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In Vitro Metabolism Considerations, Including Activity Testing of Metabolites, in the Discovery and Selection of the COX-2 Inhibitor Etoricoxib (MK-0663)

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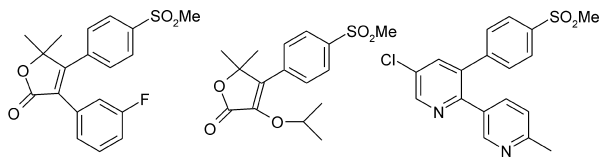
Abstract—Characterization of the metabolites of the COX-2 inhibitor etoricoxib (MK-0663 and L-791,456) produced in vitro indicate formation of an *N*-oxide pyridine and hydroxymethyl pyridine that can further be glucuronidated or oxidized to an acid. Significant turnover is observed in human hepatocytes. Several CYPs are involved in the oxidative biotranformations and, from in vitro studies, etoricoxib is not a potent CYP3A4 inducer or inhibitor. Based on an in vitro whole blood assay, none of the metabolites of etoricoxib inhibits COX-1 or contributes significantly to the inhibition of COX-2. © 2001 Published by Elsevier Science Ltd.

In recent years, efforts have been made to find potent and selective COX-2 inhibitors that could reduce pain and inflammation without affecting the cytoprotective action of COX-1.[1] This has led to the discovery of a novel class of drugs, such as rofecoxib[2,3] and celecoxib,[4] that are presently used in humans for the treatment of arthritis or pain relief. A continuous effort was made in chemistry to develop a second generation of COX-2 inhibitors, structurally different from the existing ones, that would show very high COX-2 selectivity, suitable pharmacokinetic profiles and in vivo efficacy in animal models. Based on these properties, DFP (5,5-dimethyl-3-(2-isopropoxy)-4-(4-methanesulfonylphenyl)-2(5*H*)-furanone)[5] and DFU (5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2(5*H*)-furanone)[6] were selected for human clinical trials (Fig. 1). However, during the phase I clinical studies, both of these compounds showed poor pharmacokinetic characteristics in humans: DFP had a very long half-life ($t_{1/2}=64$ h)[7] while DFU exhibited pharmacokinetics that varied significantly from individual to individual (12.2 to >72 h).[8] In addition, pharmacokinetic studies in rats showed that the clearance of DFP was significantly increased upon multiple dosing.[9] The basis of these pharmacokinetic behaviors was investigated through in vitro metabolic studies. It was found that DFP was poorly metabolized in human microsomes and hepatocytes, and a low rate of metabolism in vivo probably accounts for the very long half-life. In vitro studies in hepatocytes indicate that DFP induces its own metabolism in rat, probably through the induction of CYP3A, and this phenomenon was related to the faster clearance of DFP upon multiple dosing.[9] Regarding the metabolism of DFU, it was discovered that the compound was metabolized by a single, polymorphic cytochrome P450 (CYP2C19), which explains the variable pharmacokinetics obtained in vivo.[8] These observations indicate that differences in metabolic rates correlated with the differences in clearance of the COX-2 inhibitors. In light of these observations, it was considered critical that the metabolic fate of any potential second generation COX-2



DFU DFP Etoricoxib

Figure 1. Chemical structures of DFU, DFP, and etoricoxib.

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inhibitor should be carefully examined as early as possible.

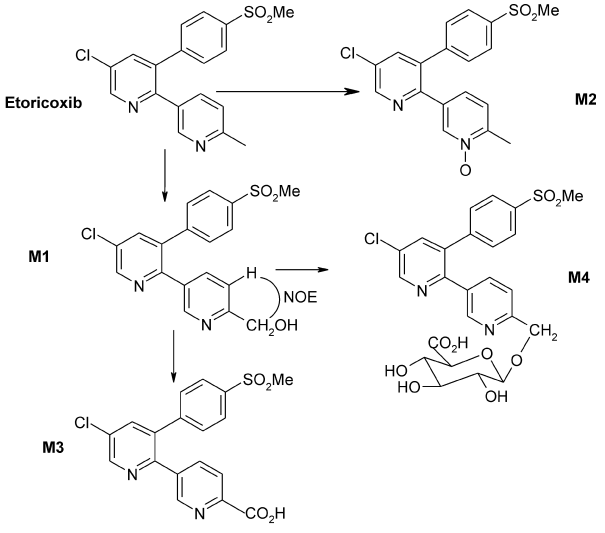
This paper reports the role of in vitro metabolism studies, performed at the drug discovery stage, in the identification of etoricoxib (MK-0663, L-791,456, [5-chloro-2-(6-methylpyridin-3-yl)-3-(4-methylsulfonylphenyl)pyridine]), a potent and selective COX-2 inhibitor[10,11] that is currently in human studies (Fig. 1). The in vitro experiments were conducted to identify etoricoxib metabolites and evaluate their effects on COX-1 and COX-2 activities. In addition, studies were performed to ensure that etoricoxib would not exhibit major metabolic liabilities in humans.

Metabolite Identification

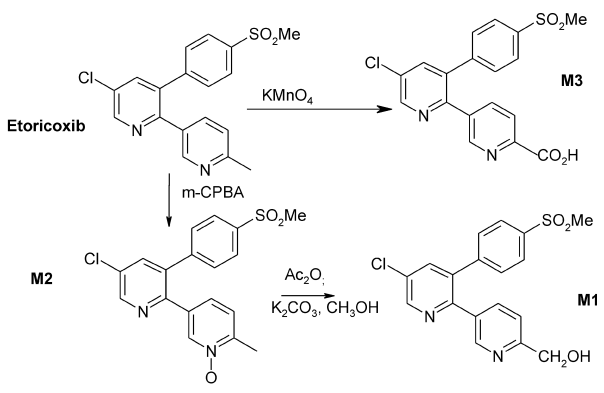
Initial in vitro biotranformation experiments were carried out with microsomes supplemented with NADPH under standard conditions.[12] HPLC analysis[13] of incubations of etoricoxib with human liver microsomes revealed the presence of one major (M1)[14] and one minor (M2)[15] metabolite. Based on the analysis by HPLC/APCI–MS, performed on a Finnigan SSQ model 7000 operated in positive ion mode, the mass (MH^+) of the two metabolites were 16 Da higher than etoricoxib ($m/z = 375$ vs 359) indicating that both metabolites resulted from the incorporation of one oxygen on the parent. The MS fragmentation pattern was not diagnostic enough to pinpoint the site of oxidation. Therefore, a large scale microsomal incubation[16] of etoricoxib was performed in an attempt to produce enough material for NMR characterization.[12] The ^1H NMR spectrum of etoricoxib (in DMSO-d_6) was fully assigned and compared to the ^1H spectrum of metabolite M1. All aromatic protons in the parent were found in M1, as were the methylsulfone protons. However, the signal for the pyridyl methyl group was not found. Two new signals at 5.42 and 4.52 ppm, integrating for one and two protons, respectively, were observed in the spectrum of M1. These results suggest that the pyridyl methyl group had been oxidized to a primary alcohol. The signals at 5.42 and 4.52 were ascribed to the hydroxyl and methylene protons, respectively. This hypothesis was confirmed by the observation of a strong NOE between the signal at 4.52 ppm that integrates for two protons and the adjacent pyridyl proton (see Scheme 1). The structure of M1 was further confirmed by comparison with an authentic standard which was prepared according to Scheme 2.

The low amount of M2 generated from the large scale incubation precluded its NMR characterization. However, its chemical structure could be determined by comparison with the 1′-*N*-oxide which was prepared according to Scheme 2. This standard co-eluted with M2 and had the same MS and diagnostic UV spectra (λ_{max} 250 nm compared to λ_{1max} and λ_{2max} 240 and 280 nm, respectively, for etoricoxib).

The overall metabolism profile of etoricoxib was also established in standard suspended hepatocyte incubations.[17] In addition to the metabolites M1 and M2 detected in microsomes, a new metabolite (M3)[18] which had a mass (MH^+) corresponding to the addition of 30 Da to that of etoricoxib (m/z 389 vs 359) was observed in human hepatocytes. The structure of M3 was speculated to be the 6-carboxy metabolite of etoricoxib and this was further confirmed by comparison of MS and chromatographic data with the synthetic material prepared according to Scheme 2. In dog hepatocytes, an additional metabolite, M4, was formed. The mass (MH^+) of M4 (m/z 551) corresponded to addition of 192 amu to the parent compound, which is consistent with an oxidation followed by a glucuronidation step. It was believed that the 6′-hydroxymethyl metabolite of etoricoxib was glucuronidated. Incubations of the synthetic 6′-hydroxymethyl analogue of etoricoxib with dog hepatocytes, followed by isolation of the metabolite, allowed its characterization. Comparison of the ^1H NMR spectrum of M4 (in DMSO-d_6) to that of etoricoxib showed that, with the exception of the methyl group, all of the parent protons were present in the metabolite. Signals corresponding to a methylene group bearing two diastereotopic protons (4.33 and 4.62 ppm) were observed as were all the protons characteristic of glucuronic acid. Comparison of the spectra from M4 to that obtained from M1 suggests that M4 is the



Scheme 1. In vitro metabolic pathways of etoricoxib.



Scheme 2. Synthesis of the oxidative metabolites of etoricoxib.

glucuronide of the 6′-hydroxymethyl metabolite of etoricoxib. Furthermore, a coupling constant of 7.8 Hz indicated that the anomeric proton was in the α-configuration consistent with a β-linked glucuronide.[17] A complete scheme of the in vitro biotransformations of etoricoxib is shown in Scheme 1.

In Vitro Metabolic Behavior in Human Microsomes and Hepatocytes

Because the initial biotransformations of etoricoxib involve an oxidation, the role of the cytochromes P450 was investigated. Incubations of etoricoxib with microsomes expressing individual CYPs (obtained from GENTEST Corp, Woburn, USA) were performed under standard conditions.[12] The metabolite M1 was formed with rCYP1A2 (17 pmol/mg•h), rCYP3A4 (68 pmol/mg•h), rCYP2D6 (170 pmol/mg•h) and rCYP2C19 (68 pmol/mg•h). Metabolite M2 was detected in incubations performed with rCYP1A2 (10 pmol/mg•h). Further studies in human liver microsomes have shown that among these various CYPs, CYP3A4 is the major contributor.[19] The fact that etoricoxib has multiple CYP enzymes involved in the various metabolic pathways reduces the likelihood of significant interindividual differences in pharmacokinetics, such as those observed with DFU.[8]

Using standard procedures,[13] in vitro studies were performed in suspended hepatocytes to determine the rate of metabolism of etoricoxib and DFP. In human hepatocytes obtained from various donors ($n=12$), the metabolism rate obtained for etoricoxob was 73 ± 35 nmol/h•10^6 cells and less than 1 nmol/h•10^6 cells for DFP. Assuming that hepatic metabolism plays a key role in the clearance of these compounds, the very long half-life of DFP observed in human[7] ($t_{1/2}=64$ h) is not anticipated for etoricoxib.

It was considered important to address the potential of etoricoxib to affect the activity of CYP3A4 knowing that this enzyme plays a significant role in the metabolism of many drugs, including itself. Inhibition studies were performed in human liver microsomes according to reported assay procedures, using testosterone as a CYP3A marker.[20] It was found that etoricoxib does not cause significant inhibition of CYP3A4 ($IC_{50}>50$ μM). Induction of CYP3A was addressed in human hepatocytes using a published procedure.[21] At the highest doses tested (50 μM of etoricoxib), the increase in CYP-3A4 protein was only ~20% relative to rifampicin (10 μM). Rifampicin, which is metabolized by CYP3A4, has been reported to cause induction of its own metabolism (auto-induction) in patients when the plasma levels were higher than 20 μM.[22] It is unlikely that etoricoxib will reach high enough concentrations in vivo to cause induction or inhibition of CYP3A4 and, therefore, should not affect the pharmacokinetics of CYP3A4-metabolized drugs.

Table 1. COX inhibitory potency of etoricoxib and its metabolites in the human whole blood assays (results are average of at least five donors)

Compound	IC_{50} (μM)	
	COX-1	COX-2
Etoricoxib	>100	1.1±0.1
6′-Hydroxymethyl (M1)	>100	>33
1′-*N*-oxide (M2)	>100	18±4
6′-Carboxylic acid (M3)	>100	21±4
6′-Hydroxymethyl glucuronide (M4)	>100	>33

COX Inhibitory Potency of the Metabolites

The presence of active circulating metabolites can severely complicate the prediction of the dosing regimen in human clinical studies. Knowing that metabolites detected in vitro can be circulating in vivo, the various metabolites made synthetically or biosynthetically were tested for the inhibition of COX-1 and COX-2 in the standard human whole blood assays[23] and were compared to those obtained for etoricoxib (Table 1). The in vitro metabolites of etoricoxib had no significant inhibition of COX-1 in the whole blood assay. They were at least 15-fold less potent than etoricoxib in the COX-2 whole blood assay. These metabolites are, therefore, not anticipated to contribute to the therapeutic benefits of COX-2 inhibition, nor any of the potential effects associated with COX-1 inhibition.

In conclusion, the in vitro studies using microsomes and hepatocytes have shown that etoricoxib does not show major metabolic liabilities in human. There are two sites of metabolism on the molecule (oxidation of the methyl pyridine or formation of the *N*-oxide) and multiple enzymes are involved in these biotransformations, reducing the likelihood of significant interindividual differences in the pharmacokinetics. It is unlikely that etoricoxib will cause drug–drug interactions with compounds metabolized by the important CYP3A enzyme. The presence of the metabolites should not modify the selectivity profile of etoricoxib because they are not potent COX-1 or COX-2 inhibitors. All these metabolism characteristics, evaluated at a drug discovery stage, have played a crucial role in the discovery of etoricoxib, a potent COX-2 inhibitor, currently in human clinical trials.

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14. **5-Chloro-2-(2-hydroxymethyl-5-pyridinyl)-3-(4-methylsulfonyl)phenylpyridine** (**M1**). A solution of M2 (150 mg, 0.40 mmol) in acetic anhydride (5 mL) was heated at reflux for 15 min and then cooled to room temperature. Ethanol and 1 N NaOH were added and the mixture was stirred for 15 min. The mixture was extracted twice with ethyl acetate and the combined organics were washed with brine, dried ($MgSO_4$) and concentrated. The residue was dissolved in methanol and solid potassium carbonate was added, and the resulting mixture was stirred at room temperature for 1 h. Water was added to dissolve the solids and the mixture was extracted twice with ethyl acetate. The combined organics were washed with brine, dried ($MgSO_4$) and concentrated. Chromatography of the residue (silica gel; ethyl acetate/dichloromethane, 4:1, v/v) provided the title compound as a white solid (100 mg, 67%). ^{1}H NMR (300 MHz, acetone-d_6): δ 3.14 (s, 3H), 4.39 (t, 1H, J = 5.6 Hz), 4.65 (d, 2H, J = 5.6 Hz), 7.41 (dd, 1H, J = 0.8, 8.1 Hz), 7.59 (d, 2H, J = 8.7 Hz), 7.69 (dd, 1H, J = 2.3, 8.1 Hz), 7.93 (d, 2H, J = 8.7 Hz), 8.00 (d, 1H, J = 2.4 Hz), 8.44 (m, 1H), 8.75 (d, 1H, J = 2.4 Hz).
15. **5-Chloro-2-(2-methyl-5-pyridinyl-*N*-oxide)-3-(4-methylsulfonylphenyl)pyridine** (**M2**). A mixture of etoricoxib (389 mg, 1.08 mmol) and *m*-chloroperoxybenzoic acid (57–86%, 330 mg) in dichloromethane (10 mL) was stirred at room temperature for 30 min and then diluted with ethyl acetate. The organics were washed with 1 N NaOH (3×), brine, dried ($MgSO_4$) and concentrated. The residual solid was suspended in ethyl acetate, stirred vigorously for 1 h and then filtered to provide the title compound as a white solid (250 mg, 62%). ^{1}H NMR (300 MHz, acetone-d_6): δ 2.34 (s, 3H), 3.15 (s, 3H), 6.91 (dd, 1H, J = 1.7, 8.1 Hz), 7.25 (d, 1H, J = 8.1 Hz), 7.67 (d, 2H, J = 8.6 Hz), 7.97 (d, 2H, J = 8.6 Hz), 8.02 (d, 1H, J = 2.4 Hz), 8.24 (d, 1H, J = 1.7 Hz), 8.74 (d, 1H, J = 2.4 Hz).
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18. **2-(2-Carboxy-5-pyridinyl)-5-chloro-3-(4-methylsulfonylphenyl)pyridine** (**M3**). To a solution of etoricoxib (200 mg, 0.56 mmol) in *t*-butanol (2 mL) and water (4 mL) at 90 °C was added solid $KMnO_4$ (264 mg, 1.67 mmol) portionwise over 30 min. The mixture was stirred for 15 h at 90 °C and then cooled to room temperature. The mixture was filtered through a plug of Celite, washing with ethyl acetate, and the filtrate was acidified with 1 N HCl. The organic layer was separated, dried ($MgSO_4$) and concentrated to provide a white solid. The suspended solid was stirred vigorously in ether and then filtered to provide the title compound as a white solid (40 mg, 18%). ^{1}H NMR (300 MHz, acetone-d_6): δ 3.14 (s, 3H), 7.63 (d, 2H, J = 8.6 Hz), 7.94–8.00 (m, 3H), 8.05–8.09 (m, 2H), 8.60 (dd, 1H, J = 0.8, 2.1 Hz), 8.81 (d, 1H, J = 2.3 Hz).
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