CYP3A-Mediated Generation of Aldehyde and Hydrazine in Atazanavir Metabolism

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ABSTRACT:

Atazanavir (ATV) is an antiretroviral drug of the protease inhibitor class. Multiple adverse effects of ATV have been reported in clinical practice, such as jaundice, nausea, abdominal pain, and headache. The exact mechanisms of ATV-related adverse effects are unknown. It is generally accepted that a predominant pathway of drug-induced toxicity is through the generation of reactive metabolites. Our current study was designed to explore reactive metabolites of ATV. We used a metabolomic approach to profile ATV metabolism in mice and human liver microsomes. 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 co-treatment with RTV in selective PI-naïve patients. The recommended dose for treatment-naïve 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-naïve 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, 2003; Sulkowski, 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, 2006; Guengerich and MacDonald, 2007). The reactive 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 identified.
detected and characterized: one aromatic aldehyde, one α-hydroxylaldehyde, and one hydrazine. CYP3A4 was identified as the primary enzyme involved in the formation of the two aldehydes and one hydrazine. These results provide a clue for studies on ATV-related adverse effects from the aspect of metabolic activation.

Materials and Methods

Chemicals and Reagents. ATV (methyl N-(2S)-1-[(2S,3S)-2-hydroxy-3-[(2S)-2-(methoxycarbonylamo)-3,3-dimethylbutanoyllino]-4-phenylbutyl]-2-[(4-pyrindin-2-ylphenyl) methyl]hydrazinyl]-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). Isoxazolidine (INH), methoxylamine, ketocanazole (KCCZ), semicarbazide, and NADPH were obtained from Sigma-Aldrich (St. Louis, MO). 4-(Pyridin-2-yl)-benzaldehyde and 4-(pyridin-2-yl)benzoic acid were 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 12:12-h dark:light 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. 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. 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. 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.

UPLC-TOFMS Analysis. ATV and its metabolites were separated using a 1.0 × 2.1-mm (Acquity 1.7-m) UPLC BEH C-18 column (Waters). The flow rate of the mobile phase was 0.3 ml/min with a gradient ranging from 2 to 98% acetonitrile containing 0.1% formic acid in a 10-min run. TOFMS was calibrated with sodium formate and monitored by the intermittent injection of lock mass spectra. Chemical shifts are reported in parts per million, and coupling constants, J, are reported in Hertz (Supplemental Fig. 1).

Trapping 4-(Pyridin-2-yl)benzaldehyde Using Methoxylamine. One aromatic aldehyde, namely, 4-(pyridin-2-yl)benzaldehyde, was detected in our study using methoxylamine as a trapping agent. The experiment was performed in 1× PBS containing 50 μM ATV, 0.1 mg of HLM, and 20.0 μl of 50 mM methoxylamine (dissolved in 1× PBS, final concentration 5 mM) 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. The same incubations without NADPH were conducted as the control. The workup is identical to the procedure described under ATV Metabolism In Vitro.

Trapping Methyl (S)-1-[(2S,3R)-3-hydroxy-4-oxo-1-phenylbutan-2-ylamino]-3,3-dimethyl-1-oxobutan-2-ylcarbamate Using INH. One α-hydroxylaldehyde, namely, methyl (S)-1-[(2S,3R)-3-hydroxy-4-oxo-1-phenylbutan-2-ylamino]-3,3-dimethyl-1-oxobutan-2-ylcarbamate was detected in our study. INH was used to trap this α-hydroxylaldehyde. The experiment was performed in 1× PBS containing 50 μM ATV, 0.1 mg of HLM, and 20.0 μl of 50 mM INH (dissolved in H2O, final concentration 5 mM) 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. The same incubations without NADPH were conducted as the control. The reactions were quenched by adding 200 μl of ice-cold acetonitrile. The mixture was vortexed for 1 min and centrifuged at 20,000 rcf for 10 min. The 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.

Results

Profile of ATV Metabolism in Mice Using a Metabolomic Approach. ATV and its metabolites were found in the feces and urine but mainly in the feces. The results of chemo metric analysis on the ions produced by UPLC-TOFMS assay of control and ATV-treated
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 by 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 α-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 = 538 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-acetyl-cysteine, 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 CH3OH) and 155 (loss of C2H4NO). 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 α-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 α-hydroxyaldehyde (M15) was trapped by INH (Fig. 5) and semicarbazide (data not shown) in the incubation of ATV in HLM.
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 \(C_{17}H_{25}N_2O_4\)) less than that of ATV. The fragment ions at \(m/z\) 339 (loss of \(CH_3OH\)), 200 (loss of \(C_{12}H_{10}N\)), and 168 (\(C_{12}H_{10}N\)) 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

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**FIG. 2.** The metabolite map of ATV. All metabolites were determined by accurate mass and mass fragmentations. 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. Among these novel ATV metabolites, one aromatic aldehyde (m1), one \(\alpha\)-hydroxyaldehyde (M15'), and one hydrazine (M16) were detected and characterized.

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 \(C_{17}H_{25}N_2O_4\)) less than that of ATV. The fragment ions at \(m/z\) 339 (loss of \(CH_3OH\)), 200 (loss of \(C_{12}H_{10}N\)), and 168 (\(C_{12}H_{10}N\)) 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
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 \([\text{M} + \text{H}]^+\) 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 (C9H14NO2). The fragment ions were interpreted in the inlaid structural diagram (Fig. 6B).

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 coincidence 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 \(\mu\)M (Fig. 7B). In the incubation with CYP3A4, KCZ at 10 \(\mu\)M 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 \(\alpha\)-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-hydroxyaldehyde (M15/H11032) 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-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.
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References


**REACTIVE METABOLITES OF ATAZANAVIR**

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