Short Communication

LKY-047: First Selective Inhibitor of Cytochrome P450 2J2

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ABSTRACT

Highly selective cytochrome P450 CYP2J2 (CYP2J2) inhibitors suitable for reaction phenotyping are currently not available. (7S)-(−)-(4-Nitro-phenyl)-acrylic acid, 8,8-dimethyl-2-oxo-6,7-dihydro-2H,8H-pyran-3(2H)-yl-7-yl-ester (LKY-047), a decursin derivative, was synthesized, and its inhibitor potencies toward CYP2J2 as well as other cytochrome P450 (P450) enzymes in human liver microsomes (HLM) were evaluated. LKY-047 was demonstrated to be a strong competitive inhibitor of CYP2J2-mediated astemizole O-demethylation and terfenadine hydroxylase activity, with Ki values of 0.96 and 2.61 μM, respectively. It also acted as an uncompetitive inhibitor of CYP2J2-mediated ebastine hydroxylation with a Ki value of 3.61 μM. Preincubation of LKY-047 with HLMs and NADPH did not alter inhibition potency, indicating that it is not a mechanism-based inhibitor. LKY-047 was found to be a selective CYP2J2 inhibitor with no inhibitory effect on other human P450s, such as CYPs 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A (IC50 > 50 μM). These in vitro data support the use of LKY-047 as a selective CYP2J2 inhibitor with potential application in the identification of P450 isoforms responsible for drug metabolism in reaction phenotyping assays.

Introduction

Cytochrome P450 (P450) enzymes represent a family of phase I enzymes responsible for the metabolism of drugs and other xenobiotics (Nelson et al., 1996). Changes in the activity of P450 enzymes cause pharmacokinetic drug-drug interactions (DDIs). In general, DDIs are the result of inhibition of P450-mediated metabolism. Therefore, it is very important to identify P450 isoforms responsible for drug metabolism to predict interaction potential with P450 inhibitor drugs. P450 reaction phenotyping is routinely performed with this aim during the early drug discovery (White, 2000), and the process often yields candidates with lower probability of undergoing drug-drug interactions (Suzuki et al., 2002).

One method of P450 reaction phenotyping involves the incubation of P450-isoform-selective chemical inhibitors with human liver microsomes (HLM) (Williams et al., 2003). The P450-isoform-selective inhibitors available are furafylline for CYP1A2, 3-((pyridine-3-yl)-1H-pyrazol-5-yl)methanamine for CYP2A6, thiota for CYP2B6, montelukast for CYP2C8, sulfaphenazole for CYP2C9, 5-benzylirvinol for CYP2C19, quinidine for CYP2D6, 4-methylpyrazole for CYP2E1, and ketoconazole for CYP3A (Khojasteh et al., 2011) (http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm093664.htm#cytoEnzymes). CYP2J2 is known for its role in metabolizing arachidonic acid to epoxyeicosatrienoic acids, which play significant roles in maintaining the homeostasis of the kidney, heart, and lung by controlling biologic processes with, for example, vasodilatory, anti-inflammatory, and antioxidative effects (Node et al., 2001; Kroeck and Zeldin, 2002; Spector et al., 2004; Spiecker and Liao, 2005). Recent research has uncovered roles for cytochrome P450 2J2 (CYP2J2) in the metabolism of some drugs. CYP2J2 plays a major role in the metabolic clearance of albendazole (Wu et al., 2013b), anidamide (Karkanis et al., 2016), astemizole (Muramoto and Yamazoe, 2001), ebastine (Liu et al., 2006), and terfenadine (Laftie et al., 2006). To date, little data are available on strong selective CYP2J2 inhibitors. Danazol (Lee et al., 2012, 2015), hydroxyebastine (Yoon and Liu, 2011; Lee et al., 2015), and telmisartan (Ren et al., 2013; Lee et al., 2015) have been reported as possessing strong inhibitory action, but their potential against CYP2J2 activity has been evaluated primarily using the recombinant CYP2J2 isoform instead of HLMs. In addition, they showed inhibitory action against other P450 isoforms. For example, danazol inhibited CYP2C8, CYP2C9, and CYP2D6 with IC50 values of 1.95, 1.44, and 2.74 μM, respectively (Lee et al., 2012), whereas hydroxyebastine inhibited CYP2C9, CYP2C19, and CYP2D6 with Ki values of 2.74, 10.2, and 3.83 μM, respectively (Yoon and Liu, 2011). Telmisartan inhibited CYP2C9-mediated tolbutamide hydroxylase activity with an IC50 value of 4.78 μM (Ren et al., 2013). The inhibitory potential of danazol and telmisartan was not evaluated for the enzymes CYP2A6, CYP2B6, and CYP2E1.

In previous research, we screened 50 natural products obtained from medicinal plants for their inhibitory potential against CYP2J2 (Lee et al., 2014b). Decursin showed moderate inhibitory action against CYP2J2-mediated astemizole O-demethylation activity with a Ki value of 8.34 μM. We synthesized four decursin derivatives (Fig. 1) and evaluated the inhibitory potential against CYP2J2 to discover a more potent CYP2J2 inhibitor. In addition, we evaluated their inhibitory potency against the action of nine other P450 isoforms to determine their selectivity for CYP2J2. The availability of a selective inhibitor for CYP2J2 would be very beneficial in assessing the extent of CYP2J2 contribution to drug metabolism.

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ABBREVIATIONS: AST, astemizole; HLM, human liver microsomes; IT-TOF MS, ion trap time-of-flight mass spectrometry; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MBZ, mebendazole; P450, cytochrome P450; TSAHC, 4′-(p-toluenesulfonylami)de)-4-hydroxychalcone.
Materials and Methods

Chemicals and Reagents. Astemizole, O-demethylastemizole, hydroxybenzine, dextromorphan, midazolam, 1′-hydroxydextromorphan, oxemorphone, oxymorphone, tolbutamide, and hydroxytolbutamide were purchased from Toronto Research Chemicals (North York, Canada). Glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PDH), nicotinamide adenine dinucleotide phosphate (NADP+), cinnamaldehyde, 4-cinnamoylaniline, 4-cinnamoylaminopyridine, N-(3-dimethylaminopyrrolidin-2′)-ethylcarbodiimide, aceticaminophen, dextromorphan, phencyanin, bupropion, hydroxybupropion, coumarin, hydroxycoumarin, chlorzoxazone, hydroxychlorzoxazone, amiodiqua, N-desethylamiodiqua, uridine 5′-diphosphoglucuronic acid, mebendazole (MBZ), and terfenadine (internal standard) were obtained from Sigma-Aldrich (St. Louis, MO). Pooled human liver microsomes (HLMs; H9030) were purchased from Xenotech (LexINGTON, KY). Solvents were high-performance liquid chromatography grade, and the other reagents and chemicals were of analytical grade (≥98%; Fisher Scientific Co., Pittsburgh, PA).

Synthesis of N-cinnamoyl-2-deacetyl Derivatives [LKY-047 (N-OAc), 21 (H), 21 (S)-OCH3 (Ok), 446 (Br)]. (7S)-(+)-(4-Nitro-phenyl)-acrylic acid, 8,8-dimethyl-2-oxo-6,7-dihydro-2H,8H-pyrano[3,2-g]chromen-7-yl-ester (LKY-047). The mixture of 4-nitroacrylic acid (1.2 Eq), N-(3-dimethylaminopyrrolidin-2′)-ethylcarbodiimide hydrochloride (2 Eq), and 4-cinnamoylaminopyridine (0.4 Eq) was dissolved in dichloromethane anhydrous. After (d+)-deacrylic (1 Eq) was added and the reaction mixture stirred at room temperature for 8–12 hours, the solvent was removed under vacuum. The residue was purified by silica gel column chromatography (25% ethyl acetate in n-hexane) to obtain LKY-047. Yield 65.7%, light yellow solid, mp: 193–194°C (lit. mp: 193°C (Phuc et al., 2017)).

(7S)-(+)-3-(4-Bromo-phenyl)-acrylic acid, 8,8-dimethyl-2-oxo-6,7-dihydro-2H,8H-pyrano[3,2-g]chromen-7-yl-ester (LKY-046). LKY-046 was prepared from 4-bromocinnamic acid using the same procedure as for LKY-047. Yield 62.2%, white solid, mp: 120°C (lit. mp: 181°C (Yoon and Liu, 2011)).

Chemical structures of LKY-021 (A), -024 (B), -046 (C), and -047 (D).

Fig. 1. Chemical structure of LKY-021 (A), -024 (B), -046 (C), and -047 (D).
CYPs 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A. A previously developed substrate cocktail method that enables simultaneous incubation and measurement of compound inhibitory potential against each P450 isoform was used to obtain IC50 values (Kim et al., 2005; Joo and Liu, 2013). The P450-isoform-selective substrates were used at concentrations approximately equal to their respective Km values: 50 μM for phenacetin, 5 μM for coumarin, 50 μM for bupropion, 1 μM for amodiaquine, 100 μM for tolbutamide, 20 μM for omeprazole, 5 μM for dextromethorphan, 50 μM for chlorzoxazone, and 5 μM for midazolam.

Following a 15-minute incubation period in HLMs (0.25 mg/ml) in the presence or absence of the inhibitor compound, the reaction was terminated and the mixtures centrifuged. Aliquots of the supernatants were analyzed by LC-MS/MS as described previously (Kim et al., 2005; Joo and Liu, 2013), with some modifications.

Data Analysis. The IC50 values were determined using the WinNonlin software (Pharsight, Mountain View, CA). The apparent kinetic parameters for inhibitory activity (Ki) were first estimated by graphical methods, such as Lineweaver–Burk, Dixon, and secondary reciprocal plots, and were more accurately determined by nonlinear least squares regression analysis, on the basis of the best enzyme inhibition model (Segal, 1976) using the WinNonlin software. In our experiments, the inhibition data were well fitted by the competitive inhibition model. The models tested included pure and partial competitive inhibition, noncompetitive inhibition, uncompetitive inhibition, and mixed-type inhibition.

Results and Discussion

Several studies conducted in recent years have reported a variety of potent and selective CYP2J2 inhibitors. Telmisartan and flunarizine with Ki values of 0.42 and 0.94 μM, respectively, could be used as moderately selective CYP2J2 inhibitors, but both compounds also inhibit CYP2C9 and CYP2D6 with IC50 values of 4.78 and 7.89 μM, respectively (Ren et al., 2013), therefore demonstrating only a greater than 10-fold selectivity when evaluated against five major P450 isoforms (CYPs 1A2, 2C9, 2C19, 2D6, and 3A). Danazol also showed no more than 15-fold selectivity for CYP2J2 when tested against these five P450 isoforms, although it noncompetitively inhibited CYP2J2-mediated astemizole O-demethylation activity (IC50 = 0.07 μM) (Lee et al., 2015). Additionally, the selectivity measured did not take into consideration other minor but important P450 isoforms, such as CYP2A6.

<table>
<thead>
<tr>
<th>P450 enzyme</th>
<th>Marker activity</th>
<th>LKY-021</th>
<th>LKY-024</th>
<th>LKY-046</th>
<th>LKY-047</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>Phenacetin O-deethylase</td>
<td>42.1</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
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<tr>
<td>2A6</td>
<td>Coumarin 7-hydroxylase</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>2B6</td>
<td>Bupropion 4-hydroxylase</td>
<td>18.5</td>
<td>28.8</td>
<td>41.7</td>
<td>&gt;50</td>
</tr>
<tr>
<td>2C8</td>
<td>Amodiaquine N-deethylase</td>
<td>37.8</td>
<td>&gt;50</td>
<td>47.1</td>
<td>&gt;50</td>
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<tr>
<td>2C9</td>
<td>Tolbutamide 4-hydroxylase</td>
<td>&gt;50</td>
<td>16.4</td>
<td>&gt;50</td>
<td>&gt;50</td>
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<tr>
<td>2C19</td>
<td>Omeprazole 5-hydroxylase</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>2D6</td>
<td>Dextromethorphan O-demethylation</td>
<td>37.3</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
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<tr>
<td>2E1</td>
<td>Chlorzoxazone 6-hydroxylase</td>
<td>&gt;50</td>
<td>&gt;50</td>
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<td>&gt;50</td>
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<td>3A</td>
<td>Midazolam 1'-hydroxylase</td>
<td>&gt;50</td>
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<td>&gt;50</td>
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<tr>
<td>2J2</td>
<td>Astemizole O-demethylase</td>
<td>&gt;50</td>
<td>27.2</td>
<td>47.1</td>
<td>1.7</td>
</tr>
</tbody>
</table>

*Values are presented as average of triplicate determination (n = 3).

Fig. 2. Representative of Lineweaver-Burk plots obtained from a kinetic study of CYP2J2-mediated astemizole O-demethylation (A) and terfenadine hydroxylation (B) in the presence of different concentrations of LKY-B047 in pooled HLMs. An increasing concentration of astemizole and terfenadine (0.2, 1, and 5 μM) was incubated with HLMs (0.25 mg/ml, Xenotech H0630) and NADPH generating system at 37°C for 20 minutes in the presence or absence of LKY-047 (0 (●), 0.2 (○), 0.5 (■■■), 2 (□), 5 (■), and 10 μM (□)). The inhibition data were fit to a competitive inhibition model. The data are shown as average of triplicate determination (n = 3).
In this study, the inhibitory effects of four decursin derivatives (LKY-021, -024, -046, and -047) on CYP2J2-mediated astemizole O-demethylase activity were evaluated in HLMs (Table 1). LKY-047, the p-nitrobenzyl derivative of decursin, showed greater inhibitory potency against CYP2J2 activity than all the other compounds tested (IC$_{50}$ = 1.7 μM). The inhibition potency of LKY-047 was also higher than that of decursin (IC$_{50}$ = 6.95 μM) (Lee et al., 2014b). LKY-024 (p-methoxybenzyl derivative) and -046 (p-bromobenzyl derivative) showed weak inhibitory action against CYP2J2 activity, with IC$_{50}$ values of 27.2 and 47.1 μM, respectively, whereas LKY-021 (benzyl derivative) had no inhibitory effect at all (IC$_{50}$ > 50 μM).

We further studied LKY-047 to clarify its inhibition mechanism. LKY-047 inhibited CYP2J2-mediated astemizole O-demethylase and terfenadine hydroxylase activities with $K_i$ values of 0.96 ± 0.12 and 2.61 ± 0.28 μM, respectively (Fig. 2). The Lineweaver-Burk plots intersected on the y-axis (Harold, 1976; Waldrop, 2009), indicating that LKY-047 competitively inhibited the enzyme activities of both astemizole (Fig. 2A) and terfenadine (Fig. 2B) when the substrate concentration ranged between 0.2 and 5.0 μM. The inhibitory potency of LKY-047 against CYP2J2 activity in HLMs was stronger than that of previously reported CYP2J2 inhibitors, including decursin [$K_i$ = 8.3 μM (Lee et al., 2014b)], haloperidol [IC$_{50}$ = 14.5 μM (Liu, 2011)], tanshinone IIA [IC$_{50}$ = 2.5 μM (Jeon et al., 2015)], thelepohic acid [IC$_{50}$ = 3.25 μM (Wu et al., 2013a)], and TSAHC [IC$_{50}$ = 2.03 μM (Lee et al., 2014a)].

Inhibitory potential of LKY-047 against CYP2J2-mediated AST O-demethylase activity in HLMs preincubated in the presence of NADPH-generating system was a little bit lower than that in untreated HLMs (IC$_{50}$ values of 4.2 and 1.7 μM, with and without NADPH preincubation, respectively), suggesting that LKY-047 is not a time-dependent inhibitor (Supplemental Fig. 1).

Danazol (Lee et al., 2015) and telmisartan (Ren et al., 2013) showed 15- and 10-fold selectivity for CYP2J2 inhibition, respectively. To evaluate the selectivity of the tested inhibitors for CYP2J2, we examined the inhibitory activities of four LKY compounds against nine different P450 isoforms, including CYPs 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A, in a 0.25 mg/ml HLMs, under conditions common in phenotyping experiments. As shown in Table 1, LKY-047 did not exhibit inhibitory action against the other P450 isoforms tested (IC$_{50}$ > 50 μM), whereas LKY-021, -024, and -046 showed inhibitory effects against CYPs 1A2, CYP2B6, CYP2C8, CYP2C9, or CYP2D6 enzymes. LKY-047 has shown the greatest selectivity from among the CYP2J2 inhibitors investigated thus far. At 20 μM LKY-047 concentration, approximately 20-fold greater than the $K_i$ value, LKY-047 was found to inhibit CYP2J2 by 85.3%, and only slightly affecting the enzyme activities of the other P450s tested. LKY-047 weakly inhibited CYP2D6 enzyme activity (37.2%) at 20 μM concentration (Fig. 3).

In conclusion, we report that (7S)-(+)-(4-nitro-phenyl)-acrylic acid, 8,8-dimethyl-2-oxo-6,7-dihydro-2H,8H-pyranono[3,2-g]chromen-7-yl-ester (LKY-047) is a potent and selective reversible competitive inhibitor of CYP2J2. LKY-047, when evaluated for astemizole O-demethylase inhibitory activity against CYP2J2 as well as nine other P450 isoforms, exhibited 50-fold selectivity for CYP2J2. This new CYP2J2 inhibitor could be useful as a selective CYP2J2 inhibitor in P450 reaction phenotyping studies and the biologic function study of this enzyme.

**Authorship Contributions**

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**BK21 Plus KNU Multi-Omics-Based Creative Drug Research Team**

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**Participated in research design:** Song, Liu.

**Conducted experiments:** Phuc, Wu, O, Lee.
Contributed new reagents or analytic tools: Song, Liu.
Performed data analysis: Song, Oh, Liu.
Wrote or contributed to the writing of the manuscript: Song, Nguyen, Liu.

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