IN VITRO METABOLISM OF THE HIV-1 PROTEASE INHIBITOR ABT-378: SPECIES COMPARISON AND METABOLITE IDENTIFICATION

GONDI N. KUMAR, VENKATA JAYANTI, RONALD D. LEE, DAVID N. WHITTERN, JOHN UCHIC, SAMUEL THOMAS, PAULEtte JOHNSoN, BRIAN GRABOWSKI, HING SHAM, DAVID BETEBENNER, DALE J. KEMPf, ANd JON F. DENISSEN

PharmaceuticAl Products Division, Abbott Laboratories, Abbott Park, Illinois

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ABSTRACT:
HIV protease inhibitor ABT-378 (ABT-378) was metabolized very extensively and rapidly by liver microsomes from mouse, rat, dog, monkey, and humans. The rates of NADPH-dependent metabolism of ABT-378 ranged from 2.39 to 9.80 nmol/mg microsomal protein min⁻¹, with monkey liver microsomes exhibiting the highest rates of metabolism. ABT-378 was metabolized to 12 metabolites (M-1 to M-12), which were characterized by mass and NMR spectroscopy. The metabolite profile of ABT-378 in liver microsomes from all five species was similar, except that the mouse liver microsomes did not form M-9, a minor secondary metabolite. The predominant site of metabolism was the cyclic urea moiety of ABT-378. In all five species, the major metabolites were M-1 (4-oxo-ABT-378) and M-3 and M-4 (4-hydroxy-ABT-378). Metabolite M-2 (6-hydroxy-ABT-378) was formed by rodents at a faster rate than by dog, monkey, and human liver microsomes. Metabolites M-5 to M-8 were identified as monohydroxylated derivatives of ABT-378. Metabolites M-9 and M-10 were identified as dihydroxylated products of M-1. Metabolites M-11 and M-12 were identified as dihydroxylated derivatives of ABT-378. The metabolite profile in human hepatocytes and liver slices was similar to that of human liver microsomes. The results of the current study indicate that ABT-378 is highly susceptible to oxidative metabolism in vitro, and possibly in vivo, in humans.
from the International Institute for the Advancement of Medicine and came in.

![Structures of HIV protease inhibitors ABT-378 and ritonavir.](image)

Fig. 1. Structures of HIV protease inhibitors ABT-378 and ritonavir.

...buffer was substituted for the NADPH-generating system. The metabolic reaction was stopped by adding 0.25 ml of acetonitrile and vortexing. **Liver Slice Incubations.** For preparation of liver slices, human liver was obtained from the International Institute for the Advancement of Medicine and came from a 44-year-old African American male donor (subject identification no.: 416961). Cylindrical tissue cores (~10 mm in length) were taken from the liver using a sterile 8-mm biopsy punch. Following the preparation of several uniform liver cores, slices of approximately 250 μm were obtained using a Kromekide Tissue Slicer (Alabama Research and Development, Munford, AL). During preparation of the liver cores and slices, the tissues were constantly maintained in cold tissue culture medium. Two slices were floated onto a stainless steel mesh half-cylinder and loaded horizontally into glass scintillation vials containing 1.7 ml of oxygenated (5% O2:5% CO2) tissue culture medium. The vials were gently placed on a temperature-controlled (37°C) vial roller at 4 rpm for 1 h. After preincubation, the cylinders containing the liver slices were placed in fresh, oxygenated medium containing ABT-378 (final concentration 3 μM; 0.3 μCi/incubation) or positive control 7-ethoxycoumarin (final concentration 20 μM, 0.3 μCi/incubation). The cylinders were incubated for various times up to 24 h. The metabolic reactions were terminated by transferring the medium to separate tubes and immediately freezing them. The viable cell count was determined by the trypan blue exclusion method and was found to be 87% at the beginning and the end of a 2-h incubation, respectively. One milliliter of prewarmed medium were repeated. The viable cell count was determined by the trypan blue exclusion method and was found to be ~44% and ~40% at the beginning and the end of a 2-h incubation, respectively. One milliliter of medium containing hepatocytes (~0.5 million viable hepatocytes/ml) was then placed in each well of a 24-well tissue culture plate (Multiwell; Becton Dickinson Labware, Lincoln Park, NJ) and ABT-378 (5 μM, 0.3 μCi/ml) in 3 μl of methanol or positive control 7-ethoxycoumarin (20 μM, 0.3 μCi/ml) was added. Cell-free control incubations contained substrate in the medium without the hepatocytes. The culture plates were then incubated at 37°C with gentle shaking under 5% carbon dioxide and 95% air atmosphere for 1, 2, and 4 h. The metabolic reaction was stopped by transferring the contents of the well into a vial containing 1 ml of acetonitrile and then vortexing. The hepatocytes used in this study were found to be metabolically active as evidenced by oxidative and conjugative metabolism of [14C]-ethoxycoumarin.

**High-Performance Liquid Chromatography (HPLC).** All analyses were performed using an Hewlett-Packard 1050 liquid chromatography system (Hewlett-Packard, Wilmington, DE) consisting of a quaternary pump, an autosampler, and a diode array detector operated at 220 nm. Separations were achieved at ambient temperature on a Beckman Ultrasphere C18 column (5 μm 4.6 × 250 mm) (Beckman Instruments, Palo Alto, CA). A linear gradient of 25% to 55% acetonitrile in buffer (25 mM ammonium acetate, pH adjusted to 4.8 with formic acid) over 57 min was used as column eluent at a flow rate of 1 ml/min. Radioactivity in the column effluent was monitored with a Flo-One/Beta model A-500 radioactivity flow detector (Packard Instruments, Meriden, CT).

**Metabolite Isolation.** A scaled-up incubation was performed as described above for isolation of metabolites for structural identification. Separations were achieved at ambient temperature on a Beckman Ultrasphere C18 column (5 μm 10 × 150 mm) and at a flow rate of 2.8 ml/min. The HPLC effluent corresponding to individual metabolite peaks was collected and the mobile phase was evaporated under nitrogen. Each metabolite residue was redissolved in methanol, filtered to remove inorganic salts, and evaporated to dryness. For NMR spectroscopy, the metabolites were purified by rechromatographing them with a mobile phase that did not contain any added buffer. The respective metabolites, after evaporation, were reconstituted in deuterated chloroform or acetonitrile.

**Liquid Chromatography-Mass Spectrometry.** The liquid chromatography-mass spectrometric analyses were performed on a Perkin-Elmer Sciex API 300 Tandem Mass Spectrometer (Sciex Instruments, Toronto, Canada) equipped with a pneumatically assisted ion spray source and interfaced with a Hewlett-Packard Series 1050 module consisting of a quaternary liquid chromatography pump and a Rheodyne model 8125 manual injector (Rheodyne Instruments, Cotati, CA). Separations were achieved at ambient temperature with a Beckman Ultrasphere C18, 5 μm, 2.0 × 250 mm column. A linear gradient of 30 to 80% acetonitrile in 10 mM ammonium acetate (adjusted to pH 4.6 with formic acid) over 15 min, followed by 80% acetonitrile in buffer for 5 min was used at a flow rate of 200 μl/min. The HPLC effluent was split 4:1 such that a 50 μl/min flow was directed into the mass spectrometer. The ion energy used was 90 electron volts and the mass spectrometer was operated in positive ion mode.

**NMR Spectroscopy.** NMR spectra were collected at ambient temperature using a Varian Unity 500 NMR spectrophotometer operating at 499.7 MHz for proton. The carbon-observed spectra were collected using a dedicated 3-mm carbon probe. The proton-observed spectra, including all of the two-dimensional spectra, were collected using a 3-mm indirect detection probe with a z-axis pulsed field gradient coil.

**Results**

ABT-378 was converted to several metabolites in an NADPH-dependent manner in liver microsomes from all five species (Table 1 and Fig. 2). The rates of metabolism of ABT-378 ranged from 2.39 to 9.80 nmol of substrate metabolized/mg microsomal protein min-1. Twelve metabolites, named M-1 to M-12, were chromatographically resolved and structurally identified. Metabolites M-1 (range, 0.17–1.43 nmol protein min-1), M-3 (range, 0.41–1.80 nmol protein min-1) and M-4 (range, 1.02–4.33 nmol protein min-1) were the major metabolites in all five species. Metabolite M-2 was formed by mouse and rat microsomes at faster rates than dog, monkey, and human liver microsomes. Of all five species tested, the rate of metabolism of ABT-378 was highest in monkey liver microsomes. Both human liver slices and human hepatocytes converted ABT-378 to several metabolites. In all cases, C-4 oxidation products M-1, M-3, and M-4 were the major metabolites.

The mass spectral fragmentation pattern of ABT-378 and its me-
The protonated molecular ion of ABT-378 was observed at m/z 629. The major fragment ions obtained from ABT-378 were m/z 611 (MH⁻ - H₂O), m/z 183/447 (fragmentation at amide bond next to valine moiety, fragments B and C), and m/z 155 (isobutyl cyclic urea moiety, fragment A). Further fragmentation of the m/z 447 fragment ion yielded two fragment ions at m/z 120 (2,6-dimethylphenoxy moiety, fragment E) and m/z 310 (dibenzyl core moiety, fragment D).

M-1. The protonated molecular ion of M-1 was observed at m/z 643. The major fragment ions obtained from M-1 were m/z 197/447 (fragments B and C) and m/z 169 (fragment A). Further fragmentation of the m/z 447 fragment ion yielded two fragment ions at m/z 120 (fragment E) and m/z 310 (fragment D), which are similar to those obtained from ABT-378, indicating that this part of the molecule is unchanged in M-1. The fragment ion corresponding to the valine-cyclic urea moiety (m/z 197) is 14 atomic mass units (amu) greater than the fragment ion obtained from ABT-378 (m/z 183), indicating that it could be an oxidation product of ABT-378. The distinct difference in the NMR spectrum of M-1 from that of ABT-378 is the disappearance of two methylene protons assigned to one of the carbons on the cyclic urea moiety of ABT-378. Four mutually coupled proton resonances were found at 2.18, 2.42, 2.76, and 3.11 ppm, suggesting a pair of adjacent methylenes. These protons were assigned to the protons of carbon-5 and carbon-6. These findings suggest that M-1 is the 4-oxo derivative of ABT-378. This was further confirmed by comparison of HPLC retention time with that of an authentic standard.

M-2. The protonated molecular ion of M-2 was observed at m/z 645. The major fragment ions obtained from M-2 were m/z 199 and 447 (fragments B and C, respectively) and m/z 171 (fragment A). Further fragmentation of the m/z 447 fragment ion yielded two fragment ions at m/z 120 (fragment E) and m/z 310 (fragment D), which were similar to those obtained from ABT-378, indicating that this part of the molecule is unchanged in M-2. The fragment ion corresponding to the valine-cyclic urea moiety (m/z 199) is 16 amu greater than the fragment ion obtained from ABT-378 (m/z 183), indicating that M-2 is a hydroxylated product of ABT-378. The proton NMR spectrum of
M-2 differed from that of ABT-378 in the disappearance of two of the methylene proton resonances and the concomitant appearance of two new resonances well down field at 4.96 and 4.58 ppm. Homonuclear decoupling and D$_2$O exchange NMR experiments showed that the new resonances should be assigned to proton-6 and 6-OH, respectively. Thus metabolite M-2 was identified as 6-hydroxy ABT-378. Further work is needed to establish the stereochemistry of the 6-hydroxy group of M-2.

M-3 and M-4. The mass spectral data for both M-3 and M-4 were identical. The protonated molecular ion of M-3 and M-4 was observed at m/z 645. The major fragment ions obtained from M-3 and M-4 were m/z 199/447 (fragments B and C) and m/z 171 (fragment A). Further fragmentation of the m/z 447 fragment ion yielded two fragment ions at m/z 120 (fragment E) and m/z 310 (fragment D), indicating that the site of hydroxylation in M-3 is on the dibenzyl core moiety of ABT-378.

M-5. The protonated molecular ion of M-5 was observed at m/z 645, indicating that it is a hydroxylated product of ABT-378. The major fragment ions obtained from M-5 were m/z 183/463 (fragments B and C) and m/z 155 (fragment A). Further fragmentation of the m/z 463 fragment ion yielded two fragment ions at m/z 310 (fragment D) and m/z 136 (fragment E). The fragment ion for the 2,6-dimethylphenoxy moiety obtained from M-5 is 16 amu greater than that obtained from ABT-378 (m/z 120), indicating that the site of hydroxylation in M-5 is on the 2,6-dimethylphenoxy moiety.

M-6, M-7, and M-8. The protonated molecular ions of metabolites M-6, M-7, and M-8 were observed at m/z 645, indicating that they are hydroxylated products of ABT-378. The major fragment ions obtained from all three metabolites were m/z 183/463 (fragments B and C) and m/z 155 (fragment A). Further fragmentation of the m/z 463 fragment ion yielded two fragment ions at m/z 326 (fragment D) and m/z 120 (fragment E). The fragment ion for the dibenzyl core moiety (m/z 326) obtained from M-6, M-7, and M-8 is 16 amu greater than that obtained from ABT-378 (m/z 310), indicating that the site of hydroxylation in M-6, M-7, and M-8 is on the dibenzyl core moiety of ABT-378.

M-9 and M-10. The protonated molecular ions of metabolites M-9 and M-10 were observed at m/z 659, indicating that they are possibly secondary metabolites of ABT-378. The major fragment ions obtained from both metabolites were at m/z 197/463 (fragments B and C) and m/z 169 (fragment A). Further fragmentation of the m/z 463 fragment ion yielded two fragment ions at m/z 326 (fragment D) and m/z 120 (fragment E). The fragment ion for the dibenzyl core moiety (m/z 327) obtained from M-9 and M-10 is 16 amu greater than that obtained from ABT-378 (m/z 310), indicating that one of the substituents is a hydroxyl group on the dibenzyl core moiety of ABT-378. The fragment ion for the isobutyl cyclic urea moiety (m/z 169) was the same as that obtained from M-1, indicating that M-9 and M-10 are hydroxylated products of 4-oxo-ABT-378 (M-1), with the hydroxyl group located on the dibenzyl core moiety of the molecule.

M-11 and M-12. The protonated molecular ions of metabolites M-11 and M-12 were observed at m/z 661, which is 32 amu greater than the molecular ion of ABT-378, indicating that they are possibly dihydroxylated metabolites of ABT-378. The major fragment ions obtained from both metabolites were at m/z 199/463 (fragments B and C) and m/z 171 (fragment A). Further fragmentation of the m/z 463 fragment ion yielded two fragment ions at m/z 326 (fragment D) and m/z 120 (fragment E). The fragment ion for the dibenzyl core moiety (m/z 326) obtained from M-11 and M-12 was 16 amu greater than that obtained from ABT-378 (m/z 310), indicating that one of the substituents is a hydroxyl group on the dibenzyl core moiety of ABT-378. The fragment ion for the isobutyl cyclic urea moiety (m/z 171) was the same as that obtained from M-2, M-3, or M-4, indicating that M-11 and M-12 are hydroxylated products of 4-hydroxy- or 6-hydroxy-ABT-378, with the second hydroxyl group located on the dibenzyl core moiety of the molecule.

### Species Comparison of ABT-378 Metabolism

Mouse liver microsomes metabolized ABT-378 to at least 11 metabolites (Table 1).
Metabolite M-9, a 4-oxo-hydroxy secondary metabolite, was not formed by mouse liver microsomes. Rat liver microsomes metabolized ABT-378 to at least 12 metabolites. Metabolites M-9 and M-10, 4-oxo-hydroxy secondary metabolites, were formed less efficiently by rat liver microsomes. Dog, monkey, and human liver microsomes metabolized ABT-378 to at least 12 metabolites. Of all the species liver microsomes examined, monkey liver microsomes exhibited the highest rates of ABT-378 metabolism.

Metabolism by Human Liver Slices. Human liver slices (subject identification no.: 416961) efficiently metabolized ABT-378 to several metabolites (Table 1). After 24 h of incubation, ~87% of [14C]ABT-378 was metabolized. After shorter incubations (3 and 6 h) the primary metabolites M-3 and M-4 were the major components. After longer incubation periods (12 and 24 h), a majority of the radioactivity was present as secondary metabolites and also as polar unknown metabolites.

Metabolism by Human Hepatocytes. Human hepatocytes (subject identification number: 807951) efficiently metabolized ABT-378 to several metabolites (Table 1). The metabolism was linear up to 2 h of incubation. Further incubation up to 4 h did not increase the metabolism. The metabolite pattern obtained with human hepatocytes was similar to that obtained with human liver microsomes. The predominant metabolites were M-1, M-3, and M-4. In longer incubations (2 and 4 h) smaller quantities of M-2, M-5, M-6, M-8, M-9, M-10, M-11, and M-12 were also observed.

Discussion

ABT-378 was metabolized very extensively and rapidly by hepatic microsomes from all five species examined in this study. The rates of ABT-378 metabolism ranged from 2.39 to 9.80 nmol ABT-378 metabolized mg microsomal protein⁻¹ min⁻¹, with monkey liver microsomes exhibiting the highest rate of metabolism. ABT-378 was metabolized to 12 metabolites (M-1 to M-12). The metabolite profiles of ABT-378 in liver microsomes from all five species were similar, except mouse liver microsomes did not form M-9, a minor secondary metabolite. In all five species, the major metabolites were M-1, M-3, and M-4. Metabolite M-2 was formed by rodents at a faster rate than dog, monkey, and human liver microsomes. In vivo small amounts of M-1 and M-4, with an unchanged parent compound as the predominant component, were found to be present in the plasma of rats and dogs administered ABT-378 in combination with ritonavir (G. Kumar, unpublished results). The metabolism of ABT-378 is essentially a deactivation reaction, because the major metabolites (M-1, M-3, and M-4) are less potent inhibitors of the HIV protease than ABT-378. The antiviral activity of the minor metabolites has not been studied yet.

### Table 3

Proton chemical shift assignments of ABT-378 and its liver microsomal metabolites

<table>
<thead>
<tr>
<th>Proton Group</th>
<th>CDCl₃</th>
<th>CD₂CN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proton</td>
<td>ABT-378</td>
<td>M-1</td>
</tr>
<tr>
<td>3 NH</td>
<td>4.99</td>
<td>7.08</td>
</tr>
<tr>
<td>4 CH₂</td>
<td>3.14,3.08</td>
<td>3.11</td>
</tr>
<tr>
<td>4 OH</td>
<td>1.72,1.53</td>
<td>2.42,2.18</td>
</tr>
<tr>
<td>5 CH₂</td>
<td>3.06,2.94</td>
<td>3.11,2.76</td>
</tr>
<tr>
<td>6 CH₂</td>
<td>4.28</td>
<td>4.16</td>
</tr>
<tr>
<td>6 OH</td>
<td>3.14</td>
<td>3.11</td>
</tr>
<tr>
<td>7 CH</td>
<td>4.99</td>
<td>7.08</td>
</tr>
</tbody>
</table>

Chemical shift assignments are assigned relative to 7.27 ppm of CDCl₃ and 1.94 ppm of CD₂CN.
The proposed metabolic pathway for ABT-378 is summarized in Fig. 3. The predominant route of metabolism of ABT-378 was oxidation at carbon-4 of the cyclic urea group of the molecule. Oxidation at C-6 was also a major biotransformation pathway in rat and mouse. On longer incubation, the primary metabolites were found to undergo hydroxylation on the phenyl groups of the central core moiety. Overall, all five species tested, the rate of metabolism was highest by monkey liver microsomes. It is interesting to note that the plasma protein binding of ABT-378 was lower in monkey compared with other species (G. Kumar, unpublished results). It has been shown that in vitro drug metabolizing activities in the cynomolgus monkey are severalfold higher than in dogs and humans (Sharer et al., 1995). For example, erythromycin \(N\)-demethylase activity in cytochrome P450 monkey microsomes was found to be 2.9 nmol\(\text{mg}^{-1}\text{min}^{-1}\) compared with 0.15 and 0.88 nmol\(\text{mg}^{-1}\text{min}^{-1}\) in dog and human liver microsomes, respectively (Sharer et al., 1995). The total cytochrome (CYP) P450 content in the liver microsomes of cynomolgus monkeys has been reported to be 3-fold higher than in human liver microsomes (Shimada et al., 1997). Thus, the higher content of drug-metabolizing enzymes may be responsible for the faster rate of metabolism of ABT-378 by monkey liver microsomes compared with microsomes from other species. The comparatively lower protein binding combined with the faster rate of in vitro metabolism of ABT-378 by monkey liver microsomes suggests the possibility of higher clearance of ABT-378 in monkeys compared with other species. When ABT-378 was administered either alone or in combination with ritonavir, AUC values were lower in monkeys compared with rats and dogs (Marsh et al., 1997).

The metabolite profiles obtained with human liver slices and hepatocytes after short incubation periods were similar to those obtained with human liver microsomes. However, on prolonged incubation, both liver slices and hepatocytes produced several secondary metabolites and other polar unknown metabolites, which are probably tertiary metabolites of ABT-378. This type of metabolite profile was similar to that obtained in rat bile after administration of i.v. ABT-378 (G. Kumar, unpublished results). These results suggest that humans will also probably extensively metabolize ABT-378.

Structurally, the central core of ABT-378 is identical to that of ritonavir (Fig. 1). However, the terminal groups flanking the central core are different between the two inhibitors. The two termini of ritonavir contain a 2-isopropyl-4-thiazolyl group, the primary site of metabolism (Kumar et al., 1996; Denissen et al., 1997) and an unsubstituted 5-thiazolyl group. Recent studies demonstrated that the 5-thiazolyl group of ritonavir interacts with the heme of CYP 450 and leads to a higher clearance of ritonavir (Fig. 1). However, the terminal groups flanking the central core moiety of ABT-378 were characterized. Human liver slices and hepatocytes produced several secondary metabolites after short incubation periods were similar to those obtained with liver slices and hepatocytes produced several secondary metabolites in both liver slices and hepatocytes. Overproduction of the primary metabolites was found to be 2.39 nmol\(\text{mg}^{-1}\text{min}^{-1}\) compared with 0.036, 0.051, and 0.060 nmol\(\text{mg}^{-1}\text{min}^{-1}\), respectively, found for ritonavir. The extremely high rate of in vitro metabolism for ABT-378 is consistent with its poor pharmacokinetic profile when administered alone in animals (Marsh et al., 1997) or humans (Lal et al., 1997). However, when coadministered with low doses of ritonavir, high plasma levels of ABT-378 are maintained both in animals and humans and lead to a >50-fold increase in AUC of ABT-378 in humans (Marsh et al., 1997; Lal et al., 1998). In vitro studies with human liver microsomes have indicated that this pharmacokinetic enhancement is because of potent inhibition of CYP3A-dependent ABT-378 metabolism by ritonavir (Kumar et al., 1997).

In summary, ABT-378 was extensively and rapidly metabolized by liver microsomes from mouse, rat, dog, monkey, and humans, with monkey exhibiting the highest rates of metabolism. The metabolite profile was similar across all five species examined. Seven hydroxylated primary metabolites and five secondary metabolites of ABT-378 were characterized. Human liver slices and hepatocytes produced a similar metabolic profile as that of human liver microsomes.

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References


