Short Communication

Enzymatic C-Demethylation of 1-[2-(5-tert-Butyl-[1,3,4] oxadiazole-2-carbonyl)-4-fluoro-pyrrolidin-1-yl]-2-(2-hydroxy-1,1-dimethyl-ethylamino)-ethanone (LC15-0133) in Rat Liver Microsomes

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ABSTRACT:
The in vitro metabolism of 1-[2-(5-tert-butyl-[1,3,4] oxadiazole-2-carbonyl)-4-fluoro-pyrrolidin-1-yl]-2-(2-hydroxy-1,1-dimethyl-ethylamino)-ethanone (LC15-0133), a novel dipeptidyl peptidase-4 inhibitor, was investigated using a hepatic microsomal system. The structures of the metabolites were characterized using mass spectral analysis and by comparison with synthetic references. The in vitro incubation of LC15-0133 with rat liver microsomes resulted in the formation of six metabolites, with the major metabolic reactions being hydroxylation and carbonyl reduction. Of the metabolites, a C-demethylated metabolite (M4) was identified, but was only detected in rat liver microsomes; experimental evidence revealed that the C-demethylated metabolite was generated by non-enzymatic decarboxylation of the carboxyl metabolite (M1). Non-enzymatic decarboxylation is postulated to occur due to the resonance stabilization by the oxadiazole ring attached to the tert-butyl moiety.

Materials and Methods

Chemicals. LC15-0133 and LC15-0133-δ6 (internal standard), with a chemical purity >99%, were provided by LG Life Sciences R&D (Daejeon, Korea). Authentic standards of LC15-0133 metabolites (M3, M4, and M5), with chemical purity >95%, were also provided by LG Life Sciences R&D. All other chemicals were the highest grade commercially available.

Microsomes. Human liver microsomes were purchased from BD Biosciences (Bedford, MA). Rat liver samples were taken from Sprague-Dawley rats (Daehan Animal, Daejeon, Korea) and dog liver samples from Beagle dogs (Daehan Animal). The microsomal fraction was prepared according to the method previously described elsewhere (Guengerich et al., 1986).

In Vitro Microsomal Incubation. LC15-0133 (100 μM final concentration) was incubated with 2 mg/ml liver microsomes at 37°C for 2 h, in the presence of an NADPH-generating system (10 mM glucose-6-phosphate, 0.67 mM β-NADPH and 1 U/ml glucose 6-phosphate dehydrogenase), in a final incubation volume of 200 μl. The reaction was terminated by the addition of 400 μl of 0.1% acetic acid with 50 μl of LC15-0133-d6 (10 μg/ml) added as an internal standard solution. The samples were then processed through activated Sep-Pak C18 cartridges (96-well type Oasis HLB extraction cartridge), washed twice with 1 ml of distilled water, and eluted with 1 ml of methanol. The methanol eluate was dried under nitrogen gas. The residue was redissovled in 50 μl of acetonitrile with 10 μl injected on to an HPLC column for HPLC/MS analyses.

HPLC/MS Analysis. The HPLC/MS system consisted of an HP 1100 series binary pump HPLC system (Agilent, Palo Alto, CA) with a diode array detector (Agilent) and an ion-trap mass spectrometer equipped with an electrospray ionization source (Agilent). The chromatographic separation of LC15-0133 and its metabolites was achieved on a Capcell Pak C8 column (2.0 mm × 15 cm, 5 μm; Shiseido, Tokyo, Japan) using a linear gradient program. The mobile phases consisted of 10 mM ammonium formate at pH 6.0 (A) and 90% acetonitrile (B). The initial composition was 15% (B), programmed linearly to 65% (B) over 15 min, at a flow rate of 0.2 ml/min. Mass spectrometry and tandem mass spectrometry analyses were performed using an ion trap mass spectrometer. The entire column eluent was directly introduced into an electrospray ionization interface via a 50-cm-long section of polyetherketone tubing (0.13 mm i.d.). Nitrogen was used as both the nebulizing and drying gas at 35 psi and a flow rate of 8 l/min, respectively, with a temperature of 350°C. The mass spectrometer was operated in the positive ion mode. Helium was used as the collision gas for the tandem mass spectrometric experiments.

ABBREVIATIONS: LC15-0133, 1-[2-(5-tert-butyl-[1,3,4] oxadiazole-2-carbonyl)-4-fluoro-pyrrolidin-1-yl]-2-(2-hydroxy-1,1-dimethyl-ethylamino)-ethanone; HPLC, high-performance liquid chromatography; MS, mass spectrometry.

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Fragmentation was induced with a resonant excitation amplitude of 0.85, following isolation of the desired precursor ion over a selected mass window of 1 Da. All instrumental controls and data processing were performed using the LC/MSD Trap Software (version 4.1).

Results and Discussion

A representative extracted ion chromatogram obtained from the analysis of rat liver microsomal incubation is shown in Fig. 1. The incubation of LC15-0133 with rat liver microsomes in the presence of the NADPH-generating system yielded six different metabolites, the incubation of LC15-0133 with rat liver microsomes in the presence of analysis of rat liver microsomal incubation is shown in Fig. 1. The structures of LC15-0133 and its metabolites were characterized and assigned based on their product ion mass spectra and fragmentation patterns obtained from collision-induced dissociation ion trap mass spectrometry. Metabolites M3, M4, and M5 were further confirmed by comparison with authentic compounds. The protonated molecular and characteristic product ions of LC15-0133 and its metabolites, along with HPLC retention times, are summarized in Table 1.

Metabolite M3 produced a protonated molecule at m/z 387, indicating that this compound was a monohydroxylated derivative of LC15-0133. M3 generated characteristic product ions at m/z 357, 313, 285, and 228. This metabolite was postulated to be a carboxylic acid derivative generated due to further oxidation of M3. The product ion at m/z 357 was postulated to have been generated due to dissociation of a carboxyl moiety from the protonated molecular ion. The MS² spectrum of the ion at m/z 357 was consistent with the MS² spectrum of M4, the C-decarboxylated metabolite (Fig. 2, B and C), which strongly indicated that M1 was a carboxylic acid metabolite.

M5 and M6 produced [M + H]⁺ ions at m/z 373, and both generated characteristic product ions at m/z 355, 301, 283, 244, and 175, suggesting that M6 was a stereoisomer of M5. The protonated molecular and the product ions of these metabolites were 2 Da higher than those of the corresponding ions of the parent compounds, suggesting that these metabolites were carbonyl-reduced metabolites. M2 produced a [M + H]⁺ ion at m/z 403, which was 32 Da higher relative to that of LC15-0133, with major product ions at m/z 385 and 357. This metabolite was postulated to be a dihydroxylated metabolite. M4 generated an [M + H]⁺ ion at m/z 375, which was 14 Da less than that of LC15-0133, suggesting that this metabolite was a demethylated derivative. All the product ions were also 14 Da less than the corresponding ions of the parent compounds, suggesting that demethylation had occurred at the tert-butyl moiety. The structure of M4 was confirmed by comparison with a synthetic standard.

The structure of M4 was somewhat unexpected, as C-demethylation is not common during metabolism, whereas O- and N-demethylation are frequently observed in the biotransformation of xenobiotics (Burke et al., 1994; Coutts et al., 1994; Ertl et al., 1999). In the case of terfenadine possessing a tert-butyl moiety on the backbone of the compound, similarly to LC15-0133, both alcohol and acid metabolites have been identified, but the C-demethylated metabolite has never been reported (Jurima-Romet et al., 1994; Ling et al., 1995; Rodrigues

<table>
<thead>
<tr>
<th>Compound</th>
<th>RT (min)</th>
<th>[M + H]⁺</th>
<th>MS/MS fragmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC15-0133</td>
<td>12.0</td>
<td>371</td>
<td>353, 299, 281, 242, 173, 127</td>
</tr>
<tr>
<td>M1</td>
<td>3.5</td>
<td>401</td>
<td>357, 247, 228</td>
</tr>
<tr>
<td>M2</td>
<td>6.1</td>
<td>403</td>
<td>385, 357, 313, 285, 263, 228</td>
</tr>
<tr>
<td>M3</td>
<td>8.5</td>
<td>387</td>
<td>369, 340, 315, 258, 173, 143</td>
</tr>
<tr>
<td>M4</td>
<td>10.3</td>
<td>357</td>
<td>339, 285, 267, 228, 173, 113</td>
</tr>
<tr>
<td>M5</td>
<td>10.7</td>
<td>373</td>
<td>355, 301, 283, 244, 214, 175, 157</td>
</tr>
<tr>
<td>M6</td>
<td>10.7</td>
<td>373</td>
<td>355, 301, 283, 244, 214, 175, 157</td>
</tr>
</tbody>
</table>

RT retention time.
et al., 1995; Terhechte and Blaschke, 1995). Initially, it was postulated that M4 was able to be generated via the sulfate conjugate of M1, from which the carboxyl sulfate moiety might easily be dissociated. However, this possibility was ruled out, as not even a trace amount of a sulfate conjugate was detected in the incubation along with phosphoadenosyl phosphosulfate (data not shown).

It was subsequently hypothesized that the C-demethylated metabolite (M4) could be spontaneously generated from the acid metabolite (M1) via decarboxylation, where the removal of the carboxyl group was attributed to the oxadiazole ring attached to the tert-butyl moiety.

To test this hypothesis, the acid metabolite (M1) was collected using preparative HPLC, treated, and then reinjected under different conditions. The treatment of M1 under high pH or high temperature conditions accelerated the formation of M4, supporting the notion that the C-demethylated metabolite was generated by the nonenzymatic decarboxylation of M1 (Fig. 3).

The oxadiazole ring attached to the tert-butyl moiety supposedly contributes to the resonance, which stabilizes the electrons by conjugation, for decarboxylation of the tert-butyl site. As shown in Scheme 1, the electron pairs forming the oxygen-hydrogen bond in the carboxyl group of M1 might be stabilized over the oxadiazole ring up to the carbonyl group. This resonance stabilization would facilitate the decarboxylation in M1.

For testing this hypothesis, we carried out microsomal incubation of M3 in D2O medium. On the assumption that M4 is formed from M3 via an intermediate of an arylketone enolate, the metabolite of which isopropyl proton was replaced with deuterium was expected to be detected, as deuterium would be inserted to the tertiary carbon of the isopropyl group in the intermediate form of arylketone enolate. When M3 was incubated in D2O medium, the MS spectrum corresponding to the peak of M4 showed a major ion at m/z 358 instead of m/z 357 as expected (Supplemental Data Fig. 1). These data proved indirectly...
that M4 was formed from an intermediate of an arylketone enolate as postulated.

A considerable amount of the C-demethylated metabolite M4 (~10% of the parent drug found) was observed in the urine and plasma samples from rats administered LC15-0133 (data not shown). The apparent kinetic parameters for M4 formation from LC15-0133 in rat liver microsomes were $K_m = 72.9 \mu M$ and $V_{max} = 5.03 \text{ pmol/min/mg protein}$ (Supplemental Data Fig. 2). However, neither the acid or demethylated metabolite (M1 and M4, respectively) was found in the incubations with dog and human liver microsomes (Supplemental Data Fig. 3), which suggests this biotransformation reaction, alcohol to carboxylic acid, is species-specific. The research on the enzyme(s) involved in carboxyl formation is progressing to identify which enzyme reaction is species-specific.

In conclusion, LC15-0133 was metabolized to a carboxylic acid metabolite, via an alcohol metabolite, by a rat microsomal enzyme, which subsequently decarboxylated to yield a demethylated metab-


![Scheme 1](image)

FIG. 3. Extracted ion chromatograms of the M1-containing fraction after incubation at pH 4 (A), pH 7.4 (B), and pH 9 (C) and at 50°C (D). The fraction containing M1 was collected after separation of the microsomal incubation mixture by HPLC, which was further incubated for 30 min under the conditions described above, and then reinjected onto the column.

**References**


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