Metabolism Mediated Drug interactions Associated with Ritonavir-Boosted Tipranavir in Mice

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Abbreviations:
P450, cytochrome P450; HLM, human liver microsomes; MLM, mouse liver microsomes; TPV, tipranavir; RTV, ritonavir; TPV/r, tipranavir and ritonavir; PI, protease inhibitor; PBS, phosphate-buffered saline; IC50, half maximal inhibitory concentration; rcf, relative centrifugal force; TOFMS, time of flight mass spectrometry; UPLC, ultra performance liquid chromatography; PCA, principal components analysis; OPLS-DA, orthogonal projection to latent structures-discriminant analysis.
Abstract

Tipranavir (TPV) is the first non-peptidic protease inhibitor used for the treatment of drug-resistant HIV infection. Clinically, TPV is co-administered with ritonavir (RTV) to boost blood concentrations and increase therapeutic efficacy. The mechanism of metabolism-mediated drug interactions associated with RTV-boosted TPV is not fully understood. In the current study, TPV metabolism was investigated in mice using a metabolomic approach. TPV and its metabolites were found in the feces of mice, but not in the urine. Principal component analysis of the feces metabolome uncovered eight TPV metabolites, including three monohydroxylated, three desaturated, one dealkylated, and one dihydroxylated metabolites. In vitro study using human liver microsomes recapitulated five TPV metabolites, all of which were suppressed by RTV. CYP3A4 was identified as the primary enzyme contributing to the formation of four TPV metabolites (metabolites II, IV, V and VI), including an unusual dealkylated product arising from carbon-carbon bond cleavage. Multiple cytochromes P450 (2C19, 2D6, and 3A4) contributed to the formation of a monohydroxylated metabolite (metabolite III). In vivo, RTV co-treatment significantly inhibited eight TPV metabolic pathways. In summary, metabolomic analysis revealed two known and six novel TPV metabolites in mice, and all of which were suppressed by RTV. The current study provides solid evidence that the RTV-mediated boosting of TPV is due to the modulation of P450-dependent metabolism.
Introduction

Tipranavir (TPV) is a non-peptidic HIV protease inhibitor (PI) displaying high enzymatic inhibition and potent antiviral activity. TPV was approved by the Food and Drug Administration in 2005 and extended for pediatric use in 2008. TPV exhibits a different therapeutic profile from that of other currently available PIs, rendering it a potential option for treatment experienced patients with resistance to multiple PIs (Pham, 2005; Courter et al., 2008). Systematic bioavailability of TPV is low. Clinically, TPV is administered orally twice daily and must be given in combination with low-dose ritonavir (RTV) to boost TPV bioavailability (Cahn et al., 2006). RTV was originally developed as an HIV protease inhibitor. It is now rarely used for its antiviral activity, but it is used as a cytochrome P450 (P450) inhibitor to boost other PIs (Kempf et al., 1997; Hsu et al., 1998). In a phase I clinical trial with healthy adult volunteers, it was noted that co-administration of TPV and RTV (TPV/r) resulted in a significant increase in steady-state TPV trough concentrations as compared with TPV at a steady state alone. The mean of the TPV trough concentrations were above a preliminary target threshold with most of the RTV-boosted doses. Without the RTV co-administration, none of the TPV-alone doses exceeded the threshold (MacGregor et al., 2004).

The mechanism of drug-drug interactions associated with RTV-boosted TPV is not fully understood. In vitro study with human liver microsomes (HLM) suggested that CYP3A4 is the predominant enzyme involved in TPV metabolism. RTV strongly inhibits CYP3A4, and it was thus proposed that the boosted level of TPV by RTV was mediated by CYP3A4 inhibition (MacGregor et al., 2004; McCallister et al., 2004). Illustration of TPV metabolic pathways would provide valuable information for this proposal. In a recent study using Sprague-Dawley
rats, the rats were administered a single dose of \(^{14}\text{C}\) TPV with the co-administration of RTV. The most abundant metabolite in feces was an oxidation metabolite. In urine, no single metabolite was found to be significantly present (Macha et al., 2007). In a human study, subjects received 500 mg TPV with 200 mg RTV twice daily for six days. On day seven, these subjects received a single oral dose of 551 mg of TPV containing 90 microCi of \(^{14}\text{C}\) TPV with 200 mg of RTV, followed by twice-daily doses of unlabeled 500 mg TPV with 200 mg of RTV for up to 20 days. Metabolites were identified using a flow scintillation analyzer in conjunction with liquid chromatography-tandem mass spectrometry. The most abundant metabolite in feces was identified as an oxidation metabolite, while a TPV glucuronide metabolite was identified in urine (Chen et al., 2007b). In these two studies, two monohydroxylation, a dehydrogenation and a glucuronide conjugate metabolite of TPV were observed (Chen et al., 2007b; Macha et al., 2007). However, neither the contributions of P450s in TPV metabolism nor the effects of RTV on TPV metabolism are clear.

Metabolomics is a rapid and systematical study of small molecule metabolites found in an organism (Thomas, 2001; Weckwerth, 2003). By integrating the resolving power of ultra performance liquid chromatography (UPLC) with the accurate mass determination of time-of-flight mass spectrometry (TOFMS) and multivariate data analysis, it is possible to determine the small changes in the metabolome that take place in different groups of organisms (Chen et al., 2007a). The implication of this new technology in drug metabolism has been well established, for example, the metabolomic analysis of aminoflavone, areca alkaloids, and melatonin (Chen et al., 2006; Giri et al., 2006; Ma et al., 2008). In these studies, a number of novel metabolites were discovered. In the current study, metabolomic approach was employed to study TPV
metabolism. The role of P450s in metabolic pathways of TPV was determined by using cDNA-expressed human P450s. In addition, the inhibitory effect of RTV on TPV metabolism and distribution was investigated.

Materials and Methods

Chemicals and Reagents

Tipranavir (TPV, N-{3-[(1R)-1-[(2R)-6-hydroxy-4-oxo-2-(2-phenylethyl)-2-propyl-3,4-dihydro-2H-pyran-5-yl]propyl]phenyl}-5-(trifluoromethyl)pyridine-2-sulfonamide) and ritonavir (RTV, 1,3-thiazol-5-ylmethyl N-[(2S,3S,5S)-3-hydroxy-5-[(2S)-3-methyl-2-[(methyl[[2-(propan-2-yl)-1,3-thiazol-4-yl]methyl]carbamoyl]amino]butanamido]-1,6-diphenylhexan-2-yl]carbamate) were 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). NADPH was obtained from Sigma-Aldrich (St. Louis, MO). 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-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. For metabolomic analysis, TPV (40 mg/kg) was administered via ball-tipped gavages needle and the mice were housed in separate metabolic cages for 18 hours. Urine and faece samples were collected and stored at -20 °C for further analysis. For tissue distribution and inhibition studies, three groups of mice were utilized and orally treated with TPV (100 mg/kg),
RTV (40 mg/kg), and TPV/r (100 mg/kg TPV, and 40 mg/kg RTV), respectively. Tissues including the liver, brain, lung, kidney, spleen, and eyes were collected 30 minutes after treatment and stored at -20 °C for further analysis.

*Incubation of TPV in HLM, MLM, and Recombinant P450s*

The incubation was conducted in 1X phosphate-buffered saline (PBS, pH 7.4), containing 50 µM TPV, 0.1 mg HLM or 0.1 mg mouse liver microsomes (MLM) 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 200 µl. After 5 minutes of pre-incubation 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 minutes with gentle shaking. The same experiment was carried out without NADPH. All reactions were terminated by adding 200 µl of acetonitrile.

*Inhibition by RTV on TPV metabolism in vitro*

RTV (0-100 µM) was used for the inhibitory test. The co-incubation of RTV (0.01, 0.1, 1, 10, 20, 50 and 100 µM, separately) was performed in 1X phosphate-buffered saline (PBS, pH 7.4), containing 50 µM TPV, 0.1 mg HLM in a final volume of 200 µl. After 5 minutes of pre-incubation 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 minutes with gentle shaking. The pre-incubation of RTV (1.0, 20, and 50 µM) was carried out in PBS (1X, pH 7.4) containing 0.1 mg HLM or MLM or 2 pmol P450 (2C19, 2D6 or 3A4), 1.0 mM NADPH in a final volume of 198 µl. After 30 minutes of incubation, 2.0 µl of TPV (final concentration 50 µM) was added and the resulting
mixture was further incubated for 30 minutes. All reactions were terminated by adding 200 µl of acetonitrile.

Sample Preparation

Urinary samples were prepared by mixing 40 µl of urine with 160 µl of 50% acetonitrile and were centrifuged at 20,000 relative centrifugal force (rcf) for 10 minutes. Feces were homogenized by adding 1X PBS (1 mg feces in 10 µl of PBS). Subsequently, 200 µl of acetonitrile was added to 200 µl of the resulting mixture, followed by vortexing and centrifugation at 20,000 rcf for 10 minutes. The supernatant was transferred to a new Eppendorf vial for a second centrifugation (20,000 rcf for 10 minutes). Serum samples were prepared by mixing 75 µl of serum with 75 µl of acetonitrile, followed by vortexing and centrifugation at 15,000 rcf for 10 minutes. Various tissues (brain, liver, kidney, lung, spleen and eyes) were weighed and homogenized in water (100 mg tissues in 400 µl water). 100 µl of each mixture were added to 100 µl of acetonitrile, followed by vortexing and centrifugation at 20,000 rcf for 10 minutes. The supernatant was transferred to a new Eppendorf vial for a second centrifugation (20,000 rcf for 10 minutes). The extraction recoveries of TPV and RTV have been performed in different tissues and the extraction rates across the tissues are comparable. The in vitro incubation was terminated by adding 200 µl of acetonitrile and vortexed for one minute and centrifuged at 20,000 rcf for 10 minutes. Each supernatant was transferred to an auto sampler vial and 5 µl was injected to a system (Waters, Milford, MA) combining UPLC and TOFMS for metabolite analysis.

UPLC-TOFMS Analysis
A 100 mm x 2.1 mm (Acquity 1.7 µm) UPLC BEH C-18 column (Waters, Milford, MA) was used for metabolite separation. 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-minute 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 liters/hour), and desolvation gas (700 liters/hour) and argon as the collision gas. TOFMS was calibrated with [glu1]-fibrinopeptide 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 and identification of major metabolites were performed by using MarkerLynx software (Waters, Milford, MA) based on accurate mass measurement (mass errors less than 10 ppm). The structures of TPV and its metabolites were elucidated by tandem mass spectrometry fragmentation with collision energy ramp ranging from 10 to 30 eV.

Data Analysis

Mass chromatograms and mass spectra were acquired by MassLynx software in centroid format from m/z 50 to m/z 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
TPV Metabolic Profiles in Mice

TPV and its metabolites were found in feces but not in urine. The results of chemometric analysis on the ions produced by UPLC-TOFMS assay of control and TPV-treated mouse feces are shown in figure 1. The unsupervised PCA analysis score plot (Fig. 1A) revealed two clusters corresponding to the control and TPV-treated groups. The corresponding S-plot (Fig. 1B) generated from OPLS-DA, displays the ion contribution to this group separation. The ions associated with TPV treatment that contributed to group separation were TPV and its metabolites, which were marked in the S-plot (Fig. 1B). The chromatograms of TPV and its metabolites were presented in figure 1C.

Identification of TPV Metabolites in Feces

Monohydroxylated TPV Metabolites

Three TPV monohydroxylated metabolites II, III, and IV were observed. Metabolite II, eluted at 6.32 minutes, had a protonated molecular ion at $m/z$ 619, 16 daltons higher than that of TPV (Fig. 2A). MS/MS analysis of metabolite II produced daughter ions at $m/z$ 427 (loss of C$_{13}$H$_{18}$ and H$_2$O) and 391 (loss of C$_6$H$_3$F$_3$NO$_2$S and H$_2$O). The daughter ion at $m/z$ 362 is derived from the daughter ion at $m/z$ 391 by loss of an ethyl group. In addition, the major daughter ions at $m/z$ 319 and 109 suggest that the oxidation occurs on the ring of aniline moiety. The daughter ions of metabolite II were interpreted in the inlaid structural diagram (Fig. 2B).

Metabolite III was detected at a retention time of 5.88 minutes in the positive ion mode, having a mass of [M+H]$^+$ = 619 $m/z$ (C$_{31}$H$_{33}$F$_3$N$_2$O$_6$S). MS/MS analysis of metabolite III suggested
daughter ions at $m/z$ 411 and 201 via the same fragmentation patterns as TPV (Fig. 2A). Beyond these ions, new fragments were observed at $m/z$ 557 (loss of CO$_2$ and H$_2$O) and 495 (loss of C$_7$H$_6$O and H$_2$O). The segment at $m/z$ 107 implied that the oxidation occurred in the benzyl group. The other ions at $m/z$ 601, 391, 349, and 133 were interpreted in figure 2C.

Metabolite IV (at a retention time of 6.57 minutes) had a mass of $[\text{M+H}]^+ = 619$ $m/z$ (C$_{31}$H$_{33}$F$_3$N$_2$O$_6$S). The corresponding MS/MS analysis showed the major daughter ions at $m/z$ 409 (loss of C$_6$H$_3$F$_3$NO$_2$S) and 391 (loss of C$_6$H$_3$F$_3$NO$_2$S and H$_2$O). The fragments at $m/z$ 191 and 149 implied that the phenethyl moiety was oxidized. The other daughter ions were interpreted in the inlaid structural diagram (Fig. 2D).

**Depropylated TPV Metabolite**

The structural elucidation of depropyl-TPV (metabolite V) was shown in figure 2E, with major daughter ions at $m/z$ 543 (loss of H$_2$O), 479 (loss of C$_2$F$_3$), 333 (loss of C$_6$H$_3$F$_3$NO$_2$S and H$_2$O) and 291 (loss of C$_6$H$_3$F$_3$NO$_2$S, CO$_2$, and H$_2$O). The fragmentations of TPV was depicted in figure 2A, with daughters ion at $m/z$ 585 (loss of H$_2$O), 521 (loss of C$_2$F$_3$), 375 (loss of C$_6$H$_3$F$_3$NO$_2$S and H$_2$O) and 333 (loss of C$_6$H$_3$F$_3$NO$_2$S, CO$_2$, and H$_2$O). After the loss of the same moieties, all the peaks as above in metabolite V, had 42 mass units less than those of TPV so it was tentatively identified as depropyl-TPV.

**Dehydrogenated TPV Metabolites**
The three dehydrogenated metabolites (VI, VII, and VIII) were detected in the feces. Metabolite VI (at a retention time of 7.44 minutes) corresponded to a protonated molecular ion at $m/z$ 601, 2 mass units less than that of TPV. Therefore, metabolite VI appeared to be a dehydrogenated metabolite. As shown in the figure 2F, MS/MS of metabolite VI produced the daughter ions at $m/z$ 391 (loss of C$_6$H$_3$F$_3$NO$_2$S), 173, and 91 (benzyl), which suggested that the dehydrogenation might take place in the propyl moiety of the molecule.

Metabolite VII was eluted at 6.01 minutes and had a [M+H]$^+$ = 601 $m/z$ (C$_{31}$H$_{31}$F$_3$N$_2$O$_5$S). The daughter ions at $m/z$ 391 (loss of C$_6$H$_3$F$_3$NO$_2$S), 343, 259, and 133 were interpreted in the inlaid structural diagram. The dehydrogenation of metabolite VII may occur in the encircled unit in figure 2G.

The third desaturated metabolite (VIII) was observed at a retention time of 6.11 minutes, having a protonated molecular ion at $m/z$ 601. The fragment ions at $m/z$ 391 (loss of C$_6$H$_3$F$_3$NO$_2$S) and 213 were interpreted in figure 2H. The formation of daughter ions at $m/z$ 199 and 133 suggested that dehydrogenation probably took place in the phenylethyl moiety of the molecule.

**Dihyroxylated TPV Metabolite**

A mass of [M+H]$^+$ = 635 $m/z$ was noted and corresponded to the empirical formula (C$_{31}$H$_{33}$F$_3$N$_2$O$_7$S) for di-hydroxytiprvanvir (metabolite IX). The dihydroxylation metabolite had a shorter retention time (5.76 minutes) than all of the TPV monohydroxylation metabolites. MS/MS of metabolite IX produced the daughter ions at $m/z$ 573 (-CO$_2$, -H$_2$O), 425 (loss of C$_6$H$_3$F$_3$NO$_2$S), 407 (loss of C$_6$H$_3$F$_3$NO$_2$S and H$_2$O), 191, and 149. The formation of daughter
ions at m/z 425, 191, 107, and 149 suggested that the hydroxylations occurred in the aniline moiety and benzyl unit, respectively (Fig. 2I).

Role of P450s in TPV Metabolism and Inhibition by RTV

Among these eight TPV metabolites, five of them were recapitulated in the in vitro study using HLM (Fig. 3A), including three monohydroxylated (metabolites II, III, and IV), one desaturated (metabolite VI), and one dealkylated (metabolite V) metabolites. All these TPV metabolic pathways in HLM were NADPH-dependant. The incubation of TPV with nine different human cDNA-expressed P450s revealed that CYP3A4 was the primary enzyme contributing to the metabolic pathways of metabolites II, IV, V, and VI (Table 1). All other P450s showed little or no activity for metabolites II, IV, V, and VI. Multiple enzymes were involved in the metabolic pathway of metabolite III with the metabolic rate mediated by CYP2D6 > CYP2C19 > CYP3A4 > CYP2C8 (Table 1). The inhibitory effect of RTV on these TPV metabolic pathways was verified by pre-incubation and co-inhibition of RTV with HLM, MLM, and cDNA-expressed CYP3A4, CYP2D6, and CYP2C19. RTV strongly inhibited metabolic pathways of metabolites II, IV, V, and VI as half maximal inhibitory concentration (IC50) less than 0.5 µM in HLM (Fig. 3A). RTV at 1 µM, greater than 80% of metabolites II, IV, V, and VI formations in HLM were suppressed (Fig. 3B). All five CYP3A4-mediated TPV metabolic pathways were significantly suppressed by co-incubation of RTV at 1 µM (Fig. 3C). The pre-incubation of RTV (1.0 µM) with HLM or CYP3A4 more effectively inhibited the formation of metabolites than the co-incubation for metabolites II, IV, V, and VI (Fig. 3C). At 1 µM, the CYP3A4-mediated
formation of metabolites II, III, IV, V, and VI was almost 100% inhibited by pre-incubation of RTV (Fig 3C).

Inhibitory effect of RTV on metabolite III formation was different from that of metabolites II, IV, V, and VI. RTV at 1 µM only inhibited ~40% of metabolite III formation in HLM (Fig. 3B). IC50 of RTV for metabolite III formation was ~10-fold higher than that of metabolites II, IV, V, and VI in HLM (Fig. 3A). Pre-incubation and co-incubation of RTV had a similar inhibitory effect on the formation of metabolite III in HLM (Fig. 3B). 1 µM RTV had no significant effect on the formation of metabolite III when it is co-incubated with CYP2D6 and CYP2C19 (Fig. 3D). When the RTV concentration was increased to 50 µM, the formation of metabolite III was suppressed by ~50% during incubation with CYP2D6, and ~80% during incubation with CYP2C19 (Fig. 3D). The more significant inhibition on metabolite III formation was noted when RTV was pre-incubated with CYP2C19 and 2D6 (Fig. 2E).

Five metabolites of TPV were also observed in MLM. Similar level of TPV metabolism in HLM and in MLM was noted (Fig. 3F). Mimicked to human dose ratio of TPV and RTV (5:2), inhibitory experiments were performed in HLM and MLM with 50 µM TPV and 20 µM RTV. The formation of TPV metabolites II, IV, V, and VI in HLM was completely suppressed by pre- or co- incubation with 20 µM RTV, and over 80% formation of metabolite III was inhibited (Fig. 3G). In MLM, the formation of metabolites II, III, IV, and V in MLM was totally inhibited by pre- or co- incubation with 20 µM RTV, and ~ 90% of formation of metabolite VI was inhibited (Fig. 3H).
Effect of RTV on TPV Metabolism and Tissue Distribution

The tissue distribution of TPV was illustrated in figure 4A. The highest distribution is in the liver, followed by the kidney, spleen, and lung. A small amount of TPV was detected in the brain and eyes. RTV had a similar tissue distribution as TPV, with the liver having the highest concentration (Fig. 4B). In TPV/r co-treated mice, the TPV abundance in the liver, spleen, and eyes were significantly higher than that in TPV alone treated mice (Fig. 4A). All eight TPV metabolites identified from the feces were detected in the serum and liver of the mice. Assuming a basis of 100% for TPV and its metabolites in each tissue, TPV metabolites account for 31% and 38% in the serum and liver in the TPV alone group (Fig. 5A and B). In TPV/r co-treated mice, only 1% and 2% metabolites were detected in the serum and liver (Fig. 5C and D).

Discussion

Traditionally, radiotracing is an efficient method for performing drug metabolism. However, its application is limited due to the source of radiolabeled compound, as well as the concerns about environmental contamination and safety. In recent several years, the implication of metabolomic approach in drug metabolism has been well established, which does not need radiolabeled compound (Chen et al., 2007a). In the current study, TPV metabolism in mice was investigated using a metabolomic approach. Analysis of urine and feces revealed that TPV and its metabolites were mainly excreted in the feces. This finding is in accordance with previous studies using radio-labeled TPV. In previous studies, two monohydroxylated, one dehydrogenated, and one glucuronide conjugated metabolites have been reported (Chen et al., 2007b; Macha et al., 2007). In these reports, one hydroxylation took place in a benzyl group,
and the other hydroxylation occurred in a trifloromethyl substituted pyridinyl ring. Our data indicated that one monohydroxylation took place on an aniline ring (II) and the other two occurred in a benzyl group (metabolites III and IV). In addition, three dehydrogenated metabolites were observed with one of them (metabolite VII) corresponding to the previously reported metabolite and the other two (metabolites VI and VIII) were novel metabolites. Metabolite VIII may derive from the hydroxylated metabolites III or IV by loss of water. Furthermore, dihydroxylated TPV (metabolite IX) was uncovered. Interestingly, a CYP3A-mediated carbon-carbon bond cleavage (metabolite V) was found in TPV metabolism in both the mouse and HLM. This type of carbon-carbon cleavage has been reported by Brandon (Brandon et al., 2005; Brandon et al., 2006), but the mechanism underlying this type of carbon-carbon cleavage is not clear.

Among the eight metabolites observed in the feces of mice, five metabolites were recapitulated in HLM, which consisted of three dehydroxylated (metabolites II, III, and IV), one desaturated (metabolite VI), and one depropylated metabolites (metabolite V). Incubation of TPV with recombinant P450s demonstrated that CYP3A4 was the primary enzyme contributing to the metabolic pathways of metabolites II, IV, V, and VI. Multiple P450s involved in the metabolic pathway of metabolite III, included CYP2D6, 2C19, 3A4, and 2C8. TPV metabolites VII, VIII and IX were only detected in the in vivo study, and the roles of P450s on these pathways were not confirmed. However, the data from TPV/r co-treatment suggest that the enzymes contributing to the formations of metabolites VII, VIII, and IX could have been inhibited by RTV. RTV inhibition on TPV metabolic pathway of metabolite III was different from that of metabolites II, IV, V, and VI. This was due to different enzymes contributing to the metabolic
pathways. RTV selectively inhibits CYP3A activity at low concentrations. With increasing concentrations, RTV inhibits CYP2C, 2D, and 3A activities (Eagling et al., 1997; von Moltke et al., 1998; Vourvahis and Kashuba, 2007). The mechanism of inhibition of RTV was identified by the pre-exposure of RTV in HLM, MLM, cDNA-expressed enzymes (CYP3A4, 2C19, and 2D6) respectively. Our data suggested that RTV-mediated inhibition on TPV metabolism is in a mechanism-based manner, which is consistent with the previous report (Koudriaková et al., 1998; Ernest et al., 2005). In addition, we identified that TPV metabolism in HLM and MLM is similar; therefore the experiment in mice can be extrapolated to humans.

TPV is an efficient drug for the treatment of experienced patients resistant to other PIs (Temesgen and Feinberg, 2007). However, TPV/r co-therapy can lead to intracranial hemorrhage and hepatotoxicity. Over 10 cases of intracranial hemorrhage and 12 cases of liver-associated deaths were reported from the Food and Drug Administration. Assessment of risk/benefit of TPV/r regimen is suggested for patients at risk for intracranial hemorrhage and hepatic failure (Chan-Tack et al., 2008). The mechanism of TPV/r toxicity is not completely clear. We noted that TPV was highly concentrated in the liver, especially when co-treated with RTV. In addition, biliary excretion appears to be critical for TPV and its metabolites, because they were only detected in feces. These data indicate that disruption of biliary function may predispose a risk for TPV-induced liver injury. Compared to the liver, the brain has extremely low TPV distribution which suggests that TPV-related intracranial hemorrhage may be due to indirect toxicity of TPV/r. Further studies are needed to establish a mechanism of TPV/r induced intracranial hemorrhage and hepatotoxicity.
In summary, TPV metabolism was thoroughly investigated in mice by using a metabolomic approach, and the TPV metabolic map was extended to include two known and six novel pathways (Fig. 6). CYP3A was identified as the primary enzyme contributing to the formation of four TPV metabolites, including one with an unusual carbon-carbon bond cleavage. CYP2C, 2D, and 3A have collaborately contributed to the formation of a monohydroxylated metabolite (metabolite III). RTV inhibited CYP2C, 2D, and 3A mediated TPV metabolism. \textit{In vivo}, RTV co-treatment significantly inhibited all eight TPV metabolic pathways, which indicated that the RTV-mediated boosting of TPV is due to the modulation of P450-dependent metabolism.
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References


Footnotes

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Figure Legends

Figure 1. Metabolomic Analysis of Control and TPV-treated Mouse Feces. Wild-type (WT) mice (n=3) were treated with 40 mg/kg TPV (po) and 18 hour urine and feces samples were collected for analysis. (A). Separation of control and TPV-treated mouse feces in PCA scores plot. The t[1] and t[2] values represent the scores of each sample in principal component 1 and 2, respectively. (B). Loading S-plot generated by OPLS-DA analysis. 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 A. Top ranking ions were marked. * stands for sodium adduct of original ion. The number of ions (metabolite ID) was accordant with that in Fig 2 and Fig 6. (C). The chromatograms of TPV and its metabolites: I (TPV); II, III, and IV (mono-hydroxylated metabolite); V (dealkylated metabolite); VI, VII, and VIII (dehydrogenated metabolites); IX (dihydroxylated metabolite).

Figure 2. MS/MS Structural Elucidation of TPV Metabolites in Mouse Feces. Feces samples from mice were collected for 18 hour after po administration of 40 mg/kg TPV. Screening and identification of major metabolites were performed by using MakerLynx software based on accurate mass measurement (mass errors less than 10 ppm). MS/MS fragmentation was conducted with collision energy ramping from 10-30 eV. Major daughter ions from fragmentation were interpreted in the inlaid structural diagrams. (A). TPV I (m/z 603+), retention time at 6.62 minutes. (B). Monohydroxylated metabolite II (m/z 619+), retention time at 6.30 minutes. (C). Monohydroxylated metabolite III (m/z 619+), retention time at 5.87 minutes. (D). Monohydroxylated metabolite IV (m/z 619+), retention time at 6.58 minutes. (E). Depropylated
metabolite V (m/z 561$^+$), retention time at 6.23 minutes.  (F). Dehydrogenated metabolite VI (m/z 601$^+$), retention time at 7.45 minutes.  (G). Dehydrogenated metabolite VII (m/z 601$^+$), retention time at 6.01 minutes.  (H). Dehydrogenated metabolite VIII (m/z 601$^+$), retention time at 6.11 minutes.  (I). Dihydroxylated metabolite IX (m/z 635$^+$), retention time at 5.75 minutes.

Figure 3. TPV Metabolism in vitro and Inhibition by RTV. Duplicate incubations were conducted in 1X PBS (pH 7.4), containing TPV (50 µM), NADPH (1.0 mM), HLM (0.5 g protein/L) or MLM (0.5 g protein/L) or cDNA-expressed CYP3A4 (10 nM), 2D6 (10 nM) and 2C19 (10 nM), with co-incubation of RTV (RTV co), or pre-incubation of RTV (RTV pre) respectively. Metabolites II, III, IV, V, and VI were analyzed by UPLC-TOFMS.  (A). Effect of RTV (0-100 µM) on TPV metabolism in HLM.  (B). Effect of pre-incubation of RTV and co-incubation of RTV (1 µM) on TPV metabolism in HLM.  (C). Effect of pre-incubation of RTV and co-incubation of RTV (1 µM) on TPV metabolism in cDNA-expressed CYP3A4.  (D). Effect of 1 µM and 50 µM RTV on TPV metabolism in cDNA-expressed CYP2C19 and 2D6.  (E) Effect of pre-incubation of RTV and co-incubation of RTV (50 µM) on TPV metabolism in cDNA-expressed CYP2C19 and 2D6 (10 nM).  (F). Comparison of TPV metabolism in HLM and in MLM. The overall abundance of TPV metabolites was set as 100% in the incubation of HLM and MLM. The relative abundance of each metabolite was compared and presented as relative ratio (MLM vs HLM).  (G). Effect of pre-incubation of RTV and co-incubation of RTV (20 µM) on TPV metabolism in HLM.  (H). Effect of pre-incubation of RTV and co-incubation of RTV (20 µM) on TPV metabolism in MLM (10 nM). The data was expressed as a mean. For each metabolic pathway, the incubation without RTV was set as 100%. N.D., not detected. The metabolite ID was accordant with that in Fig 2 and Fig 6.
Figure 4. TPV and RTV Tissue Distribution in Mice. Mice were orally treated with TPV (100 mg/kg), RTV (40 mg/kg), or TPV/r (100/40 mg/kg), respectively. Tissues including liver, brain, lung, kidney, spleen, and eyes were collected 30 minutes after treatment. TPV and RTV were extracted and analyzed by UPLC-TOFMS. (A). TPV tissue distribution in TPV treated and TPV/r co-treated mice. (B). RTV tissue distribution in TPV/r co-treated mice. The data was expressed as a mean ± SD (n=4). * P<0.05 vs TPV treated mice.

Figure 5. Relative Quantification of TPV and its Metabolites in Liver and Serum from TPV Treated and TPV/r Co-treated Mice. TPV and its metabolites were analyzed by UPLC-TOFMS. The overall abundance of TPV and its metabolites were set as 100% in each tissue. The data was expressed as a mean (n=4). The metabolite ID was accordant with that in Fig 2 and Fig 6. (A). TPV and its metabolites in the serum of TPV-treated mice. (B). TPV and its metabolites in the liver of TPV-treated mice. (C). TPV and its metabolites in the serum of TPV/r co-treated mice. (D). TPV and its metabolites in the liver of TPV/r co-treated mice.

Figure 6. TPV Metabolism and Inhibition by RTV in Mice. By using metabolomic approach, the TPV metabolic map was extended to include two known and six novel pathways. CYP3A was identified as the primary enzyme contributing to the formation of four TPV metabolites (II, IV, V, and VI). CYP2C, 2D, and 3A have collaboratively contributed to the formation of a monohydroxylated metabolite (III). All TPV metabolic pathways are significantly inhibited by RTV in vivo.
TABLE 1. **Roles of P450s in TPV metabolic pathways.** Duplicate incubations were conducted in 1X PBS (pH 7.4), containing TPV (50 µM), NADPH (1.0 mM), each cDNA-expressed P450 enzyme (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 others was compared to this enzyme. The data was expressed as a mean. The metabolite ID was accordant with Fig 2 and Fig 6.

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Figure 5

A

Serum TPV

B

Liver TPV

C

Serum TPV (+RTV)

D

Liver TPV (+RTV)
Figure 6