ABSTRACT

Imrecoxib is a typical cyclooxygenase-2 inhibitor and the benzylic carbon motif is its major site of oxidative metabolism, producing a hydroxymethyl metabolite (M1) and a carboxylic acid metabolite (M2). The plasma exposure of M2 is four times higher than those of both M0 and M1 in humans. However, this metabolite is rarely formed in in vitro experiments. Therefore, this study aims to investigate the formation mechanism of M2 and to further elucidate the reason for the discrepancy between in vitro and in vivo metabolic data. By employing human hepatocytes, human liver microsomes (HLMs), human liver cytosols (HLCs), recombinant enzymes, and selective enzyme inhibitors, the metabolic map of imrecoxib was elaborated as follows: the parent drug was initially hydroxylated to form M1 in HLMs, mainly mediated by CYP3A4 and CYP2D6, and to subsequently form aldehyde imrecoxib (M-CHO) in HLMs and HLCs. The latter process is the rate-limiting step in generating the end-product M2. In further M-CHO metabolism, two opposite reactions (namely, rapid oxidation catalyzed by CYP3A4, CYP2D6, and cytosolic aldehyde oxidase to form M2 versus reduction to regenerate M1 mediated by NADPH-dependent reductases in HLMs and HLCs, such as cytochrome P450 reductase) led to marked underestimation of the M2 amount in static oxidation metabolism in in vitro experiments. The findings provided a possible explanation for the difference between in vitro and in vivo metabolism of imrecoxib, suggesting that the effect of competitive reduction on the static oxidation metabolism in in vitro metabolic experiments should be considered.

Introduction

Coxibs are widely used in clinical practice as nonsteroidal anti-inflammatory drugs for alleviating pain based on their inhibition of cyclooxygenase-2. Typical marketed coxibs such as celecoxib and etoricoxib both contain the aromatic methyl group, which is their major site of oxidative metabolism, producing a hydroxymethyl metabolite with subsequent oxidation to the corresponding carboxylic acid metabolite (Paulson et al., 2000; Rodrigues et al., 2003). CYP2C9 is reportedly involved in the formation of hydroxymethyl celecoxib (Tang et al., 2000, 2001), and alcohol dehydrogenase is responsible for further metabolism to carboxy-celecoxib (Sandberg et al., 2002).

Similar to celecoxib, imrecoxib (another moderately selective cyclooxygenase-2 inhibitor approved in 2011) also contains a para-benzyl fraction. The metabolism of imrecoxib in rats has been investigated (Xu et al., 2006a,b). Imrecoxib undergoes a two-step oxidation of benzylic carbon and successively forms a hydroxymethyl metabolite (M1) and a carboxylic acid metabolite (M2), which are both confirmed to exhibit similar pharmacological activity to the parent drug (Feng et al., 2009). However, the metabolism of imrecoxib in humans has not been reported.

The design of drug-drug interaction clinical trials is essential for the clinical use of imrecoxib. Thus, understanding the principal metabolizing routes and identifying the enzymes involved are of considerable importance. Our pilot experiment indicated that the major metabolites of imrecoxib in humans after an oral administration are M1 and M2 by a two-step successive oxidation. M2 is the prominent drug-related component in both plasma and urine. However, this metabolite is seldom formed in human liver microsomes (HLMs) in vitro experiments. Therefore, another objective of the study was to explore the formation mechanism of M2 so as to further elaborate the cause for the discrepancy between in vitro and in vivo metabolic data.
Cryopreserved primary human hepatocytes were obtained from XenoTech (Lenexa, KS). NADPH, NAD, NADP, α-lipoic acid, and 1-aminobenzotriazole (ABT) were purchased from Sigma-Aldrich (St. Louis, MO). References of menadione and methoxyamine were obtained from Meilunbio (Dalian, China). All other reagents and solvents were either analytical or high-performance liquid chromatography grade.

Aldehyde imrecoxib (M-CHO) was synthesized as follows: 1,1-dihydro-1,1,3-triacetyloxy-1,2-benzooxazolo-3(1H)-one (D-3-TAO reagent, 11 mg, 0.026 mmol) was added to a solution of M1 (5 mg, 0.013 mmol) in dichloromethane at 0°C, and the mixture was stirred for 30 minutes. Then the reaction was quenched with NaHCO₃-saturated aqueous solution. The organic layer was separated and washed with NaCl saturated aqueous solution, dried over MgSO₄, then filtered and concentrated. The obtained residues were purified with preparative thin-layer chromatography [dichloromethane/methanol = 20:1 (v/v)] to yield a white solid. The structure was confirmed based on nuclear magnetic resonance (NMR) and mass spectrometry (MS) data. 1H NMR (400 MHz, chloroform-d) δ 10.03 (s, 1H), 7.92–7.85 (m, 4H), 7.58 (d, J = 8.2 Hz, 2H), 7.45 (d, J = 8.5 Hz, 2H), 4.37 (s, 2H), 3.58 (t, J = 7.4 Hz, 2H), 3.08 (s, 3H), 1.73 (s, J = 7.4 Hz, 2H), and 1.01 (t, J = 7.4 Hz, 3H). 13C NMR (126 MHz, Chloroform-d) δ 191.98, 169.39, 146.82, 141.37, 138.49, 137.56, 136.34, 134.76, 130.4 (2C), 130.10 (2C), 128.75 (2C), 128.25 (2C), 52.84, 44.58, 44.56, 22.11, 11.64. High-resolution MS (electrospray ionization): mass-to-charge ratio (m/z) calculated for C₉H₉NO₅S, 382.1119; found, 382.1120.

Individual stock solutions of imrecoxib, M1, M-CHO, and other inhibitors were prepared by dissolving each compound in dimethylsulfoxide. The stock solutions were then diluted with 100 mM phosphate-buffered saline (PBS; pH 7.4) containing 0.05% (v/v) of α-tocopherol and 2.1 mm lipoic acid, respectively.

**Determination of Imrecoxib, M1, M2, and M-CHO.** After acetonitrile-induced protein precipitation of samples, 10 μl of the mixture was injected into the liquid chromatography–MS/MS system for analysis. The analytes and IS were separated on an Eclipse Plus C18 column (100 × 4.6 mm i.d., 3.5 μm; Agilent, Santa Clara, CA). Column temperature was maintained at 40°C. The mobile phase consisted of 5% methanol ammonium acetate, formic acid, and acetonitrile [35:0.1:65 (v/v/v)] at the flow rate of 0.7 ml/min. Detection was performed in the positive multiple reaction monitoring scan mode by using a dwell time of 100 milliseconds per transition to detect ions pairs at m/z 370→236 (imrecoxib), 386→236 (M1), 402→236 (M2), 384→236[M-CHO] + (M1 + IS). The spray voltage and source temperature were set to 5500 V and 500°C, respectively.

**In Vitro Incubations of Imrecoxib and M1.** NADPH (2.0 mM) was preincubated with HLMs (0.5 mg/ml) or PBS for the control at 37°C for 10 minutes; then 10 μm imrecoxib and M1 were separately added to initiate their individual reactions. After 90 minutes of incubation, the reactions were terminated by adding an equal volume of ice-cold acetonitrile. To further confirm the participation of P450 enzymes, 10 isoforms of recombinant human P450 enzymes (50 pm; namely, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5) were separately incubated with imrecoxib (3.0 μM) in the presence of NADPH for 90 minutes. The operation procedures and conditions were similar to those for incubation with HLMs, except HLMs were replaced by each recombinant human P450 enzyme. The normalized relative contribution of each P450 was calculated according to the method reported previously (Rodrigues, 1999). Incubations of specific-chemical inhibitors (2 μM α-naphthoflavone, 6 μM quercetin, 1 mM ABT, 6 μM sulfaphenazole, 8 μM quinidine, 24 μM ticlopidine, 24 μM clomethiazole, and 2 μM ketocoznole) of each P450 enzyme in HLMs with imrecoxib were conducted separately using the similar protocol mentioned above.

To determine the effects of HLC enzymes, HLC (0.5 mg/ml) was preincubated with or without NAD at 37°C for 10 minutes. Afterward, 10 μM imrecoxib or M1 was added to start the reaction. After 90 minutes of incubation, the reactions were quenched by the addition of ice-cold acetonitrile.

The metabolism of imrecoxib and M1 was also evaluated in human hepatocytes. Each compound (10 μM) was incubated with human hepatocytes (1.0 × 10⁶ cells/ml) in Williams’ E medium for 3 hours at 37°C, in the absence or presence of ABT. The reactions were quenched by an equal volume of ice-cold acetonitrile. Each experiment was performed in duplicate.

In vitro incubations, probe substrates [phenacetin, buproprion, paclitaxel, tolbutamide, S-mephenytoin, dextromethorphan, midazolam, testosterone, and SGX523 (6-[(1-(benzyl[bipyrazolyl]-4-yl)]-[1,2,4]triazolo[4,3-b][1,2,4]-pyridazin-3-yl]-sulfanyl]quinoline) were selected as positive controls to prove the activity of each enzyme as well as the potency and selectivity of the chemical inhibitors.

**Trapping of M-CHO in HLMs, HLCs, and Recombinant P450s.** Trapping experiments were performed in duplicate.

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**Trapping of M-CHO in HLMs, HLCs, and Recombinant P450s.** Trapping experiments were performed in duplicate.
**In Vitro Incubations of M-CHO.** M-CHO (10 μM) was selected as the substrate to initiate the reactions, which were conducted similar to the procedure for the in vitro incubations of imrecoxib or M1. The incubations included PBS (without or with NAD, NADP, and NADPH), NADPH-fortified HLMs, and HLCs (with or without NADPH). In addition, to assess the involvement of aldehyde oxidase (AOX) on the oxidation of M-CHO, specific-chemical inhibitor menadione (20 and 100 μM) was added to HLC incubations. Reaction phenotyping studies of M-CHO were conducted as described for imrecoxib.

**M-CHO Reduction or Oxidation in HLMs.** M-CHO (10 μM) was incubated separately with recombinant POR, human CYP3A4R, and human CYP3A4BL in the presence of NADPH; these conditions were similar to the protocol for HLM incubations of M-CHO. The effect of α-lipoic acid, a selective inhibitor of POR, was assessed at 0, 5, 10, 25, and 50 mM.

**Kinetic Analysis.** In this study, kinetic analyses involved 1) the conversion of the parent drug to M1 in HLMs; 2) its subsequent oxidation to M-CHO in HLMs or HLCs; 3) conversion of M-CHO to M2 in HLMs (c1), HLCs (c2), or NADPH-fortified HLCs (c3); and 4) competing reduction of M-CHO back to the primary M1 in HLMs or NADPH-fortified HLCs. For V_{max} and K_{m} determinations, all substrates (imrecoxib, M1, and M-CHO) were incubated at the initial rate in selected concentration ranges, after optimization of incubation time and protein concentrations for the linearity of metabolite formation and <20% of parent depletion (Table 1).

Nonlinear regression analysis of kinetic data was performed using GraphPad Prism software (version 6.01; GraphPad Software Inc., La Jolla, CA). The models fitted included allosteric sigmoidal, substrate inhibition, and Michaelis-Menten models. The goodness-of-fit criteria included a high level of squared correlation coefficients (R²) as well as appropriate visual inspection of the data. Intrinsic clearance values were calculated as V_{max}/K_{m}, where V_{max} is the maximum velocity and K_{m} is the Michaelis constant.

### Results

**Characterization of Imrecoxib Metabolites in Human Plasma and Urine.** A total of 22 metabolites in humans were detected after a single oral administration of 100 mg imrecoxib (Supplemental Table 1). Metabolic profiles of imrecoxib in human plasma and urine are shown in Fig. 1. M1 and M2 were confirmed using reference standards. In plasma, M1 and M2 were the major circulating substances in addition to the parent drug. The systemic exposure ratio of imrecoxib, M1, and M2 was approximately 1:1:4 (Supplemental Table 2). Other minor pathways, such as the hydroxylation of pyrrole and the N-alkyl chain, were also identified.

**Investigation of the Metabolism of Imrecoxib and M1 In Vitro.** HLMs, HLCs, and human hepatocytes were selected as the incubation models for exploring the metabolism of imrecoxib in vitro. M0 was mostly oxidized to M1 in HLMs supplemented with NADPH and was accompanied by minor amounts of M2, which accounted for less than 2% of the total metabolites. The addition of ABT in HLMs significantly decreased the formation of the two metabolites by almost 97%, indicating the contribution of P450s to the conversion. The same phenomenon could be observed in the incubation of M0 with human hepatocytes, in which more than 30% of the parent drug was oxidized to M1. By contrast, no metabolites could be detected in HLC incubation with M0, showing that HLC enzymes are not involved in this biotransformation (Fig. 2A).

The formation of M2 originated from the direct conversion of M1 but not of M0, as demonstrated by the limited amount of M2 detected in NADPH-supplemented HLMs or human hepatocytes with M0. To investigate this possibility, M1 was incubated in the same in vitro systems as M0. Compared with those observed in M0 incubations, M2 yields increased, and M2 could be detected in HLC incubation (Fig. 2B). These observations confirmed that M2 was generated from M1 instead of M0. However, regardless of the incubation conditions, the M2 yield remained extremely low.

**Elucidation of the Formation of M-CHO.** In general, the metabolic intermediates or metabolites with an aldehyde group are generated when the hydroxyl fraction of the compound is oxidized to its corresponding carboxylic acid forms (Diao et al., 2013; Liu et al., 2015; Zhu et al., 2016). Thus, trapping experiments were performed to probe the presence of the aldehyde intermediate. Methoxamine was used as the trapping agent. A chromatographic peak with the expected [M+H]+ ion at m/z 413.150 (Fig. 3A) and the corresponding supportive fragment ions of m/z 381.128, 355.125, and 278.084 (Fig. 3C) were observed when M1 and NADPH-fortified HLMs were coincubated with methoxamine. A significant decrease in M2 yield relative to that of the incubations in the absence of the trapping agent was also observed. The M-CHO–methoxamine adduct was also detected when the same incubation was conducted with HLCs (Fig. 3B). The results confirmed that M1 is oxidized to M-CHO prior to M2 formation in both HLM and HLC incubations.

To explore the formation of M-CHO, M1 was incubated under different conditions, and the formed M-CHO (m/z 384.2→236.2) and M2 (m/z 400.2→236.2) were directly detected using a liquid chromatography–MS/MS system in multiple reaction monitoring scan mode. M-CHO was not able to be detected when M1 was incubated in PBS (Fig. 4A), regardless of the addition of coenzyme NAD, NADP, and NADPH. Meanwhile, no M2 was formed (data not shown). In the NADPH-fortified HLM incubations with M1, M-CHO was observed to accompany the M2 yield (Fig. 4B); upon the addition of ABT, the levels of the two metabolites in HLMs markedly decreased (Fig. 4C). In the M1 HLC incubations without NADPH, M2 was detected rather than M-CHO (Fig. 4D), indicating that M-CHO was immediately oxidized to M2 upon formation. These results showed that P450 and HLC enzymes were both responsible for the M-CHO formation.

**Metabolic Characteristics of M-CHO.** To obtain further insight into M-CHO metabolism, this metabolic intermediate was synthesized to initiate the incubation experiments. An unexpectedly large amount of M2 was generated when M-CHO was incubated with HLCs, whereas M2 together with M1 was considerably formed in NADPH-fortified

### Table 1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Metabolite</th>
<th>Incubation</th>
<th>Protein Concentration</th>
<th>Incubation Time</th>
<th>Concentration Range</th>
<th>V_{max} (µM)</th>
<th>K_{m} (µM)</th>
<th>Intrinsic Clearance (µl/min per mg protein)</th>
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<tbody>
<tr>
<td>Imrecoxib</td>
<td>M1</td>
<td>HLM (NADPH)</td>
<td>0.02</td>
<td>0</td>
<td>0.01–100</td>
<td>296</td>
<td>3.72</td>
<td>79.6</td>
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<tr>
<td></td>
<td>M1</td>
<td>HLM (NADPH)</td>
<td>0.10</td>
<td>60</td>
<td>0.25–2500</td>
<td>137</td>
<td>9.79</td>
<td>0.14</td>
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<tr>
<td></td>
<td>M1</td>
<td>HLM (NADPH)</td>
<td>0.10</td>
<td>60</td>
<td>0.25–2500</td>
<td>33.9</td>
<td>1299</td>
<td>0.0261</td>
</tr>
<tr>
<td>M-CHO</td>
<td>M1</td>
<td>HLM (NADPH)</td>
<td>0.04</td>
<td>45</td>
<td>0.01–100</td>
<td>725</td>
<td>8.01</td>
<td>90.5</td>
</tr>
<tr>
<td>M-CHO</td>
<td>M2</td>
<td>HLM (NADPH)</td>
<td>0.04</td>
<td>45</td>
<td>0.01–100</td>
<td>2780</td>
<td>56.9</td>
<td>48.9</td>
</tr>
<tr>
<td>M-CHO</td>
<td>M1</td>
<td>HLC</td>
<td>0.04</td>
<td>45</td>
<td>0.01–100</td>
<td>973</td>
<td>1.34</td>
<td>72.7</td>
</tr>
<tr>
<td>M-CHO</td>
<td>M2</td>
<td>HLC</td>
<td>0.04</td>
<td>10</td>
<td>0.01–100</td>
<td>39,195</td>
<td>430</td>
<td>91.2</td>
</tr>
</tbody>
</table>
HLM incubations (Fig. 5). Moreover, M-CHO was almost depleted in these in vitro incubations. By striking contrast, less M1 was metabolized when it was selected as the substrate. Thus, we concluded that in enzymatic catalysis, the generation of M-CHO from M1 is the rate-limiting step for M2 formation. The in vitro kinetic study provided more direct evidence (Table 1). Intrinsic clearance values for M1 oxidation to M-CHO were minimal whether in HLM or HLC incubations, and the \( K_m \) values of this rate-limiting step were markedly higher than those of other metabolic processes.

**NADPH-Dependent Effects in Different Subcellular Fractions for M2 Formation.** Interestingly, M1 and M-CHO metabolism were influenced significantly by coenzymes, especially NADPH (Figs. 4E and 5). In HLC incubations, M-CHO was almost completely biotransformed into M2. However, upon the addition of NADPH in HLC...
incubations, M2 formation was markedly reduced and accompanied by M1 production (Fig. 5A).

When M-CHO was added to NADPH-fortified HLM incubations, M1 was also detected in addition to M2 at a ratio of 1:2 (Fig. 5B). However, in NADPH-supplemented PBS incubations with M-CHO, neither M1 nor M2 was detected. These data indicated that M-CHO underwent opposite metabolic reactions (i.e., oxidation versus reduction). In addition, the formation of reductive metabolite M1 in these subcellular fractions was dependent on the presence of the cofactor NADPH.

The formation mechanism of M1 in the incubations of M-CHO with NADPH-supplemented HLCs was further investigated. In recombinant NADPH-POR incubations, M-CHO was completely metabolized to M1 instead of M2, suggesting that POR was responsible for the reduction of M-CHO to M1. α-Lipoic acid was effective in inhibiting M1 formation when a 10-fold molar excess of the inhibitor over M-CHO was used (Fig. 6), further supporting the role of POR in the reduction of M-CHO in HLMs.

In addition, in common NADPH-fortified recombinant human CYP3A4 (containing POR and cytochrome-b5) incubation with M-CHO, both M2 and M1 could be detected at a ratio of 36:1; in human CYP3A4R (without cytochrome-b5) and human CYP3A4BLR (containing cytochrome-b5 but low POR activity) incubations in the presence of NADPH, a significant decrease in M2 was observed and a large amount of M1 was obtained instead with the generation ratios of M2 and M1 of 0.4:1 and 0.1:1, respectively (Fig. 7). The results suggested that the oxidation to M2 and reduction to M1 from M-CHO were two competitive pathways. POR and cytochrome-b5 are both indispensable electron transporters for the oxidation effect of P450 enzymes. The integrity of the electron transport chain (ETC) of P450s might accelerate the M-CHO oxidation pathway, whereas ETC deficiency (e.g., the low activity of POR or the absence of cytochrome-b5) leads to attenuated oxidation and drives the reduction reaction.

The reducing enzyme(s) involved in the formation of M1 from M-CHO in HLCs is yet to be characterized, because specific inhibitors of several HLC-reducing enzymes, including alcohol dehydrogenase, aldo-keto reductase, quinone reductase, and glutathione peroxidase, cannot inhibit the formation of M1 when M-CHO is incubated in NADPH-supplemented HLCs (data not shown).

**Identification of Oxidative Enzymes Responsible for the Formation of M1, M-CHO, and M2.** The formation of M1 is P450 mediated. To identify the P450 isoforms, 10 isoforms of recombinant human P450 isozymes were individually incubated with M0. Given that M1 is both the metabolite of M0 and the substrate of M2 in HLMs, the same metabolic enzymes might be shared by the two metabolic steps, causing underestimation of M1 yield. Therefore, the reduction of M0 was monitored to calculate the contribution of individual human P450 enzymes under the condition that the other minor metabolic pathways of M0 were ignored. CYP3A4, CYP2D6, and CYP3A5 were identified as the principal catalyzing enzymes (Fig. 8A). After normalization for the relative hepatic abundance of P450 enzymes, the relative contributions of CYP3A4 and CYP2D6 were calculated as 68% and 32%, respectively. Specific-chemical inhibition tests also provided further supporting data (Supplemental Fig. 1).

The same method was applied to evaluate P450 enzyme(s) catalysis of M-CHO to M2. CYP3A4 was confirmed to be the most active enzyme for M2 formation, followed by CYP2D6, with contributions of 61% and 38%, respectively (Fig. 8B). M2 can be detected when M-CHO is incubated with HLC. Aldehyde dehydrogenase (ALDH), AOX, and xanthine oxidase (XO) are the main oxidases for aldehyde compounds in liver cytosol. XO always catalyzes the metabolism of endogenous substances or purine analogs, whereas the structures of imrecoxib and its metabolites do not meet the requirement. In addition, ALDH was noneffective for oxidation in the absence of NADP, whereas M-CHO could be depleted in HLCs without any cofactors. Thus, XO and ALDH were disregarded in this experiment. A specific inhibitor was added in HLCs to only identify the possible AOX being responsible for M2 formation. The yield of M2 in HLCs was attenuated by up to 25% and 95% by 20 and 100 μM menadione (an AOX inhibitor), respectively. The data indicated that AOX was the most efficient enzyme involved in the formation of M2 in HLCs.

To determine which P450 enzyme(s) preferentially oxidize M1 to M-CHO, we performed trapping experiments and investigated the formation of M-CHO adduct in different recombinant human P450 enzymes. The results indicated that CYP3A4, CYP2D6, and CYP3A5 were the key P450 enzymes responsible for the formation of M-CHO, with a ratio of 2:6:1. However, the contributions of the three P450s might be overestimated, because these isoforms can also catalyze further oxidation of M-CHO. In the HLC incubations with M1, M-CHO could not be detected because the formation rate of M2 was considerably higher than that of M-CHO. As a result, the cytosolic enzyme(s) responsible for the oxidation of M1 to M-CHO could not be identified.

**Investigation of M2 Formation in Rats.** To characterize M2 formation in vivo, imrecoxib, M1, and M-CHO were directly administered...
by intravenous injection to Sprague-Dawley rats, respectively. The mean plasma concentration-time curves of M2 are presented in Supplemental Fig. 2. After intravenous administration of M-CHO, M1, and imrecoxib, peak plasma concentrations were reached at 0.083, 0.5, and 1 hours postdose with mean values of 1013, 902, and 437 ng/ml. The area under the plasma concentration-time curve from zero to 10 hour values were comparable, with 1073, 1193, and 736 ng/h/ml, respectively.

Fig. 4. (A–E) Chromatograms of liquid chromatography–MS analyses of M1 (1.5 μM) metabolism in PBS (A), NADPH-fortified HLMs (B), NADPH– and ABT-supplemented HLMs (C), HLCs (D), and NADPH-fortified HLCs (E). Peaks I–III represent M1, M-CHO, and M2, respectively. MRM, multiple reaction monitoring; Rel. Int., relative intensity.
Discussion

The carboxylic acid metabolite M2 appears to be the major metabolite of imrecoxib in plasma and urine, accounting for 68% of the systemic exposure and 77% for the total clearance, respectively. However, M2 is not generated to any significant extent in typical in vitro metabolic models, including HLMs, HLCs, and human hepatocytes. The key metabolic process being responsible for M2 formation is expected to be investigated.

The origin of M2 should be defined first because the end-stage metabolite may be directly generated from the parent drug (Xie et al., 2013; Mu et al., 2014) or may undergo successive multistep metabolic pathways (Zhou et al., 2015). In this study, the major metabolic pathway of imrecoxib was characterized as M0 being successively oxidized to M1, to M-CHO, and ultimately to M2, because more M2 was observed in the M1 incubations relative to M0 incubations, and trapping experiments with methoxyamine provided further evidence. The kinetic analyses indicated that the biotransformation from M1 to M-CHO was the rate-limiting step for M2 generation. On the contrary, in in vivo conditions, the formation of M2 was comparable after intravenous administration of imrecoxib, M1, and M-CHO, suggesting that the barrier of M-CHO formation was easy to overcome under dynamic and physiologic situations. Thus, the discrepancy between in vivo and in vitro for M-CHO formation may be the key point for explaining why only minor amounts of M2 were formed during in vitro incubation of imrecoxib.

NADPH is often used as a cofactor in in vitro metabolism investigations for donating electrons. NADPH participates in the oxidation or the reduction based on the electron transport. In NADPH-fortified HLM incubations with M-CHO, the oxidative metabolite M2, as well as the reductive metabolite M1, could be detected. The phenomenon also emerged in recombinant CYP3A4 incubations. In general, most marketed recombinant human P450 enzymes contain NADPH-POR and cytochrome-b5 to enhance the activity of enzymes because the two components are the major electron transporters of ETC (Aigrain et al., 2011; Kenaan et al., 2011). Meanwhile, POR can directly mediate the one-electron reductive biotransformation, such as the nitro reduction of aristolochic acid (Henderson et al., 2003; Stiborová et al., 2005, 2011). In this study, POR could catalyze the reduction of M-CHO back to M1. Compared with CYP3A4 incubation, M2 production significantly decreased with the increase in M1 in human CYP3A4R and CYP3A4BLR incubations with M-CHO. Therefore, it was inferred that the NADPH-dependent POR expressed dual effects, including both oxidation and reduction on M-CHO in HLMs by donating electrons to microsomal P450s or directly to the substrate M-CHO. M1 incubation in NADPH-fortified HLMs generated M-CHO, which can be further oxidized to M2 and reduced back to M1 simultaneously in two competitive pathways.

M-CHO was rapidly metabolized with AOX mediation to M2 in HLCs, whereas a significant decrease existed in M2 formation in NADPH-fortified HLCs. Holm et al. (2016) reported that JWH-018 [1-pentyl-3-(1-naphthoyl)indole], a marketed synthetic cannabinoid, can be oxidized to JWH-018 α-COOH in HLCs, whereas the process can be nearly terminated completely by adding NADPH. However, the authors did not explain the cause of prevention by this cofactor. As seen in this study, M-CHO can be reduced back to M1 in HLCs in the presence of NADPH, causing the attenuation of the oxidation to M2. However, the NADPH-dependent enzyme(s) in HLCs responsible for the M-CHO reduction remains to be identified.

Drugs containing the benzylic carbon motif are prone to be oxidized to its corresponding benzylic alcohol (Mamidi et al., 2014; Ryu et al., 2014) and/or further to the end-product carboxylic acids in vivo (Hvenegaard et al., 2012; Walles et al., 2013; Pozo et al., 2015). It is recognized that there is an aldehyde intermediate in metabolic processing from alcohol to carboxylic acid, and some in vitro trapping tests with methoxyamine or semicarbazide also...
provided convincing evidence (Li et al., 2014; Inoue et al., 2015; Liu et al., 2015). However, further metabolism of aldehyde intermediates is rarely reported in publications. Dopamine is a special case that undergoes oxidative deamination initially to aldehyde (3,4-dihydroxyphenylacetaldehyde), followed by ALDH-mediated oxidation to phenylacetic acid metabolite and reductase-mediated reduction to phenylethanol metabolite (Jinsmaa et al., 2009). Different from M-CHO, 3,4-dihydroxyphenylacetaldehyde was reduced to a new forward metabolite, not its precursor dopamine.

In vitro metabolism incubations (e.g., subcellular fractions) occur in a static system, representing only a few of several dimensions of a complex in vivo system (Wang et al., 2010; Subramanian et al., 2013). Several drugs, such as linezolid and ziprasidone, are metabolized through multiple sequential reactions in vivo, whereas they can only undergo one or two sequential reactions in in vitro systems (Wang et al., 2010), especially when a metabolic intermediate is generated. Furthermore, even if the intermediate M-CHO can be partially reduced to M1 in vivo, this reduced product might enter into other subcellular fractions, such as kidney microsomes or cytosol, where M1 might be easily oxidized again in the absence of NADPH-dependent cytosolic reductase(s) similar to the liver (Brandon et al., 2003; Marchitti et al., 2008; Argikar et al., 2016). This was further supported by the in vitro data. M2 could be generated in M1 incubations with human renal homogenate, as well as rat renal homogenate (Supplemental Fig. 3). In rats, peak time of M2 plasma concentrations was gradually postponed after intravenous administration of M-CHO, M1, and imrecoxib, and no significant difference in plasma exposure of M2 was observed among three groups. These results indicated that the metabolite M1 derived from imrecoxib could circulate continuously into the liver or other metabolic organs and could be removed from the body in the form of the end-product M2. By contrast, in static in vitro incubations, M1 failed to accomplish a similar process to the dynamic in vivo system, and the formed M1 was not reused.

It was observed that M1 and M-CHO could interconvert into each other via oxidation or reduction in HLCs as well as in HLMs. However, the two metabolic processes are not real reversible reaction steps, because these conversions occurred under different conditions. In HLMs, the conversion of M1 to M-CHO was catalyzed by P450s, whereas the reduction of M-CHO to M1 was catalyzed by POR. In HLCs, these two metabolic pathways were mediated by NADPH-independent enzymes and NADPH-dependent enzymes, respectively.

Li et al. (2005) reported that CYP2C9 was the major enzyme involved in imrecoxib hydroxylation metabolism, along with CYP2D6 and CYP3A4. In our study, recombinant human CYP2C9 incubation assays and the HLM inhibition experiment with specific-chemical inhibitor sulfaphenazole both confirmed that CYP2C9 was not responsible for the oxidation of imrecoxib as well as M1 and M-CHO.

In conclusion, this study demonstrates that the extensive oxidative metabolism of imrecoxib occurred primarily on its benzylic carbon motif to generate M1 and M2 by a two-step successive oxidization (Fig. 9). The initial hydroxylation was mainly mediated by CYP3A4 and CYP2D6, with relative contributions of 68% and 32%, respectively, to form M1 in HLMs. M1 was then oxidized to M-CHO in HLMs and HLCs. Meanwhile, this process was the rate-limiting step in the generation of the end-product M2. Two opposite reactions occurred in further M-CHO metabolism—namely, rapid oxidation to form M2, which was catalyzed by CYP3A4, CYP2D6, and cytosol enzyme AOX, versus reduction to regenerate M1, which was catalyzed by an NADPH-dependent reductase in HLMs and HLCs, such as P450 reductase. Thus, the amount of M2 in static in vitro incubations was
considerably underestimated. These findings presented one of the explanations for the discrepancy between in vitro and in vivo metabolic data, and the investigation prompted that the effect of competitive reduction on the static oxidation metabolism should be considered in in vitro metabolic experiments.

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Authorship Contributions


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