CYP3A-Mediated Generation of Aldehyde and Hydrazine in Atazanavir Metabolism

Feng Li, Jie Lu, Laiyou Wang, and Xiaochao Ma

ABSTRACT:

Atazanavir (ATV) is a protease inhibitor (PI) used for the treatment of the human immunodeficiency virus (HIV) infection. Although advantages over other PIs because of its moderate resistance profile, minimal effect on lipid profiles, low capsule burden, and once-daily dosing (Rivas et al., 2009). ATV has particular advantages in selective PI-naive patients who cannot tolerate RTV (Rivas et al., 2009). ATV is associated with various adverse drug reactions (Busti et al., 2004; Havlir and O’Marro, 2004). Nausea was reported in ~35% of the patients receiving ATV, followed by abdominal pain, headache, and diarrhea (Goldsmith and Perry, 2003). The most common laboratory abnormality is hyperbilirubinemia, which was reported in ~40% of patients who received 400 mg of ATV once daily. ATV-induced hyperbilirubinemia rarely led to discontinuation of treatment; however, ~8% of patients developed clinical jaundice (Goldsmith and Perry, 2004). In addition, elevation of alanine aminotransferase and aspartate aminotransferase activity was noted in ~14% of the patients receiving ATV and was unrelated to bilirubin levels (Goldsmith and Perry, 2003). Monitoring of liver function is recommended for ATV-treated patients, in particular for the patients with existing liver diseases (Eholié et al., 2004). The exact mechanisms of ATV-related adverse effects are unknown.

It is generally accepted that a predominant pathway of drug-induced toxicity is via the generation of reactive metabolites (Baillie, 2006; Guengerich and MacDonald, 2007). The reactive metabolites, such as aldehyde, epoxide, quinone methide, and hydroxylamine, can cause various adverse side effects (O’Brien et al., 2005; Tang and Lu, 2010). For instance, felbamate, a broad-spectrum antiepileptic agent, resulted in hepatotoxicity by way of its metabolite atropaldehyde (Dieckhaus et al., 2002). Until now, limited information has been available on ATV metabolism. In 2009, five ATV metabolites were reported, which included one N-dealkylation product, two metabolites resulting from carbamate hydrolysis, one hydroxylated product, and one keto metabolite (ter Heine et al., 2009). Reactive metabolites of ATV have not been identified.

In the current study, we used a metabolomic approach, which has been proved to be a powerful tool in studying drug metabolism (Chen et al., 2006; Li et al., 2010), to investigate the metabolism of ATV in mice and human liver microsomes (HLM). We identified 5 known and 13 novel ATV metabolites. Three potential reactive metabolites were detected and characterized for the first time: one aromatic aldehyde, one α-hydroxyaldehyde, and one hydrazine. These potential reactive metabolites were primarily generated by CYP3A. Our results provide a clue for studies on ATV-related adverse effects from the aspect of metabolic activation. Further studies are suggested to illustrate the impact of these potential reactive metabolites on ATV-related adverse effects.

Introduction

Atazanavir (Reyataz) (ATV), is a protease inhibitor (PI) used for the treatment of the human immunodeficiency virus (HIV) infection (Swainston Harrison and Scott, 2005; Croom et al., 2009). ATV was approved by the U.S. Food and Drug Administration in 2003 and is used in combination with other antiretroviral agents, such as ritonavir (RTV). The Food and Drug Administration also approved the use of ATV without cotreatment with RTV in selective PI-naive patients. The recommended dose for treatment-naive patients is 300 mg of ATV with 100 mg of RTV once daily. ATV can be given in a dose of 400 mg once daily without RTV for selected PI-naive patients who cannot tolerate RTV (Rivas et al., 2009). ATV has particular advantages over other PIs because of its moderate resistance profile, minimal effect on lipid profiles, low capsule burden, and once-daily dosing (Rivas et al., 2009).

Despite these advantages, ATV is associated with various adverse drug reactions (Busti et al., 2004; Havlir and O’Marro, 2004). Nausea was reported in ~35% of the patients receiving ATV, followed by abdominal pain, headache, and diarrhea (Goldsmith and Perry, 2003). The most common laboratory abnormality is hyperbilirubinemia, which was reported in ~40% of patients who received 400 mg of ATV once daily. ATV-induced hyperbilirubinemia rarely led to discontinuation of treatment; however, ~8% of patients developed clinical jaundice (Goldsmith and Perry, 2004). In addition, elevation of alanine aminotransferase and aspartate aminotransferase activity was noted in ~14% of the patients receiving ATV and was unrelated to bilirubin levels (Goldsmith and Perry, 2003). Monitoring of liver function is recommended for ATV-treated patients, in particular for the patients with existing liver diseases (Eholié et al., 2004). The exact mechanisms of ATV-related adverse effects are unknown.

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**Materials and Methods**

**Chemicals and Reagents.** ATV (methyl-N-[(2S)-1-[2-[(2S,3S)-2-hydroxy-3-[(2S)-2-(methoxycarbonylamino)-3,3-dimethylbutanoyl]amino]-4-phenylbutyl]-1-[4-(pyridin-2-yl)phenyl]methyl[hydradynyl]-3,3-dimethyl-1-oxobutan-2-yl]carbamate) was supplied by the National Institutes of Health AIDS Research and Reference Reagent Program. The recombinant human P450s and HLM were purchased from Xenotech, LLC (Lenexa, KS). Semicarbazide, which predicts potential binding with cellular proform adducts with trapping agents, such as GSH, potassium cyanide, methoxylamine, ketoconazole (KCZ), semicarbazide, and NADPH were obtained from Sigma-Aldrich (St. Louis, MO). 4-(Pyridin-2-yl)-2-(methoxycarbonylamino)-3,3-dimethyl-1-oxobutan-2-ylcarbamate was purchased from SynChem, Inc. (Des Plaines, IL). All the solvents for liquid chromatography and mass spectrometry were of the highest grade commercially available.

**Animals and Treatments.** All mice (2–4 months old) were maintained under a standard 12-h dark/flight cycle with water and chow provided ad libitum. Handling was in accordance with study protocols approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee. The mice were treated (orally) with ATV (50 mg/kg) or 4-(pyridin-2-yl)benzaldehyde (12 mg/kg) and housed separately in metabolic cages for 18 h. Urine and feces were collected for metabolite analysis. In brief, urinary samples were prepared by mixing 40 μl of urine with 160 μl of 50% acetonitrile and were centrifuged at 20,000 relative centrifugal forces (rcf) for 10 min. Feces were homogenized in water (1 mg of feces in 10 μl of H2O). Then 200 μl of acetonitrile was added to 200 μl of the resulting mixture, followed by centrifugation at 20,000 rcf for 10 min. The supernatant was transferred to a new Eppendorf vial for a second centrifugation (20,000 rcf for 10 min). Each supernatant was transferred to an autosampler vial, and 5.0 μl was injected into a system (Waters, Milford, MA) combining ultraperformance liquid chromatography (UPLC) and time-of-flight mass spectrometry (TOFMS) for metabolite analysis.

**ATV Metabolism In Vitro.** Incubations were conducted in 1× phosphate-buffered saline (PBS) (pH 7.4) containing 50 μM ATV, 0.1 mg of HLM, or 2 pmol of each cDNA-expressed P450 enzyme (control, CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 1E1, and CYP3A4) in a final volume of 190 μl. After 5 min of preincubation at 37°C, the reaction was initiated by the addition of 10 μl of 20 mM NADPH (final concentration 1.0 mM) and continued for 30 min with gentle shaking. Incubations in the absence of NADPH were also conducted as controls. Coincubations of KCZ (10 μM) were performed to determine the role of CYP3A in ATV metabolism. Incubations were terminated by adding 200 μl of acetonitrile and vortexing for 1 min and centrifuging at 20,000 rcf for 10 min. Each supernatant was transferred to an autosampler vial, and 5.0 μl was injected into the UPLC-TOFMS system for metabolite analysis. All incubations were performed in duplicate.

**Biomarkers of Metabolic Activation.** Because most reactive metabolites are not stable, it is difficult to detect them directly. Reactive intermediates can form adducts with trapping agents, such as GSH, potassium cyanide, methoxylamine, and semicarbazide, which predict potential binding with cellular proteins and/or some other molecules. For example, methoxylamine can form a Schiff base with aldehydes, a process mimicking reactions between aldehyde metabolites and lysine residues on proteins (Evans et al., 2004). UPLC-TOFMS can be used to detect adducts of reactive metabolites and trapping agents. In the current study, methoxylamine, semicarbazide, and INH were used as trapping agents.

**Synthesis and Characterization of Methyl Oxime of 4-(Pyridin-2-yl) benzaldehyde.** Methoxylamine hydrochloride (172 mg, 2.1 mmol) and pyridine (167 mg, 2.1 mmol) were added to a solution of 4-(pyridin-2-yl)benzaldehyde (366 mg, 2.0 mmol) in methanol (10 ml). The reaction mixture was refluxed for 30 min in a water bath. After most of the methanol had been removed in vacuo, water (5.0 ml) was added to the residue, and the mixture was extracted with CH2Cl2 (two 15-ml extractions). The organic phases were washed with H2O (two 10-ml washes) and dried with MgSO4. After removal of the organic solvent under vacuum, the residue was subject to flash chro
mouse urine and feces are shown in Fig. 1. The unsupervised PCA analysis score plot of the feces (Fig. 1A) revealed two clusters corresponding to the control and ATV-treated groups. The S-plots (Fig. 1, B and C) generated from OPLS-DA display the ion contribution to the group separation in the feces and urine, respectively. The top ranking ions were identified as ATV and its metabolites, which were marked in the S-plots (Fig. 1, B and C). The MS/MS spectra of M2–M14 and their structural elucidations are provided in Supplemental Fig. 2. The patterns of ATV metabolites in urine and feces are similar, but most of them are much more abundant in feces (Supplemental Figs. 3 and 4). The metabolic map of ATV in mice is summarized in Fig. 2. Overall, 18 ATV metabolites were identified, including 5 previously reported metabolites (M1, M2, M6, M13, and M14) (ter Heine et al., 2009) and 13 novel metabolites (Fig. 2). Among these novel ATV metabolites, 3 potential reactive metabolites were detected and characterized: one aromatic aldehyde (m1), one \( \alpha \)-hydroxyaldehyde (M15\(^{+}\)), and one hydrazine (M16).

**Identification of an Aromatic Aldehyde (m1) in ATV Metabolism.** A dealkylated ATV metabolite (M1) was reported in a previous study (ter Heine et al., 2009). In theory, an aldehyde should be generated together with the formation of M1. We confirmed the existence of the aldehyde (m1) in ATV metabolism (Figs. 3 and 4). In the ATV-treated mouse urine samples, the dealkylated ATV metabolite (M1) was detected, but the expected aldehyde (m1) was not found. However, further metabolites of m1 were detected, which included an acid (m2), a glycine-conjugate product (m3), and an \( N \)-acetylcysteine-conjugate product (m5) (Fig. 3). In addition, the existence of m1 was confirmed in incubations with HLM using methoxylamine as a trapping agent. The evidence for m1 formation is summarized in Fig. 4. The chromatograms of M1, m2, m3, and m5 are presented in Fig. 4A. M1 was eluted at 5.51 min and had a protonated molecule \([M + H]^{+}\) at \( m/z = 358 \) Da. Compared with the MS/MS of ATV (Fig. 4B), M1 had the same fragments at \( m/z = 335 \) and 144. The fragment ion at \( m/z = 367 \) and the absence of ion at \( m/z = 168 \) suggested that the 4-(pyridin-2-yl)-benzyl moiety was lost from ATV. The other fragment ions are interpreted in the inlaid structural diagram (Fig. 4C).

**4-(Pyridin-2-yl)benzoic Acid (m2).** m2 is an oxidized metabolite of m1. In both the ATV-treated and 4-(pyridin-2-yl)benzaldehyde (m1)-treated mouse urine samples, m2, which had a protonated molecule \([M + H]^{+}\) at \( m/z = 200 \) Da, was detected. The fragment ions of m2, at \( m/z = 182, 154, 127, \) and 78, are interpreted in the inlaid diagram (Fig. 4D). In addition, m2 was confirmed by comparison of retention time and accurate mass with an authentic standard sample.

**2-(4-(Pyridin-2-yl)benzamido)acetic Acid (m3).** m3 is a glycine-conjugated product of m2. Metabolite m3 was eluted at 3.42 min, having a protonated molecule \([M + H]^{+}\) at \( m/z = 257 \) Da. The major ions at \( m/z = 182, 154, 127, \) and 78 are interpreted in Fig. 4E. The ion at \( m/z = 211 \) was formed by loss of the HCOOH moiety.

**2-Acetamido-3-(4-(pyridin-2-yl)benzylthio)propanoic acid (m5).** m5 is a further metabolite of m1, which is conjugated with \( N \)-acetylcysteine, a moiety of GSH. Metabolite m5, eluted at 3.76 min, had a protonated molecule \([M + H]^{+}\) at \( m/z = 331 \) Da. MS/MS of m5 produced the major ions at \( m/z = 289, 243, 200, \) and 168. The structural elucidation was interpreted in Fig. 4F. Metabolite m5 was observed in both ATV and 4-(pyridin-2-yl)benzaldehyde (m1)-treated mouse urine samples.

**4-(Pyridin-2-yl)benzaldehyde (m1).** In the incubation with HLM and ATV, 4-(pyridin-2-yl)benzaldehyde (m1) was trapped using methoxylamine. The formed oxime was eluted at 5.47 min (Fig. 4G), having a mass of \([M + H]^{+}\) = 213 \( m/z \). MS/MS of the m1-oxime generated the ions at 181 (loss of \( CH_{2}OH \)) and 155 (loss of \( C_{2}H_{4}NO \)). The ions were interpreted in the inlaid structural diagram (Fig. 4H). The structure of m1-oxime was confirmed by comparing the retention time and accurate mass with those of the synthetic authentic standard sample, which was characterized by NMR.

**Identification of an \( \alpha \)-Hydroxyaldehyde (M15\(^{+}\)) in ATV Metabolism.** In ATV-treated mouse urine samples and in vitro study using HLM, a novel dealkylated metabolite (M15) of ATV was identified. Meanwhile, an \( \alpha \)-hydroxyaldehyde (M15\(^{+}\)) was trapped by INH (Fig. 5) and semicarbazide (data not shown) in the incubation of ATV in HLM.

**FIG. 1.** Metabolomic analysis of control and ATV-treated mouse urine and feces. Wild-type mice (n = 4) were treated with 50 mg/kg ATV (orally). Urine and feces were collected for analysis. A, separation of control and ATV-treated mouse feces in a PCA score plot. The \( t[1] \) and \( t[2] \) values represent the score of each sample in principal component 1 and 2, respectively. B, loading S-plot generated by OPLS-DA analysis of metabolome in ATV-treated mouse feces. C, loading S-plot generated by OPLS-DA analysis of metabolome in ATV-treated mouse urine. The \( x \)-axis is a measure of the relative abundance of ions, and the \( y \)-axis is a measure of the correlation of each ion to the model. These loading plots represent the relationship between variables (ions) in relation to the first and second components present in the PCA score plot. ATV and its metabolites were labeled. The number of ions (metabolite identification) was accordant with that in Fig. 2.
The formations of M15 and M15’ were NADPH-dependent (Fig. 5A). The chromatograms of M15 and M15’-hydrazone are depicted in Fig. 5A. Metabolite M15 was eluted at 4.23 min, having a protonated molecule [M + H]⁺ at m/z 371 Da, 321 Da (loss of C₁₇H₂₅N₂O₄) less than that of ATV. The fragment ions at m/z 339 (loss of CH₃OH), 200 (loss of C₁₂H₁₀N), and 168 (C₁₂H₁₀N) were interpreted in the inlaid structural diagram (Fig. 5B). The formed M15’-hydrazone of INH, eluted at 4.67 min, had a protonated molecule [M + H]⁺ at m/z 415 Da, 365 Da (loss of C₁₇H₂₅N₂O₄) less than that of M15.

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Fig. 4. Identification of metabolite m1. Urine and feces from mice were collected for 18 h after ATV treatment (50 mg/kg p.o.). In addition, in vitro studies were performed to trap m1 using methoxylamine. Structural elucidations were performed on the basis of accurate mass measurement (mass errors less than 10 ppm) and MS/MS fragmentations. MS/MS fragmentation was conducted with collision energy ramping from 10 to 40 eV. Major daughter ions from fragmentation were interpreted in the inlaid structural diagrams. A, chromatograms of metabolite M1, m2, m3, and m5 in urine. B, MS/MS of ATV. C, MS/MS of M1. D, MS/MS of m2. E, MS/MS of m3. F, MS/MS of m5. G, chromatograms of m1-oxime in the incubation with HLM. H, MS/MS of m1-oxime.
Identification of a Hydrazine (M16) in ATV Metabolism. In the incubation with ATV and HLM, a hydrazine (M16), resulting from the dealkylation and hydrolysis, was identified (Fig. 6). The formation of M16 was NADPH-dependent (Fig. 6A). M16, eluted at 4.18 min, had a protonated molecule \([M + H]^+\) at \(m/z = 367\) Da, 171 (C₈H₁₃NO₃) Da less than that of M1. MS/MS of M16 produced the major fragment ions at \(m/z\) 349 (loss of H₂O), 179 (C₁₀H₁₂NO), and 144 (C₆H₄NO₂). The fragment ions were interpreted in the inlaid structural diagram (Fig. 5C).

Role of P450s in ATV Metabolism. The incubation of ATV with nine different human cDNA-expressed P450s (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4) revealed that CYP3A4 is the primary enzyme contributing to ATV metabolism (Supplemental Table 1). CYP3A4 and CYP2D6 cocontributed to the metabolic pathway of metabolite M1, but CYP3A4 was more important than CYP2D6 (Supplemental Table 1). CYP3A4 was the dominant enzyme responsible for the formation of metabolite M15 (Fig. 7, B and C). The inhibitory effect on the M1 and M15 metabolic pathways was further verified by coincubation of KCZ with HLM and cDNA-expressed CYP3A4. In the incubation with HLM, the formations of M1 and M15 were suppressed up to 90% by KCZ at 10 μM (Fig. 7B). In the incubation with CYP3A4, KCZ at 10 μM inhibited the formations of M1 to 95% and M15 to 90% (Fig. 7C).

Discussion

In our current study, we used a liquid chromatography-mass spectrometry-based metabolomic approach to profile ATV metabolism. We identified 18 ATV metabolites, including 5 previously reported metabolites (M1, M2, M6, M13, and M14) (ter Heine et al., 2009) and 13 novel metabolites (Fig. 2; Supplemental Fig. 2). Three potential reactive metabolites, one aromatic aldehyde (m1), one α-hydroxyaldehyde (M15'), and one hydrazine (M16), were detected and characterized for the first time. The levels of the two aldehydes (m1 and M15') were high because they were generated from two primary metabolic pathways of ATV, M1, and M15 (Figs. 2 and 7A). The aromatic aldehyde (m1) that we identified in ATV metabolism (Figs. 3 and 4) was further oxidized to the corresponding acid m2 and then conjugated with glycine to yield the metabolite m3. This pathway may contribute to the detoxication of aldehyde m1. On the other hand, an N-acetylcysteine-conjugate product (m5) was detected in the ATV-treated mouse urines. The formation of metabolite m5 indicates that the further metabolite of m1 can interact and form an adduct with GSH (m4) (Fig. 3). The exact mechanism of m5 formation was not determined in the current study. On the basis of a previous report (Ji et al., 2007), an intermediate sulfate is proposed. In brief, m1 can be reduced to an alcohol, and subsequently sulfated to form a sulfate that serves as a leaving group. The resulting sulfate reacts with GSH to form m4, which is further metabolized to an N-acetylcysteine-conjugate product (m5). Clarification of the mechanism of this interaction with GSH and its implication in ATV-related adverse effects is desired in future studies.
The hydrazine (M16) that we identified is a further metabolite of M1. It has been reported that hydrazine can cause hepatic lesions and neurotoxicity (Waterfield et al., 1993; Nicholls et al., 2001). The detection of metabolite M16 provided more insight for the study of ATV toxicity. The \( /H9251/H_{9251} \)-hydroxyaldehyde (M15) is predicted as the most active metabolite of ATV, which cannot be directly detected in mouse urine or the HLM incubation system. Previous studies have suggested that \( /H9251/H_{9251} \)-hydroxyaldehyde can form a Schiff base with the amino group on the protein. The unstable imine can be converted into a stable 1-amino-2-keto protein adduct by an intramolecular Amadori rearrangement, resulting in the toxicity (Spahn-Langguth and Benet, 1992; Tang and Lu, 2010).

CYP3A4 was determined as the primary enzyme contributing to metabolic pathways of M1 and M15. Thus, cotreatment with ATV and a CYP3A4 inducer will increase the formation of reactive metabolites M15, m1, and M16, which may augment ATV toxicity. Rifampicin, which is also a CYP3A4 inducer, is a first-line drug for tuberculosis (TB) treatment. According to the guideline of the World Health Organization, HIV/TB coinfected patients are required to be treated with anti-TB drugs for at least 2 weeks before the initiation of anti-HIV treatment. In a recent clinical trial, nausea, vomiting, and elevation of alanine aminotransferase activity were reported in all healthy volunteers who were pretreated with rifampicin followed by treatment with ATV and RTV (Haas et al., 2009). It is possible that rifampicin induces CYP3A4 expression, which accelerates ATV metabolism to reactive metabolites, such as m1, M15, and M16, resulting in liver injury.

In summary, we identified three potential reactive metabolites of ATV, which included two aldehydes and one hydrazine. CYP3A4 was determined to be the primary enzyme contributing to the formation of these aldehydes and hydrazine. Further studies are suggested to illustrate the role of these potential reactive metabolites in ATV-related adverse effects.

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Authorship Contributions

Participated in research design: Li and Ma.

Conducted experiments: Li, Lu, and Wang.

Contributed new reagents or analytic tools: Li.

Performed data analysis: Li and Ma.

Wrote or contributed to the writing of the manuscript: Li and Ma.

References


Address correspondence to: Dr. Xiaochao Ma, Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS 66160. E-mail: xma2@kumc.edu