Characterization of Two Cyclic Metabolites of Sitagliptin

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ABBREVIATIONS: CID, collision-induced dissociation; NMR, nuclear magnetic resonance spectroscopy; NOE, nuclear Overhauser effect; DPP-4, dipeptidyl-peptidase 4; GLP-1, glucagon-like peptide-1; H/D, hydrogen/deuterium; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MK-0431, (2R)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-α]pyrazin-7(8H)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine; MRL, Merck Research Laboratories.
ABSTRACT:

Two novel metabolites of the dipeptidyl peptidase inhibitor sitagliptin (MK-0431, (2R)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl]-1-(2,4,5-trifluorophenyl) butan-2-amine), were identified after purification from dog urine. The metabolites (referred to as M2 and M5) were characterized by hydrogen/deuterium exchange MS/MS and NMR NOESY experiments as the cis and trans stereoisomers formed by cyclization of the primary amino group with the alpha carbon of the piperazine ring, following oxidative desaturation.
Sitagliptin (Januvia™), also known as MK-0431, (2R)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine (Fig. 1), is a selective, potent DPP-4 inhibitor (Kim et al. 2005), recently approved for the treatment of type 2 diabetes. Sitagliptin has been shown to inhibit plasma DPP-4 activity in normal volunteers (Bergman 2005, 2006; Herman et al., 2005a) and patients with type 2 diabetes (Herman 2004), and to significantly reduce HbA1c and fasting plasma glucose in patients with type 2 diabetes (Herman 2005b; Scott et al., 2005). The pharmacokinetics, metabolism and excretion of sitagliptin in rats, dogs, and humans are described elsewhere (Beconi et al.; Bergman et al, 2006; Vincent et al.). The objective of the present studies was to characterize two metabolites of sitagliptin, referred to as M2 and M5. As discussed by Beconi et al. and Vincent et al., M2 and M5 were minor metabolites in vitro (rat, dog, monkey and human liver microsomes and hepatocytes) and in vivo, where they were detected at very low levels in rat and human plasma, and rat, dog and human excreta. However, M2 and M5 were relatively abundant in dog plasma, especially at the later time points, comprising 4-56% of circulating radioactivity between 1-24 hr following oral administration of [14C]sitagliptin. M2 and M5 were, therefore, purified for further characterization, including evaluation of their pharmacological activity.
Materials and Methods

Chemicals. Sitagliptin (Fig. 1), (2R)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine, was prepared by Process Research at Merck Research Laboratories (MRL). \[^{14}C\]Sitagliptin was synthesized by the Labeled Compound Synthesis Group at MRL. The radiochemical purity was 98.8%, as determined by HPLC, with a specific activity of 132 µCi/mg. For dosing of dogs, \[^{14}C\]sitagliptin was diluted with unlabeled material to a final specific activity of ~20 µCi/mg. All other reagents were obtained from commercial sources.

Purification of M2 and M5 by Solid Phase Extraction and HPLC. Urine collected at 0-24 hr from 3 dogs dosed with \[^{14}C\]sitagliptin at 2 mg/kg p.o. (550 ml) was concentrated on six Phenomenex Strata-E 10-g C18 solid phase extraction cartridges using methanol as the elution solvent. The methanol extracts were evaporated to dryness, reconstituted in 10 ml water, and extracted with 40 ml of acetonitrile and ethyl acetate (1:1). The organic phase was evaporated to dryness, reconstituted in water containing 0.1% formic acid, and subjected to solid phase extraction using a Strata-E 10-g C18 cartridge, which was eluted with mixtures of 0.1% of formic acid and increasing percentage of acetonitrile. The cartridge eluates were analyzed by HPLC using a ThermoHypersil fluophase PFP analytical column, as described below, and the fractions enriched with M2 and M5 were purified on a Keystone semi-preparative HPLC column (Betasil C18, 10 x 150 mm). The M2 and M5 fractions were purified further on a ThermoHypersil fluophase PFP analytical column (4.6 x 250 mm). The mobile phase consisted of 5 mM ammonium acetate and 0.05% acetic acid in water (A) and 5 mM ammonium acetate and 0.05% acetic acid in methanol (B). The elution gradient was begun with 18% B for 2 min, increased linearly to 80% B in 33 min, and then to 95% B in 5 min, followed by a hold at 95% B.
for 5 min. The purity of the isolated metabolites was evaluated by HPLC with UV (220 nm) and radiometric detection.

**Hydrogen/Deuterium (H/D) Exchange MS/MS Experiments.** The procedure used for these experiments was as described previously (Liu et al., 2001). Briefly, small quantities of the purified M2 and M5 were dissolved in D$_2$O and then infused into a ThermoFinnigan LCQ Deca XP ion trap mass spectrometer (San Jose, CA, USA). The LCQ was operated in the electrospray positive ion mode. The source voltage was maintained at 5 kV. Data acquisition and reduction were carried out using Xcalibur software (version 1.2).

**NMR Analysis.** Proton and NOESY NMR spectral analyses of M2 and M5 were carried out at room temperature on a Varian Inova 600 MHz NMR spectrometer. The solvent used was deuterated methanol.

**DPP-4 Activity.** The purified M2 and M5 were tested for DPP-4 inhibition using a published procedure (Leiting et al., 2003). Briefly, the metabolites were tested for inhibition of hydrolysis of 50 µM Gly-Pro-AMC (substrate) by 50 pM DPP-4 at 37°C for 30 min in a 100 mM HEPES buffer (pH 7.5) in the presence of 0.1 mg/ml BSA.
Results and Discussion

Mass Spectra of Sitagliptin and Metabolites M2 and M5. Sitagliptin exhibited a protonated molecule at $m/z$ 408 [M+H]$^+$ in electrospray positive ionization mode. Upon collision induced-dissociation (CID), it afforded three major fragments at $m/z$ 235, 193, and 174, which were assigned as indicated in Fig. 2. The product ion at $m/z$ 193, assigned to a fragment derived from the triazolopiperazine moiety was useful in the determination of modifications to this portion of the molecule. A minor fragment was observed at $m/z$ 391, attributed to the loss of NH$_3$. Metabolites M2 and M5 exhibited the same protonated molecule with $m/z$ 406, which was two mass units lower than that of the parent compound, and identical CID spectra. The most intense fragment ion was detected at $m/z$ 191; this fragment was two mass units lower than the corresponding fragment of the parent compound. Also, there was no evidence of loss of NH$_3$.

Three possible structures can be proposed based on the LC-MS/MS data. M2 and M5 could have been formed either through desaturation of the carbon-carbon bond of the piperazine ring or by desaturation of the C-N bonds of the piperazine ring followed by cyclization via the primary amine. C-N desaturation can occur with either one of the two carbons at the alpha position to the amide nitrogen. The change in the fragmentation pattern of M2 and M5, including the absence of [M+H-NH$_3$]$^+$ ion ($m/z$ 389), in comparing to the parent compound support the latter.

Characterization of M2 and M5 by H/D Exchange MS/MS and NMR. Due to the limited information obtained from the mass spectral fragmentation pattern, the structures of M2 and M5 could not be established. Therefore, M2 and M5 were purified from dog urine for additional mass spectrometry studies using hydrogen/deuterium exchange and NMR analysis. Following several solid-phase extractions, liquid-liquid extraction and HPLC purification steps, ~26 µg M2 and 60 µg M5 were isolated.
The hydrogen/deuterium (H/D) exchange MS/MS experiment was conducted as described previously (Liu et al., 2001). Small quantities of the purified M2 and M5 were dissolved in D_2O and infused into an ion trap mass spectrometer for analysis using an electrospray probe. If M2 and M5 were formed through desaturation of the carbon-carbon bond of the piperazine ring, the neutral molecules would contain two exchangeable hydrogens from the primary amino group. On the other hand, if the primary amine had been converted to a secondary amine via C-N desaturation followed by cyclization, only one exchangeable hydrogen would be present in the neutral molecules. The experiments proved that the latter was true for both M2 and M5. The protonated molecules [M+H]^+ in H_2O were observed at \textit{m/z} 406, while their deuterated ions in D_2O were observed at \textit{m/z} 408 (Fig. 3). This confirmed the cyclic structures for M2 and M5. A similar conclusion was derived from the H/D exchange MS/MS data on the fragment at \textit{m/z} 191, which became \textit{m/z} 192 in D_2O, indicating that it did not contain any exchangeable hydrogens (aside from the proton charge) since it contained a C-N double bond (Fig. 3). On the other hand, one would expect to see \textit{m/z} 193 (one exchangeable hydrogen) if it contained a carbon-carbon double bond.

Proton NMR spectral data of sitagliptin, M2 and M5 are summarized in Table 1. In the proton NMR spectrum of sitagliptin, the methylene \textit{f} protons of the triazolopiperazine ring were observed at 4.94 and 5.02 ppm. These signals were missing from the spectra of M2 and M5, which contained, instead, a methine signal at 5.74 and 5.83 ppm, respectively. This indicated that one of the protons of the original methylene \textit{f} in the parent compound had been lost. In the NOESY spectrum of M2, this isolated methine proton \textit{H}_f showed NOE with the \textit{g} proton at 3.23 ppm, as well as the methine proton \textit{H}_d, where the amino group is attached. These data allowed the unambiguous assignment of the structure for M2 shown in Table 1, where \textit{H}_f and \textit{H}_d are in
the cis configuration. M5 exhibited a similar proton NMR spectrum as M2, with slight differences in the chemical shifts of some of the protons (Table 3). M5 lacked NOE between H$_f$ and H$_d$, which suggested that M5 is the trans isomer of M2, i.e., H$_f$ and H$_d$ protons were in the trans configuration.

**DPP-4 Inhibition by M2 and M5.** The purified M2 and M5 showed less than 50% inhibition of DPP-4 activity at the concentration tested (20 µM), indicating that their IC$_{50}$ values were greater than 20 µM. Given that the parent sitagliptin has an IC$_{50}$ of 18 nM (Kim et al., 2005), M2 and M5 are at least 1000-fold less potent than sitagliptin.

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References


**Figure legends**

Figure 1. Proposed biotransformation pathways for sitagliptin in dogs.

Figure 2. CID spectrum of sitagliptin

Figure 3 A) full scan MS spectrum of M5 in D$_2$O showing a gain of two mass units due to two exchangeable hydrogens in the structure; and B) CID spectrum of M5 in D$_2$O.
TABLE 1

Assignments of $^1$H NMR chemical shifts$^1$ of sitagliptin and M2 and M5 (integral, multiplicity, coupling constants in Hz).

<table>
<thead>
<tr>
<th>Protons</th>
<th>Sitagliptin</th>
<th>M2</th>
<th>M5</th>
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<tr>
<td>a</td>
<td>7.19 (1H, m)$^2$</td>
<td>7.13 (1H, m)</td>
<td>7.09 (1H, m)</td>
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<tr>
<td>b</td>
<td>7.38 (1H, m)</td>
<td>7.36 (1H, m)</td>
<td>7.37 (1H, m)</td>
</tr>
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<td>c</td>
<td>3.08 (2H, m)</td>
<td>2.87 (1H, dd, 14.1, 7.3)</td>
<td>2.91 (1H, dd, 14.4, 7.0)</td>
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<td></td>
<td>2.81 (1H, dd, 14.1, 6.2)</td>
<td>2.76 (1H, dd, 14.4, 7.0)</td>
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<tr>
<td>d</td>
<td>3.85 (1H, m)</td>
<td>3.47 (1H, m)</td>
<td>3.21 (1H, m)</td>
</tr>
<tr>
<td>e</td>
<td>2.92 (2H, d, 6.2)</td>
<td>2.44 (1H, dd, 17.2, 3.8)</td>
<td>2.29 (1H, dd, 16.7, 3.7)</td>
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<td></td>
<td></td>
<td>2.21 (1H, dd, 17.2, 11.7)</td>
<td>2.21 (1H, dd, 16.7, 10.6)</td>
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<tr>
<td>f</td>
<td>4.94 (1H, d, 17.5)$^3$</td>
<td>5.02 (1H, d, 17.5)</td>
<td>5.74 (1H, s)</td>
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<td></td>
<td></td>
<td>5.83 (1H, s)</td>
<td>5.83 (1H, s)</td>
</tr>
<tr>
<td>g</td>
<td>4.0 (1H, m)$^3$</td>
<td>4.98 (1H, dd, 14.3, 3.6)</td>
<td>4.90 (1H, dd, 14.2, 4.3)</td>
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<td>4.11 (1H, m)</td>
<td>3.23 (1H, ddd, 14.3, 12.5, 4.2)</td>
<td>3.38 (1H, ddd, 14.2, 12.1, 5.1)</td>
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<tr>
<td>h</td>
<td>4.29 (2H, t, 5.4)$^3$</td>
<td>4.33 (1H, dd, 12.4, 3.8)</td>
<td>4.19 (1H, dd, 12.4, 4.7)</td>
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<td>4.07 (1H, td, 12.3, 4.5)</td>
<td>4.13 (1H, td, 12.2, 5.0)</td>
</tr>
</tbody>
</table>

$^1$ppm in CD3OD. $^2$Signal splitting patterns: s = singlet, d = doublet, t = triplet, dd = double doublet, td = triple doublet, ddd = double double doublet, and m = multiplet. $^3$Signals become complex due to existence of two forms of sitagliptin arising from restricted rotation of the amide bond.
Figure 1

Sitagliptin → dehydrogenation → cyclization → M2 cis + M5 trans
Figure 2

MS/MS of \( m/z \) 408 in H\(_2\)O

- \( m/z \) 174
- \( m/z \) 235
- \( m/z \) 193
- \( m/z \) 408 [M+H]⁺
Figure 3A
Figure 3B

A
MS in D₂O

B
MS/MS of m/z 408