Identification of metabolites of \(N\)-(5-benzoyl-2-(4-(2-methoxyphenyl)piperazin-1-yl)thiazol-4-yl)pivalamide including CYP3A4-mediated \(C\)-demethylation in human liver microsomes with high-resolution/high-accuracy tandem mass spectrometry

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Abstract

KRO-105714, \(N\)-(5-benzoyl-2-(4-(2-methoxyphenyl)piperazin-1-yl)thiazol-4-yl)pivalamide, is a 2,4,5-trisubstituted 1,3-thiazole derivative that exerts anti-atopic dermatitis activity via robust suppression of the sphingosylphosphorylcholine receptor. This study used high-resolution/high-accuracy tandem mass spectroscopy (HRMS) and recombinant complementary DNA (cDNA)-expressed cytochrome P450 isoforms (P450) to identify the metabolic pathway and metabolites of KRO-105714 in human liver microsomes (HLMs) as therapeutic agents for inflammation. The incubation of KRO-105714 with pooled HLMs in the presence of nicotinamide adenine dinucleotide phosphate generated four metabolites (M1–M4). The metabolites were identified using HRMS and confirmed using synthetic standards for M2 and M4. M1 and M2 were identified as mono-hydroxylated metabolites, and M3 and M4 were further identified as \(O\)-demethyl and \(C\)-demethyl-KRO-105714, respectively. In the inhibition study with selective CYP3A4 inhibitors and incubation in recombinant cDNA-expressed CYPs, all the metabolites of KRO-105714 were formed by CYP3A4 in HLMs. The CYP3A4-mediated formation of M4 from M2 was confirmed via incubation of M2 in HLMs. These results showed that the unusual \(C\)-demethylated metabolite M4 was generated from mono-hydroxyl metabolite M2 via a CYP3A4-mediated enzymatic reaction in HLMs.
Introduction

Sphingosylphosphorylcholine (SPC) is structurally similar to sphingosine-1-phosphate, which along with lysophosphatidic acid (LPA) is classified as a lysophospholipid. These substances act as important signaling intermediates in the cell proliferation, migration, and inflammatory responses of immune function (Nixon et al., 2008). SPC is generated by the action of sphingomyelindeacylase from sphingomyelin, a structural component of cell membranes (Higuchi et al., 2000; Nixon et al., 2008). Furthermore, SPC displays a wide range of biological activities such as angiogenesis, activation of calcium signaling, protection of cells from apoptosis, and stimulation of nitric oxide production (Desai and Spiegel, 1991; Chen et al., 1998; Boguslawski et al., 2000; Higuchi et al., 2000; Jeon et al., 2005). Atopic dermatitis is an example of an SPC-related disease (Imokawa, 2009). Patients with the disease experience pain, itching, and poor quality of life. Direct intradermal injection of SPC has shown that SPC causes itching owing to its structural similarity to LPA (Hashimoto et al., 2004).

KRO-105714, \( N-(5\text{-benzoyl}-2-(4\text{-methoxyphenyl})\text{piperazin-1-yl})\text{thiazol-4-yl})\text{pivalamide, is a 2,4,5-trisubstituted 1,3-thiazole derivative and therapeutic agent for anti-inflammatory diseases induced by SPC (Gong et al., 2009). The SPC receptor is inhibited by these novel derivatives, and the anti-inflammatory effect of KRO-105714 has been confirmed using dermal cells derived from humans and mice. As a part of the preclinical study of KRO-105714, we investigated its \textit{in vitro} metabolic pathway in human liver microsomes (HLMs) using high-resolution/high-accuracy tandem mass spectroscopy (HRMS) coupled with high-performance liquid chromatography (HPLC) and complementary DNA (cDNA)-expressed CYPs. \)
A number of analytical techniques have been developed for detection and determination in drug metabolism studies. For example, HRMS techniques such as quadrupole time-of-flight, Fourier transform ion cyclotron resonance mass spectrometry (MS), and hybrid high-resolution tandem mass spectrometry (MS/MS; Orbitrap) have been applied to identify metabolite structure. Orbitrap, the newest addition to the HRMS family (Zubarev and Makarov, 2013), is based on high resolution in excess of 1,000,000 full width at half maximum. In investigations of drug metabolites, accurate measurement using HRMS is beneficial in that the change in elemental compositions is performed with accurate masses and charge states below 5 ppm (Lee et al., 2013).

In the present study, KRO-105714 was metabolized to four metabolites including C-demethyl, O-demethyl, and two mono-hydroxyl KRO-105714s. Among these reactions, C-demethyl KRO-105714 is an uncommon product of metabolic enzymatic reactions; therefore, further investigation of the metabolism of KRO-105714 was undertaken. The purpose of the present study was to characterize the in vitro metabolism of KRO-105714 and identify the metabolic pathway of enzymatic C-demethyl KRO-105714 in HLMs.
Materials and Methods

Chemicals

KRO-105714 (purity > 99%) was prepared via chemical synthesis. 150-Donor pooled mixed gender HLMs and human recombinant cDNA-expressed CYP isoforms were purchased from BD Gentest (Woburn, MA, USA). Glucose 6-phosphate, β-glucose 6-phosphate dehydrogenase, potassium phosphate dibasic, and potassium phosphate monobasic were obtained from Sigma-Aldrich (St. Louis, MO, USA). β-NADPH was obtained from Oriental Yeast Co. (Tokyo, Japan). Solvents were HPLC grade (Merck KGaA, Darmstadt, Germany), and the other chemicals were of the highest quality available.

Biotransformation of KRO-105714

KRO-105714 (50 μM final concentration) was incubated with 1 mg/mL pooled HLMs at 37°C for 90 min in the presence of an NADPH-generating system (NGS) containing 0.1 M glucose 6-phosphate, 10 mg/ml β-NADPH, and 1.0 unit/mL β-glucose 6-phosphate dehydrogenase at a final volume of 100 μl. The reaction was stopped via addition of 1 ml of ethyl acetate, and after mixing for 1 min, the samples were centrifuged at 13,000 rpm for 5 min at 20°C. Then, 900 μl of supernatant was separated and removed via evaporation. The samples were reconstituted with 100 μl of acetonitrile in 0.1% formic acid and centrifuged at 13,000 rpm for 10 min at 20°C. The supernatants were diluted with 400-μl LC-grade water and injected into an HPLC column for linear trap quadrupole Orbitrap analysis.
Chemical inhibition studies and metabolism with cDNA-expressed CYP isoforms

The inhibitory effects of known CYP isoform-selective inhibitors and the metabolism of KRO-105714 were evaluated to determine the CYP isoforms involved in the metabolic pathway. Well-characterized CYP-selective inhibitors - i.e., α-naphthoflavone for CYP1A2 (1 and 5 μM), tranylcypromine for CYP2A6 (1 and 5 μM), quercetin for CYP2C8 (5 and 20 μM), fluconazole for CYP2C9 (5 and 10 μM), ticlopidine for CYP2C19 (2 and 10 μM), quinidine for CYP2D6 (10 and 50 μM), diethyldithiocarbamate for CYP2E1 (20 and 100 μM), and ketoconazole for CYP3A4 (2 and 10 μM) - were incubated with KRO-105714 (Newton et al., 1995; Ko et al., 2000; Lee et al., 2008; Khojasteh et al., 2011). Incubations were performed with CYP-selective inhibitor pooled HLMs (1 mg/ml) and KRO-105714 (50 μM). The activity of all the inhibitors was compared with that of inhibitor-free controls.

The incubation mixtures, including 5 μl of recombinant cDNA-expressed CYP (diluted to 10 pmol/mL with phosphate buffer, pH 7.4) and KRO-105714 (50 μM) reconstituted in 0.1 M phosphate buffer (pH 7.4), were pre-incubated for 5 min at 37°C. The reaction was started by adding the NGS, and the reaction mixtures (final volumes of 100 μl) were incubated for 60 min at 37°C in a thermo shaker. The reaction was stopped by adding 500-μl ethyl acetate after mixing and centrifuged at 13,000 rpm for 10 min at 20°C. In all experiments, KRO-105714 was dissolved in ethyl acetate; the solvent was subsequently removed via evaporation to dryness under reduced pressure. The residues were reconstituted with 100 μl of 50% acetonitrile (in formic acid 0.1%), and then the internal standard solution (reserpine, 3.0 ng/mL; 5 μl) was added. The supernatants were transferred to auto-sampler vials, and 10-μl
Aliquots were used for liquid chromatography-tandem mass spectroscopy (LC-MS/MS) analysis.

**Metabolite structures confirmation by co-elution**

To confirm structures of M2 (C-hydroxylation) and M4 (C-demethylation), the retention time on HPLC elution and MS$^2$ and MS$^3$ spectra were compared between generated metabolites in HLMs and synthetic standards (Supplemental Methods). Metabolites M2 and M4 were prepared from incubation in HLMs, reconstituted to 200 μl in 100% acetonitrile (in formic acid 0.1%), and separated into halves. Synthetic standards for M2 and M4 were mixed to 0.1 μM in 200 μL and separated into halves. Incubated samples were mixed with the synthetic mixtures, respectively. The prepared samples were submitted to LC-MS/MS analysis.

**In Vitro Enzyme Kinetics**

Kinetic parameters such as apparent $K_m$ (apparent affinity), $V_{max}$ (apparent maximum reaction velocity) and $Cl_{int}$ (apparent intrinsic clearance) for KRO-105714 C-demethylation were determined in HLMs. The reaction mixture (100 μl) was composed of 0.5 mg protein/mL HLMs containing KRO-105714 (0 to 50 μM) or synthetic standard M2 (0 to 20 μM), respectively. The concentrations of each ranged from and with the NGS. The reaction was started with incubation for 60 min at 37°C. The kinetic parameters $K_m$, $V_{max}$ and $Cl_{int}$ were determined using nonlinear regression analysis (SigmaPlot; Systat Software Inc., San Jose, CA, USA).
Instruments

HRMS experiments were conducted on a Finnigan linear trap quadrupole Orbitrap XL hybrid Fourier transform MS system (Thermo Fisher Scientific Inc., MA, USA). For LC analysis, an Inertsil ODS-3, 3 μM (2.1 × 150 mm, GL Science Inc, Tokyo) column was used. The mobile phases consisted of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in LC-grade water (B). Gradient elution was conducted as follows: B was linearly increased from 30% to 95% for 7 min, retained at 95% for an additional 5 min, and then directly decreased to 30% and held at 30% for equilibration for 5 min at a flow rate of 200 μl/min. The instrument was operated in full-scan mode. Electrospray ionization was performed in positive ion mode. The operating conditions were as follows: spray voltage, 4.5 kV; capillary voltage and temperature, 34 V and 320°C, respectively; sheath and auxiliary gas flow rates, 40 and 15 (arbitrary units); and tube lens, 100 V. Data acquisition and analysis were performed using Xcalibur (version 2.2).
Results

Metabolism of KRO-105714 in HLMs

Representative HPLC chromatograms of metabolism after incubation of KRO-105714 with HLMs and an NGS were studied, characterized, and experimentally identified using LC-MS/MS. A typical chromatogram of KRO-105714 and its four metabolites (M1–M4) is shown in Figure 1. Observation of [M+H]+ ions was performed at m/z 495 for M1, via monohydroxylation (+16 Da) for M2, at m/z 465 for M3, and via demethylation (−14 Da) for M4, with retention times of 9.0, 9.5, 10.7, and 11.2 min, respectively (Figure 1). The [M+H]+ ions of the metabolites were identified with full-scan MS detection. No metabolites were obtained without the NGS in HLM and human recombinant cDNA-expressed CYPs, thereby showing the involvement of CYP enzymes and indicating that the metabolism of KRO-105714 is NGS dependent.

Characterization of metabolites in HLMs

Metabolite structures were characterized and assigned based on their HRMS, MS2, and MS3 product ion masses (Figures 2 and 3). HRMS analysis confirmed the elemental composition of the product ions at less than 5 ppm (Table 1). The protonated molecular ion of KRO-105714 was observed at m/z 479.21138 (C26H31O3N4S1) at 11.7 min (see Figure 2). The protonated ion at m/z 395.15397 (C21H23O2N4S1) was generated as a dominant fragment ion of MS2, which indicates the loss of an alkoxy group moiety (C5H8O1). MS3 fragmentation generated a protonated ion at m/z 377.14358, indicating the loss of H2O and another at m/z
289.11227, which showed the loss of an acetophenone moiety (C₇H₆O₁). The protonated ion at \( m/z \) 246.06998 (C₁₂H₁₂O₁N₃S₁) was generated after the loss of cyclohexane (C₉H₉N₁).

The precursor ions of M1 and M2 were determined at \( m/z \) 495.20715 and 495.20709, respectively, which are 15.9945 Da higher than that of the parent KRO-105714, indicating that the mono-hydroxylated elemental compositions of protonated M1 and M2 were C₂₆H₃₁O₄N₄S₁ (Table 1). The protonated M1 at \( m/z \) 411.14911 was generated as a dominant fragment ion of MS², signaling the loss of an alkoxy group moiety, and MS³ fragmentation generated ions at \( m/z \) 393.13855 (C₂₁H₂₁O₂N₄S₁), thus indicating the loss of H₂O; generated ions of \( m/z \) 305.10730 (C₁₄H₁₇O₂N₄S₁) indicated the loss of an acetophenone moiety (Figure 3A). The protonated ion at \( m/z \) 246.07007 (C₁₂H₁₂O₁N₃S₁) was generated after cleavage of a cyclohexane (see Figure 3A). Metabolite M2, the major metabolite peak, gave an [M+H]⁺ ion at \( m/z \) 495.20715, suggesting that one oxygen atom was inserted from the parent compound. The MS² spectrum of M2 characteristic major product ions at \( m/z \) 395.15424 (C₂₁H₂₃O₂N₄S₁) suggests the loss of an alkoxy group moiety. The MS³ spectrum of ions at \( m/z \) 289.11237 (C₁₄H₁₇O₁N₄S₁) and \( m/z \) 246.07004 (C₁₂H₁₂O₁N₃S₁) was postulated to have been generated by cleavage of an acetophenone moiety and a cyclohexane moiety from the protonated molecular ion (Figure 3B).

Metabolites M3 and M4 had retention times of 10.7 and 11.2 min on HPLC and showed protonated ions at \( m/z \) 465.19577 and \( m/z \) 465.19604, respectively, which are 14.0156 Da lower than that of the parent KRO-105714 (see Table 1). The elemental compositions of protonated M3 and M4 were C₂₅H₃₀O₃N₄S₁, indicating demethylation. Metabolite M3 produced a protonated molecule at \( m/z \) 465, indicating an O-demethylated metabolite of KRO-105714. M3 generated characteristic product ions at \( m/z \) 381.13864 (C₂₀H₂₁O₂N₄S₁),
suggesting the loss of an alkoxy group moiety from MS² fragmentation. The MS³ spectrum of the ion at $m/z$ 246 ($C_{12}H_{12}O_1N_3S_1$) was postulated to have been generated by the cleavage of a cyclohexane moiety from the protonated molecular ion (Figure 3C). Metabolite M4, the minor metabolite peak, gave an $[M+H]^+$ ion at $m/z$ 465.19604, suggesting that a methyl group was dissociated from the parent compound. The MS² spectrum of characteristic M4 product ions at $m/z$ 395.15421 ($C_{21}H_{23}O_2N_4S_1$) suggests the loss of an alkoxy group moiety. The MS³ spectrum pattern identifying M2 fragmentation is the same as that of M4 (Figure 3D). Therefore, M4 was proposed as a C-demethylated metabolite with an alkoxy group moiety.

**Identification of M2 and M4 metabolism**

Metabolites M2 and M4 were further confirmed through comparison with synthetic standards (Supplemental Figure 1). These synthetic standards showed the same MS² and MS³ patterns as those of M2 and M4 (Figure 3 and Supplemental Figure 2). Co-elution with authentic metabolites using the same LC conditions revealed matching mass peaks and retention times with synthetic standards (Figure 4). After simultaneous analysis of metabolites and synthetic standards, the structures of metabolites M2 and M4 were confirmed to result from mono-hydroxylation and C-demethylation, respectively, of an alkoxy group.

We then hypothesized that C-demethylation metabolite M4 could be generated from the C-hydroxylation of metabolite M2 via removal of methanol in the alkoxy group. To confirm this hypothesis, we incubated M2 in HLMs and characterized the generated metabolites. M4 was collected using equivalent LC-MS/MS conditions, and the postulated metabolic pathway showing that M4 generated from M2 was confirmed (Supplemental Figure 3). Moreover, kinetic analyses of the formation of KRO-105714 to M2 and M2 to M4 were conducted in
HLMs, respectively. The Michaelis-Menten plots of KRO-105714 and M2 metabolism in HLMs is shown in Supplemental Figure 4. The kinetic parameters of KRO-105714 and synthetic standard M2 metabolism to M2 and M4 components, respectively, are summarized in Table 2. The $K_m$ values of M2 and M4 formation from KRO-105714 and M2 were 4.4 and 2.3 $\mu$M, respectively. The $V_{max}$ for generation of M2 and M4 were calculated as 39.4 and 7.2 pmole/min per mg protein, and the intrinsic clearance of KRO-105714 and M2 were 9.0 and 3.1 $\mu$L/min per mg protein, respectively.

**Identification of major CYP isoforms involved in C-demethylation of KRO-10714**

The CYP isozyms responsible for the oxidative metabolism of KRO-105714 were identified using both selective inhibitors of CYP enzymes and incubation with human recombinant cDNA-expressed CYPs. The effects of selective inhibitors of CYP enzymes on KRO-105714 metabolism in HLMs are shown in Table 3. Ketoconazole, a selective inhibitor of CYP3A4, strongly inhibited the formation of all metabolites in a concentration-dependent manner. The formation of M3 was slightly inhibited by quinidine as a selective CYP2D6 inhibitor. Other CYP-specific inhibitors did not significantly inhibit the formation of KRO-105714 metabolites at the concentration used in the current study. For further confirmation of the CYPs primarily responsible for the metabolism of KRO-105714, we incubated KRO-105714 with human recombinant cDNA-expressed CYPs. CYP3A4 clearly resulted in the formation of M1, M2, M3, and M4, suggesting that CYP3A4 is the main CYP responsible for the metabolism of KRO-105714 (Figure 5 and Supplemental Figure 5). The formation of metabolites indicated the involvement of CYP3A4 in the metabolism of KRO-105714 in HLMs.
**Discussion**

The metabolic disposition of KRO-105714 in HLMs had not been researched previously. In this study, we attempted to determine the postulated metabolites, metabolic enzymes, and metabolic pathway of KRO-105714 in HLMs. KRO-105714 is primarily metabolized to mono-hydroxylation (M1), C-hydroxylation (M2), demethylation (M3), and C-demethylation (M4) metabolites in HLMs (Figure 6). In particular, the structure of M4 was partly unexpected, as C-demethylation is uncommon during drug metabolism, whereas O- and N-demethylations are frequently observed in the biotransformation of xenobiotics (Burke et al., 1994; Coutts et al., 1994; Ertl et al., 1999).

To confirm the unusual C-demethylation of KRO-105714, we analyzed the elemental composition of generated metabolites using Orbitrap MS. A loss of 14.01534 Da in M4 compared to the composition of KRO-105714 indicated that the former is a demethylated metabolite, and MS² and MS³ analyses using HRMS confirmed the C-methylation of KRO-105714. In addition, we prepared synthetic C-demethylated KRO-105714 as a gold standard, and the structure of M4 was confirmed through comparison of MS² and MS³ fragmentation and chromatographic co-elution. Moreover, the generation mechanism of C-demethylation in an alkoxy group metabolite was proved by observing the enzymatic formation of M4 in HLMs after incubation with synthetic M2 and cDNA-expressed CYP3A4.

Several previous studies have demonstrated the mechanism of C-demethylation during drug metabolism. A C-demethylated metabolite is generated via mono-hydroxylation by non-enzymatic decarboxylation of a carboxyl metabolite in rat liver microsomes (Yoo et al., 2008). Another possible mechanism involves the oxidative metabolite to form aldehyde metabolites and an olefin metabolite followed by CYP-mediated deformylation (Prakash et al., 2008).
Aplidine, a potent marine-derived anti-cancer drug, is C-demethylated at the (R)-N(methyl)-leucine group by CYP2A6 in pooled HLMs (Brandon et al., 2007). In the present study, CYP-reaction-enzyme phenotyping strongly suggested that KRO-105714 is primarily metabolized by mono-hydroxylation and C-demethylation at the alkoxy group moiety by CYP3A4 in HLMs. In short, the most dominant CYP3A4 in HLMs might contribute to the unusual drug metabolism depending on the partial structure of the target.

In conclusion, we demonstrated that KRO-105714 is metabolized to two mono-hydroxyl metabolites (M1 and M2), an O-demethyl metabolite (M3), and a C-demethyl metabolite (M4) in HLMs. The unusual C-demethylation of KRO-105714 occurs in two steps via CYP3A4 and is enzyme dependent.
Authorship Contributions

Participated in research design: Song, M., Lee, S.

Conducted experiments: Song, M., Lee, D., Kim, S.J.

Contributed new reagents or analytic tools: Lee, T., Lee, J.

Performed data analysis: Bae, J., Lee, J., Gong, Y., Lee, S.

Wrote or contributed to the writing of the manuscript: Song, M. Lee, S.
DMD #57570

References


Hashimoto T, Ohata H, and Momose K (2004) Itch-scratch responses induced by


Footnotes

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Legends for Figures

Figure 1. Extracted ion chromatograms for \( N-(5\text{-benzoyl}-2\-(4\-(2\text{-methoxyphenyl})piperazin-1-yl)thiazol-4-yl)pivalamide \) (KRO-105714) and its metabolites after incubation with human liver microsomes in the presence of a nicotinamide adenine dinucleotide phosphate (NADPH)-generating system.

Figure 2. MS\(^2\) and MS\(^3\) spectra and proposed mechanism for the formation of the product ions of protonated KRO-105714.

Figure 3. MS\(^2\) and MS\(^3\) spectra and proposed mechanism for the formation of the product ions of protonated M1 (A), M2 (B), M3 (C), and M4 (D).

Figure 4. Extracted ion chromatograms for M2 (A) and M4 (B) of enzyme-derived KRO-105714 metabolites (human liver microsome [HLM]), the synthetic standard (STD), and their mixture (Mixture).

Figure 5. Representative ion chromatograms of the extracts resulting from the incubation of KRO-105714 with human recombinant complementary DNA-expressed CYP2C9 (A), CYP2D6, (B), CYP3A4 (C), and CYP3A5 (D). Incubations were conducted for 1 h with 10 pmol/mL of CYP and 50 \( \mu \)M KRO-105714 in the presence of an NADPH-generating system. An asterisk indicates interference peaks. CYP, cytochrome P450.

Figure 6. Postulated metabolic pathways of KRO-105714 in human liver microsomes. CYP, cytochrome P450.
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<td>C_{14}H_{17}O_{2}N_{4}S_{1}</td>
<td>2.198</td>
</tr>
<tr>
<td>M4</td>
<td>465.19604</td>
<td>C_{37}H_{37}O_{3}N_{5}S_{1}</td>
<td>1.187</td>
<td>13</td>
<td>395.15421</td>
<td>C_{21}H_{23}O_{2}N_{4}S_{1}</td>
<td>1.485</td>
</tr>
<tr>
<td></td>
<td>395.15427</td>
<td>C_{21}H_{23}O_{2}N_{4}S_{1}</td>
<td>1.637</td>
<td>21</td>
<td>378.12784</td>
<td>C_{14}H_{17}O_{2}N_{4}S_{1}</td>
<td>2.206</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>289.11234</td>
<td>C_{13}H_{16}O_{2}N_{3}S_{1}</td>
<td>2.012</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>246.07004</td>
<td>C_{14}H_{17}O_{2}N_{4}S_{1}</td>
<td>1.954</td>
</tr>
</tbody>
</table>

KRO-105714, N-(5-benzyol-2-(4-(2-methoxyphenyl)piperazin-1-yl)thiazol-4-yl)pivalamide; CE, collision energy.
Table 2. Kinetics of M2 and M4 formation in human liver microsomes

<table>
<thead>
<tr>
<th>Parameters</th>
<th>KRO-105714 to M2*</th>
<th>M2 to M4</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ ($\mu$M)</td>
<td>4.4</td>
<td>2.3</td>
</tr>
<tr>
<td>$V_{max}$ (pmole/min per mg protein)</td>
<td>39.4</td>
<td>7.2</td>
</tr>
<tr>
<td>$C_{int}$ ($\mu$L/min per mg protein)</td>
<td>9.0</td>
<td>3.1</td>
</tr>
</tbody>
</table>

*Amount of product is a combination of generated M2 and M4.

KRO-105714, $N$-(5-benzoyl-2-(4-(2-methoxyphenyl)piperazin-1-yl)thiazol-4-yl)pivalamide; $K_m$, apparent affinity; $V_{max}$, apparent maximum reaction velocity; $C_{int}$, apparent intrinsic clearance.
Table 3. Effects of CYP inhibitors on the metabolism of KRO-105714 in human liver microsomes

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration (μM)</th>
<th>Relative percentage of control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>M1</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>α-Naphthoflavone</td>
<td>1</td>
<td>97</td>
</tr>
<tr>
<td>(CYP1A2)</td>
<td>5</td>
<td>93</td>
</tr>
<tr>
<td>Tranylcypromine</td>
<td>1</td>
<td>90</td>
</tr>
<tr>
<td>(CYP2A6)</td>
<td>5</td>
<td>85</td>
</tr>
<tr>
<td>Quercetin</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>(CYP2C8)</td>
<td>20</td>
<td>88</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>5</td>
<td>94</td>
</tr>
<tr>
<td>(CYP2C9)</td>
<td>10</td>
<td>99</td>
</tr>
<tr>
<td>Ticlopidine</td>
<td>2</td>
<td>90</td>
</tr>
<tr>
<td>(CYP2C19)</td>
<td>10</td>
<td>87</td>
</tr>
<tr>
<td>Quinidine</td>
<td>10</td>
<td>84</td>
</tr>
<tr>
<td>(CYP2D6)</td>
<td>50</td>
<td>73</td>
</tr>
<tr>
<td>Diethyldithiocarbamate</td>
<td>20</td>
<td>91</td>
</tr>
<tr>
<td>(CYP2E1)</td>
<td>100</td>
<td>97</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>2</td>
<td>43</td>
</tr>
<tr>
<td>(CYP3A4)</td>
<td>10</td>
<td>4</td>
</tr>
</tbody>
</table>

Data represent the means of duplicate determinations.

KRO-105714, N-(5-benzoyl-2-(4-(2-methoxyphenyl)piperazin-1-yl)thiazol-4-yl)pivalamide.
Figure 1
Figure 2

KRO-105714

\([\text{M+H}]^+ 479\)

\(m/z 395\)

\((C_{21}H_{23}O_2N_4S_1)\)

\(-84\ \text{Da}\)

479 MS\(^2\) > 395 MS\(^3\)

292.11191

289.11227

-106 Da

246.06998

377.14358

395.15411

395.15397

479 MS\(^2\)

\([\text{M+H}]^+ 479.21138\)

292.11191

289.11227

-106 Da

246.06998

377.14358

395.15411

395.15397

479.21138

-84 Da
Figure 3

(A) M1

\[ [\text{M+H}]^+ 495 \rightarrow m/z 411 \rightarrow m/z 246 \]

495 MS\textsuperscript{2} > 411 MS\textsuperscript{3}

Relative Abundance

(B) M2

\[ [\text{M+H}]^+ 495 \rightarrow m/z 395 \rightarrow m/z 246 \]

495 MS\textsuperscript{2} > 395 MS\textsuperscript{3}
Figure 4

(A) M2

(B) M4

HLM

STD

Mixture

Relative Abundance

Time (min)
Figure 5

(A) CYP2C9

(B) CYP2D6

(C) CYP3A4

(D) CYP3A5
Figure 6

Diagram showing the metabolism of KRO-105714 by CYP3A4 and CYP2D6, leading to the formation of metabolites M1, M2, M3, and M4.
Identification of metabolites of N-(5-benzoyl-2-(4-(2-methoxyphenyl)piperazin-1-yl)thiazol-4-yl)pivalamide including CYP3A4-mediated C-demethylation in human liver microsomes with high-resolution/high-accuracy tandem mass spectrometry

Min Song, Doohyun Lee, Sun Ju Kim, Jong-Sup Bae, Jaeick Lee, Young-Dae Gong, Taeho Lee, and Sangkyu Lee

College of Pharmacy, Research Institute of Pharmaceutical Sciences, Kyungpook National University, Daegu, 702-701, Republic of Korea (M.S., D.L., S.J.K., J.B., T. L., S. L.); Doping Control Center, Korea Institute of Science and Technology, PO Box 131, Chungryang, Seoul, Republic of Korea (J.L.); Center for Innovative Drug Library Research, Department of Chemistry, College of Natural Science, Dongguk University, Seoul 100-715, Republic of Korea (Y.G.)
Supplemental Methods

Instruments

For the quantification of KRO-105714 and its metabolites, a Thermo AccelaTM HPLC system (Thermo Fisher Scientific) coupled to a TSQ Vantage Triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a HESI-II Spray source. For LC analysis an Inertsil ODS-3, 3μM (2.1 x 150 mm, GL Science Inc, Tokyo) column was used. Mobile phases consisted of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in LC grade water (B). Gradient elution was conducted as follows: A was linearly increased from 30% to 95% during 7 min, retained at 70% for an additional 5 min, and then directly declined to 30% for equilibration for 3 min, at a flow rate of 200 μL/min. The instrument was operated in selective reaction monitoring (SRM) mode. Electrospray ionization was performed in positive ion mode. The operating conditions were determined as follows: spray voltage, 3,500 kV; vaporizer and capillary temperatures, 300 and 300 °C; Nitrogen gas was used as the sheath and auxiliary gas, 60 and 15 (arbitrary units). Acquisition and analysis of the data were performed using Xcalibur (Version 2.1).

Synthesis of KRO-105714 and its metabolites

General Procedure for Synthesis of 4 and 5

To a solution of 4-aminothizole 1 (1.0 mmol) in MeCN (10 mL, 0.1 M) were added acid chloride (2 or 3) (1.5 mmol, 1.5 eq) and pyridine (2.0 mmol, 2.0 eq). The reaction mixture was refluxed for 4 h, and then diluted with EtOAc, washed with brine, dried over MgSO4,
filtered, and concentrated. The residue was purified by column chromatography on silica gel (EtOAc:Hex=1:3) to yield the desired products (4 and 5) (Supplement Figure 1).

**General Procedure for Synthesis of 6 and 7**

To a solution of sulfide 4 or 5 (0.6 – 0.8 mmol) in CH$_2$Cl$_2$ (10 mL) was added mCPBA (3.5 eq) at 0°C. Their action mixture was slowly warmed to room temperature and stirred for 5 h, and then quenched with aq. Na$_2$S$_2$O$_3$, washed with saturated NaHCO$_3$, brine, dried over MgSO$_4$, filtered, concentrated, and dried in vacuum. The crude product was used next step without further purification.

**General Procedure for Synthesis of 9 and 10**

To a solution of crude sulfone 6 or 7 and amine 8 (1.2 eq) in CHCl$_3$ (10 mL) was added triethylamine (1.2 eq). The reaction mixture was heated at 45°C for overnight. The residue was evaporated, and purified by column chromatography on silica gel (EtOAc:Hex=1:3) to yield the desired products (9 and 10).

**Synthesis of 11**

Aqueous lithium hydroxide solution (0.1 M, 1.0 mL) was added to a stirring solution of 3-(5-Benzoyl-2-(4-(2-methoxyphenyl)piperazin-1-yl)thiazol-4-ylamino)-2,2-dimethyl-3-oxopropyl acetate 10 (40 mg, 0.075 mmol) in THF (1.0 mL) at 0°C. The reaction mixture was slowly warmed to room temperature and stirred for 20 min. The reaction mixtures were
quenched with aq. NH$_4$Cl, extracted with EtOAc, dried over MgSO$_4$, filtered, and concentrated. The residue was purified by column chromatography on silica gel (EtOAc:Hex=1:2) to yield the desired deacetylated product 11.

**Compound 9:** $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 11.93 (br s, 1H), 7.77 (d, $J$ = 7.6 Hz, 1H), 7.53 – 7.43 (m, 2H), 7.12 – 7.03 (m, 1H), 6.96 – 6.88 (m, 2H), 3.90 – 3.72 (m, 4H), 3.88 (s, 3H), 3.16 (t, $J$ = 5.0 Hz, 4H), 2.92 – 2.63 (hept, $J$ = 6.7, 13 Hz 1H), 1.33 (d, $J$ = 6.9 Hz, 6H); $^{13}$CNMR (125MHz, CDCl$_3$) $\delta$ 186.4, 175.0, 173.2, 158.1, 152.4, 141.2, 140.4, 131.4, 128.6, 127.6, 124.0, 121.2, 118.7, 111.6, 102.4, 55.6, 50.1, 48.5, 37.3, 19.5

**Compound 11:** $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 12.28 (br s, 1H), 7.78 (d, $J$ = 7.5 Hz, 2H), 7.5 – 7.44 (m, 3H), 7.13 – 7.01 (m, 1H), 6.97 – 6.87 (m, 3H), 3.95 -3.80 (m, 7H), 3.69 (d, $J$ = 5.9 Hz, 2H), 3.17 (t, $J$ = 5.0 Hz, 4H), 2.95 (t, $J$ = 6.5 Hz, 1H), 1.42 (s, 6H); $^{13}$CNMR (125MHz, CDCl$_3$) $\delta$ 186.8, 176.4, 173.4, 157.8, 152.5, 141.1, 140.4, 131.7, 128.6, 127.7, 124.1, 121.3, 118.8, 111.7, 103.1, 70.2, 55.6, 50.1, 48.6, 45.5, 22.8
Legend of Supplemental Figures

Supplemental Figure 1. Synthesis scheme of standard KRO-105714 metabolites (M2 and M4)

Supplemental Figure 2. MS² and MS³ tandem mass spectra of the synthetic M2 (A) and M4 (B), respectively.

Supplemental Figure 3. Representative extracted ion chromatograms of the extracts after incubation of synthetic M2 in in pooled human liver microsomes.

Supplemental Figure 4. Kinetic parameters for KRO-105714 (A) and M2 (B) metabolism in human liver microsomes (HLMs). The kinetic parameters for formation of M2 and M4 were determined using an NADPH-generating system and varying substrate concentrations of KRO-105714 (0 - 50 μM) and M2 (0 – 20 μM). Each value represents the mean activity of duplicate determinations.

Supplemental Figure 5. Representative extracted ion chromatograms of the extracts after incubation of KRO-105714 with human recombinant cDNA-expressed CYP2C19 (A), CYP2E1, (B), CYP1A1 (C), CYP2A6 (D) and CYP2C8 (E), respectively. Incubations were conducted for 1 h with 10 pmol/mL of CYP and 50 μM KRO-
105714 in the presence of an NADPH-generating system. An asterisk indicates interference peaks.
Supplemental Figure 1

1. R = H (76%)
2. R = CH₂OAc (82%)
3. R = CH₂OAc (from 5, 76%)
4. R = H (from 4, 83%)
5. R = CH₂OAc (from 5, 76%)
6. R = H
7. R = CH₂OAc
8. R = H
9. R = H
10. R = CH₂OAc
11. R = CH₂OH (64%)
Supplemental Figure 2

RT: 0.00 - 15.00

Relative Abundance

EIC 495

M2

8.79

EIC 465

M4

10.60
Supplemental Figure 3

(A) 495 MS²

(B) 465 MS²

495 MS² > 395 MS³

465 MS² > 395 MS³
Supplemental Figure 4

(A) KRO-105714 to M2

(B) M2 to M4
Supplemental Figure 5

(A) CYP2C19  
(EIC495) M1  
(EIC465) M2  
(EIC465) M3  
(EIC465) M4  

(B) CYP2E1  
(EIC495) M1  
(EIC465) M2  
(EIC465) M3  

(C) CYP1A1  
(EIC495) M1  
(EIC465) M2  
(EIC465) M3  

(D) CYP2A6  
(EIC495) M1  
(EIC465) M2  
(EIC465) M3  

(E) CYP2C8  
(EIC495) M1  
(EIC465) M2  
(EIC465) M3  

Time (min)