CYP3A-Mediated Generation of Aldehyde and Hydrazine in Atazanavir Metabolism

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Abbreviations:
P450, cytochrome P450; HLM, human liver microsomes; ATV, atazanavir; RTV, ritonavir; PI, protease inhibitor; HIV, human immunodeficiency virus; TB, tuberculosis; KCZ, ketoconazole; INH, isoniazid; TOFMS, time of flight mass spectrometry; UPLC, ultra performance liquid chromatography; PCA, principal component analysis; OPLS-DA, orthogonal projection to latent structures-discriminant analysis.
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 five known and thirteen novel ATV metabolites. Three potential reactive metabolites were detected and characterized for the first time: one aromatic aldehyde, one alpha-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 (ATV), also known as Reyataz, 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 (FDA) in 2003 and is used in combination with other antiretroviral agents, such as ritonavir (RTV). The FDA recently also approved the use of ATV without cotreatment with RTV in selective PI-naïve patients. The recommended dosage for treatment-naïve patients is 300 mg of ATV with 100 mg of RTV once daily. ATV can be taken 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 ATV 400 mg 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 liver function is recommended for ATV-treated patients, especially for the patients with existing liver diseases (Eholie 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, 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 five known and thirteen novel ATV metabolites. Three potential reactive metabolites were detected and characterized: one aromatic aldehyde, one alpha-hydroxyaldehyde, 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-[2-(2S,3S)-2-hydroxy-3-[[((2S)-2-(methoxycarbonylamino)-3, 3-dimethylbutanoyl]amino]-4-phenylbutyl]2-[(4-pyridin-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 (Lenexa, KS). Isoniazid (INH), methoxylamine, ketoconazole (KCZ), 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 (two to four months old) were maintained under a standard 12-hour dark and 12-hour 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 (po) with ATV (50 mg/kg) or 4-(pyridin-2-yl)-benzaldehyde (12 mg/kg) and housed separately in metabolic cages for 18 hours. Urine and feces were collected for metabolite analysis. Briefly, 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 feces in 10 µl of H2O). Subsequently, 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 Eppendoff vial for a second centrifugation (20,000 rcf for 10 min). Each supernatant was transferred to an auto sampler vial, and 5.0 µl was injected to a system (Waters, Milford, MA) combining ultra performance liquid chromatography (UPLC) and time of flight mass spectrometry (TOFMS) for metabolite analysis.

**ATV Metabolism in vitro**

Incubations were conducted in 1X phosphate-buffered saline (PBS, pH 7.4) containing 50 µM ATV, 0.1 mg HLM or 2 pmol of each cDNA-expressed P450 enzyme (control, CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and CYP3A4) in a final volume of 190 µl. After 5 min of pre-incubation at 37 ºC, 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 used as controls. Co-incubations 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 auto sampler vial, and 5.0 µl was injected into UPLC-TOFMS for metabolite analysis. All the 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 glutathione
(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 the 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 MeOH (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 (2× 15 ml). The organic phases were washed with H₂O (2× 10 ml) and dried with MgSO₄. After removal of the organic solvent under vacuum, the residue was subject to flash chromatography on silica gel (hexane/EtOAc 6 : 1) to yield the oxime ether (338 mg, 80 %) as a colorless oil. 

$^1$H NMR (400 MHz, CDCl₃): δ 8.70 (dd, $^3$J = 8.1 Hz, $^4$J = 1.5 Hz, 1H, pyridinyl-H), 8.10 (s, 1H, CH=NOCH₃), 8.01 (d, $^3$J = 8.0 Hz, 2H, phenyl-H), 7.72-7.80 (2H, pyridinyl-H), 7.67 (d, $^3$J = 8.0 Hz, 2H, phenyl-H), 7.25 (m, 1H, pyridinyl-H), 3.99 (s, 1H, CH=NOCH₃); $^{13}$C NMR (100 MHz, CDCl₃): δ 156.5 (CH=NOCH₃), 149.7 (pyridinyl-C), 148.2 (pyridinyl-C), 140.5 (phenyl-C), 136.8 (pyridinyl-C), 132.6 (phenyl-C), 127.7, (phenyl-C), 127.1 (phenyl-C), 122.4 (pyridinyl-C), 120.5 (pyridinyl-C), 62.1 (CH=NOCH₃); HRMS (ESI, pos.): m/z
[\text{M+H}^+] \text{ calcd for C}_{13}\text{H}_{13}\text{N}_2\text{O}: \text{213.1028; found: 213.1024.} \quad ^1\text{H} \text{ and } ^{13}\text{C} \text{ NMR spectra were recorded on a 400 MHz Varian. Chemical shifts are reported in ppm and coupling constants (} J \text{) are reported in Hz (Supplemental Figure 1).} \\

\text{Trapping 4-(pyridin-2-yl)benzaldehyde using methoxylamine.} \quad \text{One aromatic aldehyde, namely, 4-(pyridin-2-yl)benzaldehyde, was detected in our study using methoxylamine as a trapping agent. The experiment was carried out in 1X PBS, containing 50 \mu M \text{ ATV, 0.1 mg HLM, and 20.0 \mu l of 50 mM methoxylamine (dissolved in 1X PBS, final concentration 5 mM) in a final volume of 190 \mu l. After 5 min of pre-incubation at 37 \degree \text{C, the reaction was initiated by the addition of 10 \mu l of 20 mM NADPH (final concentration 1.0 mM) and continued for 30 min with gentle shaking. The same incubations were conducted without NADPH as the control. The work-up is identical to the procedure described in the section on ATV metabolism in vitro.}\\

\text{Trapping methyl (S)-1-((2S,3R)-3-hydroxy-4-oxo-1-phenylbutan-2-ylamino)-3,3-dimethyl -1-oxobutan-2-ylcarbamate using INH.} \quad \text{One alpha-hydroxyaldehyde, 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 alpha-hydroxyaldehyde. The experiment was performed in 1X PBS, containing 50 \mu M \text{ ATV, 0.1 mg HLM, and 20.0 \mu l of 50 mM INH (dissolved in H}_2\text{O, final concentration 5 mM) in a final volume of 190 \mu l. After 5 min of pre-incubation at 37 \degree \text{C, the reaction was initiated by the addition of 10 \mu l of 20 mM NADPH (final concentration 1.0 mM) and continued for 1 h 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 one min and centrifuged at 20,000 rcf for 10 min. The supernatant was transferred to an auto sampler vial and 5.0 µl was injected to UPLC-TOFMS for metabolite analysis. In addition to INH, semicarbazide was also used to trap this alpha-hydroxyaldehyde.

**UPLC-TOFMS Analysis**

ATV and its metabolites were separated using a 100 mm x 2.1 mm (Acquity 1.7 µm) UPLC BEH C-18 column (Waters, Milford, MA). The flow rate of the mobile phase was 0.3 ml/min with a gradient ranging from 2% to 98% aqueous acetonitrile containing 0.1% formic acid in a 10-min run. TOFMS was operated in both positive and negative modes with electrospray ionization. The source temperature and desolvation temperature were set at 120 °C and 350 °C, respectively. Nitrogen was applied as the cone gas (10 L/hour), and desolvation gas (700 L/hour). Argon was applied as the collision gas. TOFMS was calibrated with sodium formate and monitored by the intermittent injection of lock mass leucine enkephalin in real time. The capillary voltage and the cone voltage were set at 3.5 kV and 35 V in positive ion mode. Screening of metabolites was performed by using MakerLynx software (Waters, Milford, MA) based on accurate mass measurement (mass errors less than 10 ppm). The structures of ATV and its metabolites were elucidated by tandem mass spectrometry fragmentation with collision energy ramp ranging from 10 to 40 eV.
**Data Analysis**

Mass chromatograms and mass spectra were acquired by MassLynx software in centroid format from $m/z$ 50 to 1000. Centroid and integrated mass chromatographic data were processed by MarkerLynx software to generate a multivariate data matrix. Principal component analysis (PCA) and orthogonal projection to latent structures-discriminant analysis (OPLS-DA) were conducted on Pareto-scaled data. The corresponding data matrices were then exported into SIMCA-P+12 (Umetrics, Kinnelon, NJ) for multivariate data analysis.
Results

Profile 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 chemometric analysis on the ions produced by UPLC-TOFMS assay of control and ATV-treated mouse urine and feces are shown in Figure 1. The unsupervised PCA analysis score plot of the feces (Figure 1A) revealed two clusters corresponding to the control and ATV-treated groups. The S-plots (Figures 1B and 1C) 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 (Figures 1B and 1C). The MS/MS spectra of M2-M14 and their structural elucidations are provided in Supplemental Figure 2. The patterns of ATV metabolites in urine and feces are similar, but most of them are much more abundant in feces (Supplemental Figures 3 and 4). The metabolic map of ATV in mice is summarized in Figure 2. Overall, eighteen ATV metabolites were identified, including five previously reported metabolites (M1, M2, M6, M13, and M14) (ter Heine et al., 2009) and thirteen novel metabolites (Figure 2). Among these novel ATV metabolites, three 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). Theoretically, an aldehyde should be generated together with the formation of M1. We confirmed the existence of the aldehyde (m1) in ATV metabolism (Figures 3
and 4). In the ATV-treated mouse urines, the dealkylated ATV metabolite (M1) was detected, but the expected aldehyde (m1) was not found. However, the further metabolites of m1 were detected, which included an acid (m2), a glycine-conjugate product (m3), and an N-acetylcysteine-conjugate product (m5) (Figure 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 Figure 4. The chromatograms of M1, m2, m3, and m5 are presented in Figure 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 (Figure 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 (Figure 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 urines, m2 was detected which had a protonated molecule [M + H]^+ at m/z = 200 Da. The fragment ions of m2, at m/z 182, 154, 127, and 78, were interpreted in the inlaid diagram (Figure 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
The major ions at \( m/z \) 182, 154, 127, and 78 were interpreted in Figure 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 glutathione (GSH). The 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 Figure 4F. Metabolite m5 was observed in both ATV and 4-(pyridin-2-yl)benzaldehyde (m1) treated mouse urines.

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 (Figure 4G), having a mass of \([M+H]^+\) = 213 \( m/z \). MS/MS of the m1-oxime generated the ions at 181 (loss of CH\(_3\)OH) and 155 (loss of C\(_2\)H\(_5\)NO). The ions were interpreted in the inlaid structural diagram (Figure 4H). The structure of m1-oxime was confirmed by comparing the retention time and accurate mass with the synthetic authentic standard sample, which was characterized by NMR.

Identification of an Alpha-Hydroxyaldehyde (M15’) in ATV Metabolism

In ATV-treated mouse urines and in vitro study using HLM, a novel dealkylated metabolite (M15) of ATV was identified. Meanwhile, an alpha-hydroxyaldehyde (M15’) was trapped by INH (Figure 5) and semicarbazide (data not shown) in the incubation of ATV in HLM. The formations of M15 and M15’ were NADPH dependent (Figure 5A).
The chromatograms of M15 and M15'-hydrazone are depicted in Figure 5A. Metabolite M15 was eluted at 4.23 min, having a protonated molecule \([\text{M+H}]^+\) at \(m/z = 371\) Da, 321 Daltons (loss of \(\text{C}_{17}\text{H}_{25}\text{N}_{2}\text{O}_{4}\)) less than that of ATV. The fragment ions at \(m/z\) 339 (loss of \(\text{CH}_3\text{OH}\)), 200 (loss of \(\text{C}_{12}\text{H}_{10}\text{N}\)), and 168 (\(\text{C}_{12}\text{H}_{10}\text{N}\)) were interpreted in the inlaid structural diagram (Figure 5B). The formed M15'-hydrazone of INH, eluted at 4.67 min, had a protonated molecule \([\text{M+H}]^+\) at \(m/z = 470\) Da. MS/MS of the M15'-hydrazone produced the major ions 282 (loss of \(\text{C}_{8}\text{H}_{15}\text{N}_{2}\text{O}_{3}\)) and 162 (\(\text{C}_{10}\text{H}_{12}\text{NO}\)). The structural elucidation was interpreted in the inlaid structural diagram (Figure 5C).

**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 (Figure 6). The formations of M16 were NADPH dependent (Figure 6A). M16, eluted at 4.18 min, had a protonated molecule \([\text{M+H}]^+\) at \(m/z = 367\) Da, 171 (\(\text{C}_{8}\text{H}_{13}\text{NO}_{3}\)) Da less than that of M1. MS/MS of M16 produced the major fragment ions at \(m/z\) 349 (loss of \(\text{H}_2\text{O}\)), 179 (\(\text{C}_{10}\text{H}_{15}\text{NO}_{2}\)), and 144 (\(\text{C}_{7}\text{H}_{14}\text{NO}_{2}\)). The fragment ions were interpreted in the inlaid structural diagram (Figure 6B).

**ATV Metabolism in HLM**

Fourteen ATV metabolites were identified in the incubation with HLM (Supplemental Table 1), including two dealkylated ATV metabolites (M1 and M15), two aldehydes (m1 and M15’), four monohydroxylated ATV metabolites (M2, M3, M4, and M5), one
monohydroxylated+monodehydrogenated metabolite (M6), one dihydroxylated ATV metabolite (M10), one dihydroxylated+monodehydrogenated metabolite (M12), two hydrolyzed metabolites (M13 and M14), and one hydrazine (M16). Their relative abundance is displayed in Figure 7A. M2 is the most abundant metabolite of ATV in the incubation with HLM, followed by M15 and M1. The metabolic pathways of M1 and M15 should be emphasized because (1) they are major metabolic pathways in ATV metabolism; (2) two aldehydes (m1 and M15') are generated simultaneously with the formation of metabolites M1 and M15; and (3) the generation of the hydrazine (M16) is also dependent on M1.

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 CYP3A4) revealed that CYP3A4 is the primary enzyme contributing to ATV metabolism (Supplemental Table 1). CYP3A4 and CYP2D6 co-contributed 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 (Figures 7B and 7C). The inhibitory effect on the M1 and M15 metabolic pathways was further verified by co-incubation 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 (Figure 7B). In the incubation with CYP3A4, KCZ at 10 µM inhibited the formations of M1 to 95% and M15 to 90% (Figure 7C).
Discussion

In our current study, we used an LC-MS-based metabolomic approach to profile ATV metabolism. We identified eighteen ATV metabolites, including five previously reported metabolites (M1, M2, M6, M13, and M14) (ter Heine et al., 2009) and thirteen novel metabolites (Figure 2 and Supplemental Figure 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 (Figures 2 and 7A).

The aromatic aldehyde (m1) that we identified in ATV metabolism (Figures 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 adduct with GSH (m4) (Figure 3). The exact mechanism of m5 formation was not determined in the current study. Based upon a previous report (Ji et al., 2007), an intermediate sulfate is proposed. Briefly, 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 alpha-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 alpha-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 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. Accordingly, co-treatment with ATV and a CYP3A4 inducer will increase the formation of reactive metabolites M15’, m1, and M16, which may augment ATV toxicity. Rifampicin is a first-line drug for tuberculosis (TB) treatment, which is also a CYP3A4 inducer. According to the guideline of the World Health Organization, HIV/TB co-infected patients are required to treat with anti-TB drugs for at least two 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: Xiaochao Ma and Feng Li

Conducted experiments: Feng Li, Jie Lu and Laiyou Wang

Contributed the new reagents or analytic tools: Feng Li

Performed data analysis: Feng Li and Xiaochao Ma

Wrote or contributed to the writing of manuscript: Feng Li and Xiaochao Ma
References


Footnotes

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Figure Legends

Figure 1. Metabolomic analysis of control and ATV-treated mouse urine and feces.
Wild-type (WT) mice (n=4) were treated with 50 mg/kg ATV (po). 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 Figure 2.

Figure 2. The metabolic map of ATV. All metabolites were determined by accurate mass and mass fragmentations. Overall, eighteen ATV metabolites were identified, including five previously reported metabolites (M1, M2, M6, M13, and M14) (ter Heine et al., 2009) and thirteen novel metabolites. Among these novel ATV metabolites, one aromatic aldehyde (m1), one alpha-hydroxyaldehyde (M15’), and one hydrazine (M16) were detected and characterized.
Figure 3. A scheme of m1 formation and its further metabolism. m1 was generated from ATV spontaneously with M1, and it was trapped using methoxylamine (MeONH$_2$). In mice, m1 can be further metabolized to acid (m2) and conjugated with glycine (m3). m1 may interact with GSH (m4) and be further metabolized to an N-acetylcysteine-conjugate product (m5).

Figure 4. Identification of metabolite m1. Urine and feces from mice were collected for 18 hours after ATV treatment (50 mg/kg, po). In addition, in vitro studies were performed to trap m1 using methoxylamine. Structural elucidations were performed based on accurate mass measurement (mass errors less than 10 ppm) and MS/MS fragmentations. MS/MS fragmentation was conducted with collision energy ramping from 10-40 eV. Major daughter ions from fragmentation were interpreted in the inlaid structural diagrams. (A) The chromatograms of metabolite M1, m2, m3, and m5 in the urine. (B) The MS/MS of ATV. (C) The MS/MS of M1. (D) The MS/MS of m2. (E) The MS/MS of m3. (F) The MS/MS of m5. (G) The chromatograms of m1-oxime in the incubation with HLM. (H) The MS/MS of m1-oxime.

Figure 5. Identification of metabolite M15’. Duplicate incubations were conducted in 1X PBS (pH 7.4) containing ATV (50 µM), HLM (0.5 g protein/l), INH (5.0 mM), with or without NADPH (1.0 mM). The metabolites were analyzed using UPLC-TOFMS. (A) The chromatograms of M15 and M15’-hydrazone. (B) The MS/MS of M15. (C) The MS/MS of M15’-hydrazone.
Figure 6. Identification of the metabolite M16. Duplicate incubations were conducted in 1X PBS (pH 7.4) containing ATV (50 µM), HLM (0.5 g protein/l), with or without NADPH (1.0 mM). The metabolites were analyzed using UPLC-TOFMS. (A) Chromatogram of M16. (B) The MS/MS of M16.

Figure 7. ATV metabolism in HLM and the role of CYP3A in ATV metabolism. Duplicate incubations were conducted in 1X PBS (pH 7.4) containing ATV (50 µM), NADPH (1.0 mM), HLM (0.5 g protein/l) or CYP3A4 (10 nM). KCZ (10 µM) was used in the inhibitory test. (A) Relative abundance of ATV metabolites generated from HLM. (B) The effects of KCZ (10 µM) on the formations of M1 and M15 in the incubation with HLM. (C) The effects of KCZ (10 µM) on the formations of M1 and M15 in the incubation with cDNA-expressed CYP3A4. The data are expressed as means.
Fig. 3
CYP3A-Mediated Generation of Aldehyde and Hydrazine in Atazanavir Metabolism

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

**Supplemental Table 1. Roles of P450s in ATV metabolism.** Duplicate incubations were conducted in 1X PBS (pH 7.4), containing ATV (50 μM), NADPH (1.0 mM), each cDNA-expressed P450 (10 nM). Metabolites were analyzed by UPLC-TOFMS. The highest peak area of each metabolite produced by a cDNA-expressed P450 was set as 100% and the contribution of other P450 was compared to this enzyme. The data was expressed as a mean. The metabolite numbers were accordant with those in Figure 2.

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Supplemental Figure 1. The NMR spectra of m1-Oxime. The spectra were collected on the 400 MHz Varian. (A) $^1$H NMR of m1-oxime. (B) $^{13}$C NMR of m1-oxime.
 Supplemental Figure 2. The MS/MS spectra of M2-M14 and their structural elucidations

Supplemental Figure 2A. M2, eluted at 5.22 min, had a protonated-molecule at m/z 721, 16 Daltons higher than that of ATV (Fig. 3A). MS/MS analysis of M2 produced daughter ions at m/z 703 (loss of H₂O) and 534 (loss of C₈H₁₄NO₄). The fragment ion at m/z 168 was generated from 4-(pyridin-2-yl)-benzyl group. In addition, the fragment ions at m/z 351 and 363 suggested that the oxidation occurred on the tert-butyl moiety. The fragment ions are interpreted in the inlaid structural diagram.
**Supplemental Figure 2B.** M3 was detected at 5.18 min, having a mass of \([M+H]^+ = 721 \text{ m/z}\). MS/MS analysis revealed the fragment ions at \text{m/z} 703, 351, and 168, which were generated in the same fragmental patterns as M2. The new fragment ion at \text{m/z} 550 (loss of C_8H_14NO_3), 168 (C_{12}H_{10}N), 144 (C_7H_14NO_2), and 107 suggested that the oxidation took place on the benzyl group.
Supplemental Figure 2C. M4 (5.11 min) had a mass of [M+H]^+ = 721 m/z. The corresponding MS/MS analysis showed the major fragment ions at m/z 538 (loss of C_{12}H_{10}NO) and 184 (C_{12}H_{10}NO). The fragment ion at m/z 367 is derived from the fragment ion at m/z 538 by loss of cabamate moiety (C_{8}H_{14}NO_{3}). The fragments at m/z 184 implied that the 4-(pyridin-2-yl)-benzyl group was oxidized. The other fragment ions were interpreted in the inlaid structural diagram.
Supplemental Figure 2D. M5 was eluted at 5.42 min and had a [M+H]$^+$ = 721 m/z (C$_{38}$H$_{53}$N$_6$O$_8$). The corresponding MS/MS was identical to that of the metabolite M4. The fragment ion at m/z 184 indicated that the hydroxylation occurred on the 4-(pyridin-2-yl)-benzyl moiety as well. The fragment ions were interpreted in the inlaid structural diagram.
**Supplemental Figure 2E.** M6 was observed at 5.96 min, having a protonated molecule at \( m/z \) 719. MS/MS analysis of metabolite M6 showed the main fragment ions at \( m/z \) 701 (loss of \( \text{H}_2\text{O} \)) and 168 (\( \text{C}_{12}\text{H}_{10}\text{N} \)). The formation of ions at \( m/z \) 530 and 333 indicated that the hydroxylation occurred on one of the tert-butyl moieties and the dehydrogenation happened on the middle panel. The fragment ions were interpreted in the inlaid structural diagram.
**Supplemental Figure 2F.** M7 was eluted at 5.67 min and had a mass of $[\text{M}+\text{H}]^+ = 719$ m/z. The MS/MS of metabolite M7 is the same with that of metabolite M6. The fragment ions at m/z 701 (loss of H$_2$O) and 168 (C$_{12}$H$_{16}$N) were observed. The other ions were interpreted in the inlaid structural diagram.
Supplemental Figure 2G. M8 was observed at 4.68 min, having a protonated ion at 737 m/z. The fragment ions at m/z 719 (loss of H2O) and 168 (C12H10N) were interpreted in the (S_Fig. 2G). The formation of the fragment ions at m/z 550, 363, and 168 suggested that the hydroxylation occurred on both tert-butyl moieties. The other ions were interpreted in the inlaid structural diagram.
Supplemental Figure 2H. M9 was eluted at 4.95 min and had a mass of $[\text{M+H}]^+ = 735$ $m/z$. MS/MS of metabolite M9 produced the fragment ions at 685 (loss of $\text{CH}_3\text{OH}$) and 168 ($\text{C}_{12}\text{H}_{10}\text{N}$). Compared with the spectrum of M2 (S._ Fig. 2A), the ion at $m/z$ 349 generated from metabolite M9 was 2 mass units less than the ion at $m/z$ 351 produced from metabolite M2. The formation of the ion at $m/z$ 349 suggested that the dehydrogenation happened on this framed panel. The fragment ions were interpreted in the inlaid structural diagram.
**Supplemental Figure 21.** M10 was eluted at 4.98 min, having a mass of [M+H]$^+$ = 737 m/z. MS/MS analysis of metabolite M9 showed the fragment ions at m/z 719 (loss of H$_2$O) and 168 (C$_{12}$H$_{10}$N). The formation of fragment ions at m/z 566 and 367 suggested that the dihydroxylation took place on one of the tert-butyl moieties. The ions were interpreted in the inlaid structural diagram.
**Supplemental Figure 2J.** M11 was eluted at 5.19 min, having a mass of \([M+H]^+ = 735\) m/z. MS/MS analysis of metabolite M11 showed the ions at m/z 717 (loss of H2O) and the 168 (C12H10N). Compared with the fragment ion at m/z 367 from the metabolite M9 (S_Fig. 2H), the formation of the ion at m/z 365 suggested that the dehydrogenation may take place on this framed unit.
**Supplemental Figure 2K.** M12 was eluted at 5.38 min and had a mass of [M+H]$^+$ = 735 m/z. MS/MS of M12 produced the fragment ions at 717 (loss of H$_2$O), 685 (loss of CH$_3$OH) and 168 (C$_{12}$H$_{10}$N). Compared with the spectrum of ATV (Fig. 3B), the ion at m/z 333 generated from ATV was 2 mass units less than the ion at m/z 335 produced from metabolite ATV. The ion at m/z 333 suggested that the dehydrogenation happed on this framed panel. The fragment ions were interpreted in the inlaid structural diagram.
**Supplemental Figure 2L.** Two ATV metabolites M13 and M14, resulting from the carbamate hydrolysis, were identified. M13 (4.66 min) corresponded to a protonated molecular ion at $m/z$ 647, 59 mass units less than that of ATV. The fragment ions at $m/z$ 534, 335, and 168 (C$_{12}$H$_{10}$N) were interpreted in the inlaid structural diagram.
**Supplemental Figure 2M.** M14, eluted at 4.69 min, had a mass of $[\text{M+H}]^+ = 647 \text{ m/z}$, 59 Daltons (loss of $\text{C}_2\text{H}_3\text{O}_2$) less than that of ATV. The MS/MS analysis showed that the major ions at $\text{m/z} 629$ (loss of $\text{H}_2\text{O}$) and 168 ($\text{C}_{12}\text{H}_{10}\text{N}$). The other ions at $\text{m/z} 534$, 424, and 277 were interpreted in the Supplemental Figure 2M.
Supplemental Figure 3. The chromatography of ATV metabolites in mouse urine. Urine samples from mice were collected for 18 hour after ATV treatment (50 mg/kg, po). ATV and its metabolites were analyzed using UPLC-TOFMS.
Supplemental Figure 4. Relative quantification of ATV metabolites in mouse urine (A) and feces (B). Urine and feces from mice were collected for 18 hour after ATV treatment (50 mg/kg, po). ATV and its metabolites were analyzed using UPLC-TOFMS, and relatively quantified using peak areas. The data are expressed as means ±SD (n=4).