and water and then dried (magnesium sulfate), filtered, and concentrated in vacuo. The crude product was subjected to silica gel chromatography (1:1 and then 2:1 ethyl acetate/hexanes) followed by filtration over a silica plug with ethyl acetate to furnish 950 mg of 2′-hydroxy-LM-4108 as white solid (71%). Melting point = 158–160°C; ESI-MS m/z calculated for C37H32ClN2O4 (MH+) 747, found 747. CID fragmentation: 459, 312, 174, 139, and 120. 1H NMR (CDCl3) δ 7.62–7.65 (d, 2H, J = 8.4 Hz, ArH), δ 7.47–7.49 (d, 2H, J = 8.4 Hz, ArH), 7.24–7.26 (m, 5H, ArH), 6.86–6.89 (m, 2H, ArH), 6.69–6.73 (dd, 1H, J = 9.0 and 2.5 MHz, ArH), 5.92 (bt, 1H, NH), 4.76–4.81 (m, 1H, CH2), 3.82 (s, 3H, CH3), 3.58–3.69 (m, 3H, CH2 + CH2), 3.29–3.38 (m, 1H, CHH), 3.07–3.08 (d, 1H, J = 3.7 Hz, OH), 2.32 (s, 3H, CH3).

(2S)-2-[1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl]-N-(2′- oxo-2′-phenyl-ethyl)-acetamide (2′-oxo-LM-4108). To a solution of indomethacin (1.0 g, 2.8 mmol) in 25 ml of anhydrous methylene chloride was added 2-aminoacetophenone hydrochloride (530 mg, 3.1 mmol), 1-[3-(dim-
Selective Inhibitors.

methylene chloride. The organic solution was dried (anhydrous sodium sulfate) and concentrated in vacuo. The crude amide was subjected to silica gel chromatography (3:2 ethyl acetate/hexanes) to furnish 1.2 g of 2’-oxo-LM-4108 as a pale yellow solid (87%). Melting point = 167–169°C; ESI-MS m/z calculated for C_{27}H_{23}ClN_{2}O_{3} (MH^+) = 475, found 475. CID fragmentation: 457, 312, 270, 174, 139, and 136. ^1 H NMR (CDCl3) δ 7.69–7.92 (d, 2H, CH), 7.37–7.5 (m, 5H, ArH), 6.98–6.75 (m, 2H, ArH), 6.72–6.57 (m, 2H, ArH + NH), 4.73 (s, 2H, CH2), 3.85 (s, 3H, CH3), 3.73 (s, 2H, CH2), 2.40 (s, 3H, CH3).

2-[4-Chlorobenzoyl]-5-methoxy-2-methyl-1-(4-indol-3-yl)N-steryl-acetamide (50 mg) and approximately 5 mg of p-toluene sulfonic acid monohydrate in 10 ml of anhydrous dimethyl sulfoxide under argon was heated to 160°C and stirred for 1 h at that temperature. The solution was then cooled to room temperature, diluted with ethyl acetate, and washed twice with water. The organic solution was dried (magnesium sulfate), filtered, and concentrated in vacuo. The dehydrated product was obtained after silica gel chromatography (1:9, then 1:3 ethyl acetate/hexanes) as a pale yellow solid (85%). ^1 H NMR (CDCl3) δ 7.66–7.69 (d, 2H, J = 8.5 Hz, ArH), 7.40–7.53 (m, 3H, ArH + CH), 7.13–7.30 (m, 6H, ArH + NH), 6.87–6.94 (m, 2H, ArH), 6.70–6.76 (m, 1H, ArH), 5.92–5.97 (d, 1H, J = 14.4 Hz, CH2), 3.82 (s, 3H, CH3), 3.75 (s, 2H, CH2), 2.43 (s, 3H, CH3); ESI-MS m/z calculated for C_{27}H_{23}ClN_{2}O_{3} (MH^+): 459, found 459. CID fragmentation: 312, 139, and 120.

Metabolism of Indomethacin Phenethylamide

Microsomes. Microsomes were incubated with 10 μM LM-4108 in 50 mM sodium phosphate buffer, pH 7.4, in the presence of 1 mM NADPH. Protein concentrations ranged from 0.125 to 0.5 mg/microsomal protein/mL. Results from the different species were normalized to a P450 standard (LM-5105 or LM-4106; see Fig. 1). LM-4108 and its metabolites were separated by reversed phase HPLC on either a 3.5-μm Luna C8 column, 150 × 3.0 mm (Agilent Technologies, Palo Alto, CA) or a 3-μm Luna C8 column, 150 × 2.0 mm (Phenomenex, Torrance, CA) with a mobile phase of acetonitrile/20 mM ammonium acetate, pH 4.0 (55:45) at flow rates of 0.5 ml/min or 0.25 ml/min, respectively. The ammonium acetate buffer was prepared by addition of 0.1 M ammonium acetate to 0.1 M acetic acid to obtain a pH equal to 4.0, followed by a 1:4 dilution with HPLC-grade water. The column temperature was 40°C. Compounds were detected at 260 nm on an LDC Milton Roy SM4000 variable wavelength UV detector. Disappearance rates were determined by comparison of the LM-4108/internal standard ratios relative to 30-min control incubations lacking NADPH. No metabolites were observed in control incubations.

Mass Spectrometry. Metabolites were identified by LC/tandem mass spectrometry (LC/MS/MS) on a Finnigan TSQ7000 instrument interfaced with a Waters Alliance HPLC system. LC conditions were similar to those described above. Compounds were ionized by positive-ion, atmospheric pressure ESI with a tube lens voltage of 90 V and capillary temperature of 260°C. CID spectra were generated in the second quadrupole at ~22 V at ~2.5 mTorr of argon, and the daughter ions were mass analyzed in the third quadrupole. Other mass spectral parameters were optimized by direct inlet infusion of a solution of LM-4108 (50 μg/ml in methanol) into the LC mobile phase stream at a flow rate of 10 μl/min before LC/MS/MS analysis. For further identification of the 2-hydroxy-LM-4108 and the putative 1-hydroxy-LM-4108 metabolite, tube lens voltage was varied between 30 and 120 V to explore the extent of dehydration in the gas phase. Mass spectral identification was confirmed by comparison of retention times and CID fragmentation to authentic, synthetic standards of O-desmethyl-LM-4108, 2’-hydroxy-LM-4108, and 2’-oxo-LM-4108.

Cyclooxygenase Inhibition Studies. Synthetic standards of the oxidative metabolites of LM-4108 were evaluated as selective COX-2 inhibitors. The inhibition assays were performed in duplicate by preincubating ovine COX-1 or human recombinant COX-2 (44–66 nM) and test compound (0–66 μM in DMSO) for 20 min at 25°C in a 100 mM Tris-HCl buffer, pH 8.0, containing 500 μM flavonol. The remaining cyclooxygenase activity was determined after addition of [1-^14C]arachidonic acid (50 μM) for 30 s at 37°C. The formation of radiolabeled arachidonic acid metabolites was measured by a quantitative TLC assay with a radiochromatography scanner (BioScan-AR-2000, BioScan, Washington DC) as described previously (Kalugaturu et al., 1998).

Analysis of Rat Plasma and Bile. Two 250-g rats with an exteriorized bile duct and a cannulated jugular vein were purchased from Charles River Laboratories, Inc. (Wilmington, MA). The rats were allowed to acclimate for 3 days after receipt with daily flushing of the jugular vein and duodenal cannulas. An intravenous bolus dose of LM-4108 (2 mg/kg dissolved in propylene glycol/ethanol/normal saline/DMSO [40:20:38:2]) was administered over 1 min. Serial blood samples (0.5 ml) were collected at times t = 0.083, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, and 24 h postdose and anticoagulated with K3 EDTA. The blood samples were centrifuged for 5 min at 4000 rpm at 4°C to obtain 0.2 ml
of plasma. Plasma samples were stored at −80°C until analysis. Plasma samples (pooled from two rats at each time point) were extracted on 1-ml Empore C18 solid phase extraction disk cartridges (3M Company, St. Paul, MN) after addition of an internal standard. Compounds were eluted with methanol after a 20 mM ammonium acetate buffer wash, evaporated to dryness under argon, and reconstituted in HPLC mobile phase. The plasma extracts were analyzed for the presence of LM-4108, 2′-hydroxy-LM-4108, 1′-hydroxy-LM-4108, and 2′-oxo-LM-4108 by LC/MS/MS with selective reaction monitoring at m/z 461.2 → 138.9, 477.2 → 138.9, 459.2 → 138.9, and 475.2 → 138.9, respectively. Compounds were separated with an isocratic mobile phase of acetonitrile/20 mM ammonium acetate buffer, pH 4.0 (60:40) at 40°C on a 3 μm Luna C8 column (150 × 2.1 mm; Phenomenex, Torrance, CA) at a flow rate of 0.25 ml/min. The HPLC column was interfaced to the mass spectrometer via an electrospray interface with a tube lens voltage of 90 V and capillary temperature of 260°C.

Bile was collected at 1-h intervals for 7 h directly into microcentrifuge tubes containing 100 μl of 0.1 M ammonium acetate buffer, pH 4.0. Bile was subjected to mass spectral analysis before and after treatment with β-glucuronidase/sulfatase. Bile (0.25 ml) collected from 1 to 2 h after LM-4108 administration was diluted with 250 μl of 0.1 M ammonium acetate buffer, pH 5.0, and then incubated overnight at 37°C with 500 units/ml β-glucuronidase/sulfatase (Type H2; Sigma-Aldrich). Control bile samples were incubated overnight at 37°C without enzyme. The diluted bile samples were extracted by solid phase extraction and analyzed by LC/MS/MS as described above. Bile samples also were directly analyzed by gradient LC/MS/MS. The HPLC gradient was 0 to 60% acetonitrile in 20 mM ammonium acetate buffer, pH 4.0 over 20 min with an additional 10-min hold at 60% acetonitrile. Glucuronides were initially identified by a neutral loss experiment (difference of 20 min with an additional 10-min hold at 60% acetonitrile). Glucuronides were initially identified by a neutral loss experiment (difference of 20 min with an additional 10-min hold at 60% acetonitrile). Glucuronides were initially identified by a neutral loss experiment (difference of 20 min with an additional 10-min hold at 60% acetonitrile). Glucuronides were initially identified by a neutral loss experiment (difference of 20 min with an additional 10-min hold at 60% acetonitrile). Glucuronides were initially identified by a neutral loss experiment (difference of 20 min with an additional 10-min hold at 60% acetonitrile). Glucuronides were initially identified by a neutral loss experiment (difference of 20 min with an additional 10-min hold at 60% acetonitrile).

Microsomal Stability of LM-4108. A simple isocratic mobile phase system capable of separating indomethacin, LM-4108, and its metabolites and a suitable internal standard in less than 16 min was developed (Fig. 2, top panel). Since indomethacin displayed poor peak shape in mobile phases with a pH greater than 5.0, an HPLC separation with 20 mM ammonium acetate buffer, pH 4.0, was developed. LM-4108 and its potential metabolites demonstrated a broad UV absorption plateau from 240 to 265 nm.

A comparison of the metabolic stability (disappearance rates) of LM-4108 (10 μM initial concentration) in liver microsomes from rat, human, and mouse is presented in Fig. 3. Initial experiments with rat liver microsomes at typical protein concentrations of 0.6 pmol P450/ml indicated a very rapid NADPH-dependent metabolism of LM-4108. Consequently, P450 concentrations for the rat liver microsomal incubations were reduced to 0.15 pmol P450/ml and the disappearance rates of LM-4108 were determined. Similar studies with mouse and human liver microsomes were conducted with a P450 content identical to that used in the rat microsomal incubations. Under these modified experimental conditions, the half-lives for the disappearance of LM-4108 were 11 min, 21 min, and 51 min in rat, human, and mouse liver microsomal incubations, respectively.

Metabolite Identification. HPLC analysis of the microsomal incubations at 260 nm revealed the NADPH-dependent formation of three major metabolites eluting at retention times (Rt) = 7.2, 8.4, and 10.7 min, respectively. A typical HPLC chromatogram depicting LM-4108 metabolism in human liver microsomes in the presence and absence of NADPH is shown in Fig. 2. Analysis of LM-4108 microsomal reaction mixtures incubated for more than 15 min revealed the absence of NADPH-dependent formation of an additional metabolite (Rt = 6.7 min) along with several small peaks from 4 to 6 min, suggesting sequential metabolism of the major LM-4108 metabolites. Indomethacin (5.1 min), a potential metabolite arising from amidase-mediated hydrolysis of LM-4108, was not observed in these incubations (<1% of total metabolism).

Positive-ion ESI mass spectrometry indicated a protonated molecular ion (MH)+ for LM-4108 at m/z = 461.2. The CID spectrum of LM-4108 and the structures of the major fragment ions are shown in Fig. 4. The CID spectra for LM-4108 and its major microsomal metabolites indicated major fragment ions at m/z = 312.2 and m/z = 138.9 which corresponded to cleavage at the indole nitrogen and concomitant release of the p-chlorobenzoyl moiety, respectively. Monohydroxylation/oxidation of LM-4108 by cytochrome P450 would be expected to result in metabolites with protonated molecular ions at m/z = 477.2. Selected ion monitoring at 477.2 revealed the presence of three metabolites corresponding to retention times observed in the UV traces at 7.2, 8.4, and 10.7 min, respectively. The most plausible positions for oxidative metabolism in LM-4108 were aromatic hydroxylation on the phenyl ring and oxidation of the carbons on the phenethyl side chain. The 2′-hydroxy and p-hydroxyphenyl metabolites were synthesized, and the retention times and mass spectra were compared with those of the microsomal metabolites. The synthetic standard of the p-hydroxyphenyl metabolite of LM-4108 eluted at 6.1
Molecules in addition to molecules containing naturally abundant
not shown).

**Fig. 3. Disappearance of LM-4108 in incubations from mouse, human, and rat liver microsomes.**

LM-4108 (10 μM) was incubated with microsomes (at equivalent P450 concentrations) and 1 mM NADPH (n = 3). Analysis was conducted by HPLC-UV (260 nm) as described under Materials and Methods.

min and did not correspond to any of the observed metabolites (data not shown).

Synthetic 2'-hydroxy-LM-4108 coeluted with the major metabolite at Rₜ = 7.2 min and had an identical fragmentation pattern with significant fragments at 459.2 (dehydration of the 2'-alcohol to the corresponding styryl amide) and 120.1, respectively. The 459/477 ratio was highly dependent upon the tube lens voltage with little dehydration observed at low tube lens voltages (∼50 V). The peak eluting at 10.7 min demonstrated a protonated molecular ion at m/z = 475, corresponding to a metabolite 14 mass units higher than LM-4108. The small signal observed at m/z = 477 most likely represented the percentage of molecules containing two naturally abundant ¹³C molecules in addition to molecules containing naturally abundant ³⁷Cl. The phenethylnamine side chain fragment present at m/z = 122 in LM-4108 was absent in the mass spectrum of this metabolite and a fragment with m/z = 136 appeared, suggesting the presence of a carbonyl group in the phenethyl side chain. This ketone metabolite could arise by further oxidation of the 2'-hydroxyl group in 2'-hydroxy-LM-4108. Indeed, synthetic 2'-oxo-LM-4108 coeluted with the observed metabolite at 10.7 min and had an identical fragmentation pattern compared with the microsomal metabolite.

The metabolite eluting at 8.4 min was tentatively identified as either the 1'-hydroxy or N-hydroxy metabolite of LM-4108. The CID spectrum of this metabolite indicated a major fragment ion at m/z = 459 with less than 2% abundance observed at m/z = 477. Even at low tube lens voltages, m/z = 459 predominated, suggesting rapid dehydration of the hydroxy group during ionization. This metabolite did not coelute with the synthetic phenethylamine amide derivative of indomethacin (Rₜ = 16 min), which is the expected product of dehydration of either the 2'-hydroxy- or the 1'-hydroxy-LM-4108 metabolites. In an attempt to definitively identify this metabolite, 100 μM LM-4108 was incubated with 40 μl of phenobarbital-induced rat liver microsomes for 30 min. Metabolites were extracted with ethyl acetate, concentrated, and subjected to semipreparative HPLC. The metabolite corresponding to the peak eluting at 8.4 min was further purified by liquid-liquid extraction and preparative TLC. ¹H NMR (CDCl₃) results for the metabolite eluting at 8.4 min were as follows: 8.76–7.64 (d, 2H, J = 8.6 Hz, ArH), 7.48–7.50 (d, 2H, J = 8.6 Hz, ArH), 7.09–7.15 (m, 3H, ArH), 6.91–6.93 (m, 3H, ArH), 6.71–6.76 (m, 2H, ArH), 6.11–6.13 (d, 1H, J = 6.7 Hz, CH), 5.51–5.56 (m, 1H, CH), 3.79 (s, 2H, CH₂), 3.55–3.56 (d, 2H, J = 3.11 Hz, CH₂), 2.85–2.90 (dd, 1H, J = 5.2 Hz and 13.9 Hz, CHH), 2.74–2.79 (dd, 1H, J = 6.3 Hz and 13.9 Hz, CHH), 2.19 (s, 3H, CH₃). The amide proton was particularly well resolved (6.1 ppm) and the C-1' methine proton appeared as a multiplet at 5.5 ppm (shifted downfield from 3.7 ppm in LM-4108) that integrated as a single proton. These results clearly indicate that the position of hydroxylation was on the 1'-carbon and not on the nitrogen.

O-Desmethyl-LM-4108 was also synthesized and eluted at 6.7 min, corresponding to the minor microsomal metabolite observed in LM-4108-liver microsomal mixtures incubated for longer periods of time. Overall, the disappearance of LM-4108 and the appearance of 2'-hydroxy-LM-4108, 2'-oxo-LM-4108, and 1'-hydroxy-LM-4108 metabolites in rat, human, and mouse liver microsomes is depicted in Fig. 5.

**Characterization of LM-4108 Metabolites in Rat Plasma and Bile after Intravenous Administration.** LC/MS/MS analysis of plasma extracts from rats after intravenous administration of LM-4108 (2 mg/kg) revealed the presence of three metabolites with protonated molecular ions at 477.2, 475.2, and 459.2 with characteristic daughter ions at m/z 138.9 corresponding to the p-chlorobenzoyl fragment. Metabolites at m/z 477.2 and 475.2 eluted at identical retention times compared with synthetic 2'-hydroxy-LM-4108 and 2'-oxo-LM-4108, respectively. The third metabolite present in plasma at m/z 459.2 eluted at the same retention time as 1'-hydroxy-LM-4108 with mass spectral characteristics identical to those of the biochemically generated metabolite. Quantitative analysis of systemic exposure ~5 min postdose indicated LM-4108 concentrations to be 9.4 μM, whereas those of the 2'-hydroxy- and 2'-oxo-LM-4108 metabolites were estimated to be 0.613 μM and 0.275 μM, respectively. Systemic exposure 30 min postdose indicated that ~15% of the initial LM-4108 concentrations remained. Treatment of pooled rat bile (0–30 min postdose) with β-glucuronidase/sulfatase resulted in a dramatic increase in a peak corresponding to 2'-hydroxy-LM-4108. Low levels of unconjugated LM-4108, 2'-oxo-LM-4108, and 2'-hydroxy-LM-4108 also were found in bile not treated with β-glucuronidase. The glucuronide of 2'-hydroxy-LM-4108 was identified directly by gradient LC/MS/MS. A peak eluting at 20.7 min with a protonated molecular ion at m/z = 653 was observed. The CID spectrum consisted of daughter ions at m/z 477, 459, 312, 174, 139, and 120. These are characteristic fragments for 2'-hydroxy-LM-4108, although the most abundant ion was m/z 459 for the glucuronide in contrast to ions at m/z 139 and 312 for 2'-hydroxy-LM-4108. An additional glucuronide with a molecular ion at m/z = 639 was detected in rat bile. The molecular mass was consistent with glucuronidation of O-desmethyl-
Inhibition of LM-4108 Metabolism in Human Liver Microsomes by P450 Isoform-Specific Inhibitors. A pooled human liver microsomal preparation was used to study the effect of selective cytochrome P450 inhibitors on LM-4108 metabolism (Table 1). No inhibition of the formation of 2'-hydroxy-LM-4108, 2'-oxo-LM-4108, or 1'-hydroxy-LM-4108 in human liver microsomes was discernible in the presence of 10 μM quercetin (CYP2C8-selective), 2 μM quinidine (CYP2D6-selective), 10 μM sulfaphenazole (CYP2C9-selective), or 10 μM ticlopidine (CYP2D6- and CYP2C19-selective). In contrast, greater than 70% inhibition of LM-4108 metabolism to 2'-hydroxy-LM-4108, 2'-oxo-LM-4108, or 1'-hydroxy-LM-4108 was observed in human liver microsomal incubations containing the selective CYP3A4/5 inhibitors ketoconazole (1 μM) and troleandomycin (500 μM). The formation of O-desmethyl-LM-4108 in human liver microsomes was inhibited by ~40% in the presence of the selective CYP2D6 inhibitor quinidine (2 μM). Ketoconazole (1 μM) also strongly inhibited (~80%) the formation of 2'-oxo-LM-4108 in microsomes incubated with 2'-hydroxy-LM-4108, indicating that both steps in the formation of the ketone are catalyzed by CYP3A4/5.

Metabolism of LM-4108 by Recombinant Human Cytochrome P450 Enzymes. Based on these findings, LM-4108 was incubated with microsomes from insect cells expressing CYP3A4 plus cytochrome P450 reductase and cytochrome b5 (Table 2). 2'-Hydroxy-LM-4108, 2'-oxo-LM-4108, and 1'-hydroxy-LM-4108 were all formed by cloned, expressed CYP3A4 (25 pmol/incubation) in ratios similar to those observed in human liver microsomes. No metabolism was observed when LM-4108 was incubated with cloned, expressed CYP1A2, confirming the lack of inhibition by furafylline. O-Demethylation of LM-4108 was catalyzed by CYP2D6, confirming the observed inhibition by quinidine on the production of O-desmethyl-LM-4108 in human liver microsomes. Incubation of 2'-hydroxy-LM-4108 with expressed CYP2D6 resulted in the production of a secondary metabolite, eluting at 4.4 min with a protonated molecular ion at 463.3. This metabolite has been preliminarily identified as O-desmethyl-2'-hydroxy-LM-4108 based on its CID spectrum (fragments at m/z = 445, 298, 139, and 120). Although CYP2C9 and CYP2C19 also catalyzed the formation of O-desmethyl-LM-4108, the turnover in the presence of these isoforms was lower than that observed with CYP2D6 at equivalent P450 concentrations.

Selective COX-2 Inhibition by LM-4108 and Its Metabolites. Based on our previous work with a number of analogs of LM-4108, it was suspected that metabolism in the phenethyl side chain would result in the formation of active metabolites. The selective COX-2-inhibitory activity of LM-4108 metabolites was determined with purified ovine COX-1 and human recombinant COX-2. Indomethacin demonstrated selective COX-1 inhibition [IC_{50}(COX-2) = 0.75 μM, IC_{50}(COX-1) = 0.05 μM], whereas 2'-hydroxy-LM-4108 and 2'-oxo-LM-4108 were highly selective COX-2 inhibitors with potency and selectivity comparable to LM-4108 [LM-4108: IC_{50}(COX-2) = 0.06 μM, IC_{50}(COX-1) > 66 μM; 2'-hydroxy-LM-4108: IC_{50}(COX-2) = 0.06 μM, IC_{50}(COX-1) > 66 μM; 2'-oxo-LM-4108: IC_{50}(COX-2) = 0.05 μM, IC_{50}(COX-1) > 66 μM].

Discussion

The metabolic pathways of LM-4108 in rat, mouse, and human liver microsomes and in rat plasma and bile after intravenous administration are shown in Fig. 6. LM-4108 underwent rapid microsomal
metabolism on its phenethyl amide side chain. Interestingly, the 1'-hydroxy-LM-4108 metabolite was found to be remarkably stable for a carbinolamide (several months at 4°C). This may be due to the formation of a stable six-membered ring conformation via an intramolecular hydrogen bond between the 1'-hydroxy group and the carbonyl substituent within the amide moiety.

O-Demethylation is the primary oxidative pathway of indomethacin in liver microsomes from human and preclinical species. Nakajima et
al. (1998) reported that CYP2C9 was the predominant enzyme responsible for O-demethylation of indomethacin. However, O-demethyl-LM-4108 was a minor metabolite in rat, mouse, and human liver microsomes. Furthermore, sulfaphenazole, a highly selective CYP2C9 inhibitor, did not significantly inhibit the metabolism of LM-4108 in human liver microsomes. CYP2C9 is known to preferentially bind substrates containing an anionic group, and it has been proposed that the active site contains a cationic amino acid residue (Jones et al., 1996a,b; Poli-Scaife et al., 1997; Lewis et al., 1998).

Conversion of the carboxylic acid group in indomethacin to an amide derivative results in the loss of the anionic moiety and thus may selectively bind substrates containing an anionic group, and it has been proposed that the active site contains a cationic amino acid residue (Jones et al., 1996a,b; Poli-Scaife et al., 1997; Lewis et al., 1998). Thus, neutral indomethacin amides/esters may be potentially useful probes in the pharmacophore modeling of CYP2D6. Ketoconazole and the mechanism-based inhibitor, troleandomycin, significantly inhibited the oxidation of the phenethyl side chain in LM-4108. Both of these compounds are selective CYP3A4/5 inhibitors. Given the large molecular weight of LM-4108 and the lack of an ionizable group, it is not surprising that CYP3A4 would be the primary enzyme involved in the metabolism of LM-4108.

Rat, mouse, and human liver microsomes all produced a similar metabolic profile. The rate of metabolism was investigated by normalizing to an identical P450 content. However, this procedure results in different protein concentrations in the incubations. When normalized to P450 content, the rate of LM-4108 disappearance was slower in mouse microsomes compared with human or rat liver microsomes. Differences in the production of individual metabolites with time were observed between species. At early times, hydroxylation at the 2’ position predominated in all three species. In contrast, formation of the 1’-hydroxy-LM-4108 was faster in human liver microsomes than in rodent microsomes. Forma-

### TABLE 1

**Inhibition (percentage of control) of LM-4108 oxidative metabolism in pooled human liver microsomes by selective P450 isoform inhibitors**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>CYP</th>
<th>2’-Hydroxy-LM-4108</th>
<th>2’-Oxo-LM-4108</th>
<th>1’-Hydroxy-LM-4108</th>
<th>O-Desmethyl-LM-4108</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furafylline</td>
<td>1A2</td>
<td>106 ± 15.2</td>
<td>102 ± 3.5</td>
<td>106 ± 4.1</td>
<td>101 ± 15.2</td>
</tr>
<tr>
<td>(10 μM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Furafylline</td>
<td>1A2</td>
<td>97.6 ± 2.6</td>
<td>93.2 ± 1.9</td>
<td>97.3 ± 2.9</td>
<td>82.7 ± 1.5</td>
</tr>
<tr>
<td>(50 μM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>3A</td>
<td>30.7 ± 2.7</td>
<td>4.6 ± 1.6</td>
<td>22.7 ± 4.0</td>
<td>165 ± 10.7</td>
</tr>
<tr>
<td>(1 μM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>2C8</td>
<td>91.9 ± 3.3</td>
<td>65.5 ± 2.5</td>
<td>90.0 ± 3.0</td>
<td>95.2 ± 4.7</td>
</tr>
<tr>
<td>(10 μM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quinidine</td>
<td>2D6</td>
<td>95.9 ± 2.1</td>
<td>105 ± 5.1</td>
<td>90.6 ± 4.5</td>
<td>62.2 ± 4.1</td>
</tr>
<tr>
<td>(2 μM)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Sulphaphenazole</td>
<td>2C9</td>
<td>107.2 ± 4.1</td>
<td>113 ± 3.0</td>
<td>100 ± 3.6</td>
<td>114 ± 4.6</td>
</tr>
<tr>
<td>(10 μM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ticlopidine</td>
<td>2D6</td>
<td>93.8 ± 2.0</td>
<td>89.8 ± 6.5</td>
<td>103 ± 4.0</td>
<td>92.1 ± 8.7</td>
</tr>
<tr>
<td>(10 μM)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>2C19</td>
<td>63.7 ± 6.2</td>
<td>25.5 ± 4.4</td>
<td>46.2 ± 6.2</td>
<td>121.3 ± 3.7</td>
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<tr>
<td>(50 μM)</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Troleandomycin</td>
<td>3A4/5</td>
<td>30.6 ± 2.4</td>
<td>4.0 ± 0.4</td>
<td>16.2 ± 2.8</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± S.D. (n = 3) at 10 μM LM-4108. Values are expressed as percentage of control.

### TABLE 2

**Formation (metabolite/parent ratio) of the oxidative metabolites of indomethacin phenethylamide (LM-4108) and 2’-hydroxy-LM-4108 in incubations with cloned, expressed cytochrome P450 enzymes**

Values represent the peak area ratio of the metabolite formed/parent substrate (HPLC at 260 nm), mean ± S.D. (n = 3). All incubations contained the same amount of P450 (25 pmol).

<table>
<thead>
<tr>
<th>P450 Enzyme</th>
<th>O-Desmethyl-LM-4108</th>
<th>2’-Hydroxy-LM-4108</th>
<th>2’-Oxo-LM-4108</th>
<th>1’-Hydroxy-LM-4108</th>
<th>O-Desmethyl-LM-4108</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>0.013 ± 0.001</td>
<td>0.019, 0.013°</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>0.064, 0.028°</td>
<td>0.12 ± 0.06</td>
<td>0.06 ± 0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2D6</td>
<td>2.35 ± 0.53</td>
<td>0.13 ± 0.02</td>
<td>N.D.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2D6</td>
<td>2.0 ± 0.08</td>
<td>2.73 ± 0.044</td>
<td>N.D.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2A4</td>
<td>N.D.</td>
<td>0.17 ± 0.02</td>
<td>N.D.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N.D.: not detected.

° Duplicate values.
tion of the 2'-oxo metabolite probably arises from the further oxidation of the 2'-hydroxy-LM-4108 metabolite since human liver microsomes or recombinant CYP3A4 incubated with racemic 2'-hydroxy-LM-4108 produced 2'-oxo-LM-4108 in a NADPH-dependent fashion. In human liver microsomes, this reaction also was inhibited by ketoconazole. In incubations with LM-4108, ketoconazole and troleandomycin more potently inhibited the formation of this metabolite than either of the hydroxylated metabolites (see Table 1), further suggesting that the 2'-oxo metabolite is formed by sequential metabolism.

The three major metabolites identified in rat liver microsomes were present in the plasma of bile duct-cannulated rats injected intravenously with LM-4108. The pharmacokinetics of LM-4108 and its metabolites have been the subject of a preliminary report (Remmel et al., 2000) and will be reported in greater detail elsewhere. In vivo, the 2'-hydroxy-LM-4108 metabolite was found to undergo glucuronidation and was rapidly excreted into the bile of rats. After an intravenous dose, 2'-hydroxy-LM-4108 appears within 5 min, and in the bile as a glucuronide in the first 30 min. Thus, benzylic hydroxylation resulting in the formation of 2'-hydroxy-LM-4108 appears to be the predominant oxidative pathway of LM-4108 metabolism in vivo. Several other biliary metabolites were observed, suggesting sequential metabolism. The metabolites have not been completely characterized, although one glucuronide was observed with a molecular ion at m/z = 639, which would be the expected mass for a glucuronide of O-desmethyl-2'-hydroxy-LM-4108. O-Desmethyl-2'-hydroxy-LM-4108 was produced when 2'-hydroxy-4108 was incubated with cloned, expressed CYP2D6 (Table 2).

The 2'-hydroxy-LM-4108 and 2'-oxo-LM-4108 metabolites are potent and selective inhibitors of COX-2 and were also observed in substantial concentrations in rat plasma. The amide bond of LM-4108 when bound to COX-2 is proposed to be located at the constriction site in COX-2 formed by Arg120/Tyr355/Glu524 (Kalgutkar et al., 2000a). Based on molecular modeling of the murine COX-2 crystal structure and site-directed mutagenesis data, the phenethyl side chain of LM-4108 is expected to be positioned in a spatially generous region of the COX-2 protein termed the lobby. The lobby is located just below a constriction in the active site where the amide group is likely to be positioned (Kalgutkar et al., 2000b). Hydroxylation on the phenethyl amide side chain does not appear to affect the COX-2-inhibitory potency or selectivity compared with LM-4108 and, therefore, there appears to be enough room to accommodate a hydroxyl or keto group, even though this position is thought to be near the constriction site in COX-2.

In summary, LM-4108 is rapidly metabolized by liver microsomes. CYP3A appears to be the primary enzyme involved in the oxidative metabolism of the phenethyl amide side chain of LM-4108. Since CYP3A is highly expressed in both liver and intestine (Thummel and Wilkinson, 1998), extensive first pass metabolism of LM-4108 may occur. Comparison of the metabolic stability determined in vitro to the observed clearance values in animals will be the subject of future investigations.

**Acknowledgments.** We acknowledge the assistance of Lisa Meunier and Dr. David Hachey in the Mass Spectrometry Center at Vanderbilt University. Dr. Muhamed Hashim assisted with some of the microsomal incubations.

**Fig. 6.** Proposed metabolic scheme for indomethacin phenethylamide (LM-4108).
References


