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CPY3A4-mediated lopinavir bioactivation and its inhibition by ritonavir

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Abbreviations: LPV, lopinavir; RTV, ritonavir; PI, protease inhibitor; LPV/r, ritonavir-boosted lopinavir regimen; DMP, 2,6-dimethylphenol; GSH, glutathione; HLM, human liver microsomes; PBS, phosphate-buffered saline; OPLS-DA, orthogonal projection to latent structures-discriminant analysis; TOFMS, time-of-flight mass spectrometry; UPLC, ultraperformance liquid chromatography.

Abstract

The combination of lopinavir (LPV) and ritonavir (RTV) is one of the preferred regimens for the treatment of HIV infection with confirmed efficacy and relatively low toxicity. LPV alone suffers the poor bioavailability due to its rapid and extensive metabolism. RTV boosts the plasma concentration of LPV by suppressing its metabolism and thus increasing LPV efficacy. In the current study, we found that RTV also inhibits LPV bioactivation. LPV bioactivation was investigated in human liver microsomes and cDNA-expressed human cytochromes P450. Twelve glutathione-trapped reactive metabolites of LPV were identified by using a metabolomic approach. Semicarbazide-trapped reactive metabolites of LPV were also detected. RTV effectively suppressed all pathways of LPV bioactivation via CYP3A4 inhibition. Our data together with previous reports suggest that LPV plus RTV is an ideal combination because RTV not only boosts LPV plasma concentration, but decreases LPV bioactivation.

Introduction

Lopinavir (LPV) is an HIV protease inhibitor (PI) (Sham et al., 1998; Hurst and Faulds, 2000). Because of the rapid metabolism of LPV by cytochrome P450 3A (CYP3A), LPV bioavailability is very low (Kumar et al., 1999a; Ter Heine et al., 2011). In clinical practice, LPV is co-administered with ritonavir (RTV), a PI that strongly inhibits CYP3A4 activity, to increase LPV plasma concentration and efficacy (Cvetkovic and Goa, 2003; Kumar et al., 2004; Oldfield and Plosker, 2006). In healthy volunteers, the maximum plasma concentration of LPV was shown to be ~0.1 mg/mL after a single dose of 400 mg LPV. In contrast, co-administration of 400 mg LPV with 50 mg RTV increased the maximum plasma concentration of LPV to 5.5 mg/mL (Sham et al., 1998). At present, the RTV-boosted LPV (LPV/r) regimen is the only co-formulated PI available that is approved by the Food and Drug Administration for the treatment of HIV infection in both adults and children (Croxtall and Perry, 2010).

The LPV/r regimen is generally well tolerated (Murphy et al., 2008; Gathe et al., 2009). For example, the incidence of severe liver events was relatively low in HIV patients receiving LPV/r (Bonfanti et al., 2005; Palacios et al., 2006). However, lead-in treatment with rifampicin significantly increased hepatotoxicity associated with LPV/r (Nijland et al., 2008). Rifampicin is a potent CYP3A inducer (Kolars et al., 1992), which may accelerate LPV and/or RTV bioactivation and result in liver injury. LPV is known to be metabolized by CYP3A (Kumar et al., 1999b; Kumar et al., 2004). Nevertheless, no information of LPV bioactivation is available. In a previous study, a mechanism-based inactivation of CYP3A4 by LPV has been reported (Ernest et al., 2005).

Mechanism-based P450 inactivation usually involves bioactivation of the drug to reactive intermediate(s), which covalently modify active site(s) of the P450 (Kalgutkar et al., 2007). These previous reports suggest that LPV bioactivation is likely occurring.

The current study was designed to investigate LPV bioactivation and examine the effect of RTV on LPV bioactivation. We identified glutathione (GSH)-trapped and semicarbazide-trapped reactive metabolites of LPV. RTV effectively inhibited all pathways of LPV bioactivation that are primarily mediated by CYP3A4.

Materials and methods

Materials. LPV [(2*S*)-*N*-[(2*S*,4*S*,5*S*)-5-[2-(2,6-dimethylphenoxy)acetamido]-4-hydroxy-1,6-diphenylhexan-2-yl]-3-methyl-2-(2-oxo-1,3-diazinan-1-yl)butanamide]] and RTV (1,3-thiazol-5-ylmethyl *N*-[(2*S*,3*S*,5*S*)-3-hydroxy-5-[(2*S*)-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 CYP450s and human liver microsomes (HLM) were purchased from XenoTech (Lenexa, KS). GSH, 2,6-dimethylphenol (DMP), semicarbazide and NADPH were obtained from Sigma-Aldrich (St. Louis, MO). All the solvents for liquid chromatography and mass spectrometry were of the highest grade commercially available.

LPV bioactivation. LPV bioactivation was investigated in HLM and cDNA-expressed human CYP450s. GSH and semicarbazide were used as trapping reagents to

confirm the existence of reactive metabolites of LPV. The incubation was conducted in 100 mM phosphate-buffered saline (PBS), containing 30 μ M LPV, 2.5 mM GSH, 1.0 mg/mL HLM, and 1.0 mM NADPH in a final volume of 400 μ L. The final volume of the incubation with semicarbazide (2.5 mM) was 1.5 mL. After 5 min of pre-incubation at 37 °C, the reaction was initiated by the addition of NADPH and continued for 50 min with gentle shaking. Incubations in the absence of NADPH or trapping reagent were used as controls. The reaction mixtures were quenched by adding 100 μ L of formic acid (10% in water) for GSH containing incubations and 350 μ L for the semicarbazide containing incubations. After centrifugation at 18,000 rcf for 10 min, the resulting supernatants were loaded onto Oasis HLB solid-phase extraction cartridges (Waters, Milford, MA). The cartridges were washed with 1.0 mL of water, followed by 1.0 mL of methanol. The methanol fractions were dried using nitrogen and reconstituted with 100 μ L of acetonitrile/water (v/v, 50/50). Aliquots (5.0 μ L) of the reconstituted solutions were injected into a system combining ultraperformance liquid chromatography (UPLC) and time-of-flight mass spectrometry (TOFMS) (SYNAPT G1, Waters, Milford, MA).

Effect of RTV on LPV bioactivation. Because LPV is co-administered with RTV in clinical practice, RTV was co-incubated with LPV to examine the effect of RTV on LPV bioactivation. The incubation was conducted in PBS (100 mM, pH 7.4), containing 30 μ M LPV, 2.0 μ M RTV, 2.5 mM GSH or semicarbazide, 1.0 mg/mL HLM. The reaction was initiated by the addition of NADPH and continued for 50 min with gentle shaking. The reaction mixtures were quenched by the addition of 10% formic acid. After

centrifugation at 18,000 rcf for 10 min, the resulting supernatants were extracted using solid-phase extraction cartridges and analyzed by UPLC-TOFMS.

Role of CYP450s in LPV bioactivation. cDNA-expressed CYP450s (CYP1A2, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4) were used to determine the role of individual CYP450 in LPV bioactivation. The incubation was conducted in PBS (100 mM, pH 7.4), containing 30 μ M LPV, 2.5 mM GSH, and 10 pmol of each cDNA-expressed CYP450. The reaction was initiated by the addition of NADPH and continued for 50 min with gentle shaking. The reaction mixtures were quenched by the addition of 10% formic acid. After centrifugation at 18,000 rcf for 10 min, the resulting supernatants were extracted using solid-phase extraction cartridges and analyzed by UPLC-TOFMS. The incubations were conducted in duplicate.

Role of CYP450s in DMP bioactivation. DMP was identified as a novel metabolite of LPV in the current study. cDNA-expressed CYP450s were used to determine the role of individual CYP450 in DMP bioactivation. The incubation was conducted in PBS (100 mM, pH 7.4), containing 30 μ M DMP, 2.5 mM GSH, and 20 pmol of each cDNA-expressed CYP450 in a final volume of 200 μ L. The reaction was initiated by adding of NADPH and continued for 30 min with gentle shaking. The reaction mixtures were terminated by the addition of cold acetonitrile (200 μ L). After centrifugation at 18,000 rcf for 10 min, the resulting supernatants were analyzed by UPLC-TOFMS. The incubations were conducted in duplicate.

UPLC-TOFMS analyses. Chromatographic separation of trapped reactive metabolites of LPV was achieved using a 100 mm x 2.1 mm (Acquity 1.7 μ m) 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 a positive mode 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). 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, generating a reference ion at m/z 556.2771. The capillary voltage and the cone voltage were set at 3.5 kV and 35 V. The structures of trapped reactive metabolites of LPV were elucidated by tandem mass spectrometry fragmentation with collision energy ramp ranging from 10 to 40 eV.

Data analysis. The implication of a metabolomic approach in studying xenobiotics bioactivation was recently developed and verified by examining bioactivation of pulegone, acetaminophen, and clozapine (Li et al., 2011). Briefly, mass chromatograms and mass spectra were acquired by MassLynx software (Waters Corp., Milford, MA) in centroid format from m/z 50 to 1000. Centroid and integrated mass chromatographic data were processed by MarkerLynx software (Waters Corp., Milford, MA) to generate a multivariate data matrix. The corresponding data matrices were then exported into SIMCA-P (Umetrics, Kinnelon, NJ) for multivariate data analysis. Principal component analysis and orthogonal projection to latent structures-discriminant analysis (OPLS-DA)

were conducted on Pareto-scaled data. For chemometric analysis, the matrix data were processed from m/z 700 to 1000.

Results

Profiling trapped reactive metabolites of LPV using a metabolomic approach.

Overall, 12 GSH-trapped and 3 semicarbazide-trapped reactive metabolites of LPV were identified (Table 1). The results of chemometric analysis of the incubations of LPV in HLM are shown in Figure 1. The supervised OPLS-DA analysis separated samples corresponding to two controls (without GSH or NADPH) and the analyte (with GSH and NADPH) (Figure 1A). The S-plot generated from OPLS-DA analysis revealed that LPV_GSH adducts highly contributed to the group separation (Figure 1B). Ten LPV_GSH adducts (I-X) were readily uncovered, including two GSH conjugated LPV adducts (I and II), four GSH conjugated monohydroxylated LPV adducts (III, IV, V, and VI), one GSH conjugated dihydroxylated+dehydrogenated LPV adduct (VII), one GSH conjugated dihydroxylated LPV adduct (VIII), and two GSH conjugated trihydroxylated+dehydrogenated LPV adducts (IX and X). In addition, two GSH conjugated hydroxylated DMP adducts (XI and XII) and three LPV_semicarbazide adducts (LPV_hydrazones 1, 2 and 3) were screened out from the biological matrix as expected metabolites.

Identification of GSH-trapped reactive metabolites of LPV. Structural elucidations were performed on the basis of accurate mass measurement and MS/MS fragmentations. LPV adduct I, eluted at 5.73 min (Figure 2A), had a protonated molecule $[M+H]^+$ at m/z 934.4413, 305 Daltons higher than that of LPV. Compared to the MS/MS

of LPV (Figure 2B), adduct I had the same fragments at m/z 447.2668 and 429.2541, suggesting that GSH conjugated at the right moiety. The fragment ion at m/z 488.1823 in Figure 2C is 305 Daltons higher than that of ion at 183 in Figure 2B, indicating that the GSH moiety was attached to the encircled unit (Figure 2C). Other fragments were interpreted in the inlaid structural diagram. Adduct II was eluted at 5.68 min (Figure 2A), having a protonated molecule $[M+H]^+$ at m/z 934.4384 (Figure 2D). Adduct II had similar mass spectra patterns to that of adduct I, indicating that GSH was linked to the same unit as with adduct I, but at a different position. The chromatograms and MS/MS spectra of LPV_GSH adducts III to X are provided in the supplemental data (Supplemental Figures 1 to 4).

Identification of semicarbazide-trapped reactive metabolites of LPV. Three LPV hydrazones (LPV_hydrazone_1, 2, and 3) were detected in the incubation of LPV with semicarbazide in HLM (Figure 3). LPV_hydrazone_1, eluted at 4.99 min (Figure 3A), had a protonated molecule $[M+H]^+$ at m/z 580.3199. MS/MS analysis of LPV_hydrazone_1 produced daughter ions at m/z 398.2188, 323.1753, 183.1134, and 155.1183 (Figure 3B). The mass difference between the ions at m/z 398.2188 and 323.1753 is 75 Daltons, which equals to the mass of semicarbazide, suggesting that hydrazone formed. LPV_hydrazone_2 (Figures 3A and 3C), eluted at 4.58 min, had a protonated molecule $[M+H]^+$ at m/z 578.3071. MS/MS analysis of LPV_hydrazone_2 produced daughter ions at m/z 560.3050 (loss of H₂O), 398.2188, 323.1764, 181.1008, and 153.1023. Compared to the ions at m/z 183.1134 and 155.1183 generated from LPV_hydrazone_1, the ions at 181.1008 and 153.1023 from LPV_hydrazone_2 indicate

that the dehydrogenation occurred in the encircled units (Figure 3C). LPV_hydrazone_3 had a protonated molecule $[M+H]^+$ at m/z 594.3044, 16 Daltons higher than that of LPV_hydrazone_2. MS/MS analysis of LPV_hydrazone_3 produced daughter ions at m/z 576.2954 (loss of H₂O), 398.2197, 381.1949, 323.1733, and 250.1614. Compared to the ions at m/z 181.1008 and 153.1023 produced from LPV_hydrazone_2, the ions at 197.0921 and 169.0972 from LPV_hydrazone_3 suggest that the dehydrogenation and hydroxylation occurred in the encircled units (Figure 3D).

Coupled with the generation of LPV aldehydes, DMP is produced (Figure 4). DMP is further oxidized to form O_DMP, which can be transformed into reactive intermediates, such as quinone and/or quinone methide, and then be trapped by GSH (Evans et al., 2004; Ma and Subramanian, 2006). In the incubation of LPV in HLM containing GSH, two GSH conjugated DMP adducts were identified (Figure 5). Adduct XI was detected at 3.22 min (Figure 5A), having a protonated molecule $[M+H]^+$ at m/z 444.1396. The MS/MS of adduct XI produced fragment ions at m/z 315.1006, 195.0494, 169.0330, and 130.0508. The formation of ions 315.1006 and 169.0330 suggest that the GSH was connected to monooxidized DMP (Figure 5B). Adduct XII, eluted at 2.98 min (Figure 5A), had a protonated molecule $[M+H]^+$ at m/z 444.1425. Adduct XII had a similar fragmentation pattern to that of XI, except for the ions at m/z 297.0750 and 251.0679. The slight difference in MS/MS between XI and XII suggest that the hydroxylated or GSH coupled position varied (Figure 5C). The existence of adducts XI and XII were confirmed in the incubation of authentic DMP in HLM containing GSH (data not shown).

Effect of RTV on LPV bioactivation. Twelve GSH conjugated LPV adducts were identified in the incubation with HLM (Figure 6). RTV significantly suppressed all pathways of LPV bioactivation. The formation of LPV adducts I and II was suppressed ~80% by RTV in the incubation with HLM (Figure 6). The formations of LPV_GSH adducts III to XII were completely inhibited by RTV (Figure 6). The formations of semicarbazide conjugated LPV adducts (LPV_Hydrazone_1, 2, and 3) were also entirely inhibited by RTV (Figure 7). These data suggest that CYP3A4 plays an important role in the LPV bioactivation and RTV suppresses LPV bioactivation via CYP3A4 inhibition.

Role of CYP450s in LPV bioactivation. From the inhibitory test of RTV on LPV bioactivation, we observed that RTV cannot entirely suppress LPV adducts I and II formation in HLM (Figure 6), suggesting that some other enzyme(s) are involved in LPV bioactivation. We examined LPV bioactivation in a panel of CYP450s including CYP1A2, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4. CYP3A4 was identified as the dominant enzyme in the formation of LPV adducts I and II. In addition, ~8% of the formation of LPV adducts I and II was mediated by CYP2D6 (Figure 8A). These data suggest that CYP2D6 may be involved in LPV bioactivation. We found that multiple enzymes, especially CYP1A2, are involved in DMP bioactivation (Figure 8B). These data suggest that the formation of adducts XI and XII need two metabolic steps (Figure 4): step 1 is primarily mediated by CYP3A4 and step 2 is primarily mediated by CYP1A2.

Discussion

Characterizing reactive intermediates can provide information on the structure of the reactive species, thereby defining a potential bioactivation mechanism. In addition, information on adducts of reactive metabolites can be used to predict the potential bindings of reactive metabolites with cellular proteins and/or some other molecules. Most reactive metabolites are not stable, so it is difficult to detect them directly. Reactive metabolites can form adducts with trapping reagents that make the reactive metabolites detectable (Evans et al., 2004; Li et al., 2011). GSH is commonly used to trap soft electrophiles, such as epoxides, α,β -unsaturated carbonyls, quinones, quinone imines, quinone methides, imine methide, isocyanate, isothiocyanates, aziridinium, and episulfonium (Evans et al., 2004; Ma and Subramanian, 2006). Semicarbazide is used to trap hard electrophiles, such as aldehydes (Evans et al., 2004; Li et al., 2011). In the current study, GSH and semicarbazide were used as trapping reagents. We identified 12 GSH-trapped and 3 semicarbazide-trapped reactive metabolites of LPV.

Our study showed that CYP3A4 is the primary enzyme involved in LPV bioactivation (Figures 6 and 7). CYP3A4 is one of the most important metabolic enzymes expressed in liver and intestine (Guengerich, 1999). Induction or inhibition of CYP3A4 has been known to cause significant drug-drug interactions (Guengerich, 1997; Tanaka, 1998). Because of the importance of CYP3A4 in LPV bioactivation, the effects of CYP3A4 inhibitors and inducers on LPV bioactivation are expected. RTV is a potent CYP3A4 inhibitor that is routinely co-administered with LPV. We found that RTV effectively suppressed LPV bioactivation (Figures 6 and 7). To our knowledge, no clinical studies

have been done to compare the safety profile of LPV/r to LPV alone. However, there is a case report in which LPV alone caused serious multi-organ hypersensitivity (Manfredi and Sabbatani, 2006). These data suggest that RTV may reduce the toxic risk resulting from LPV bioactivation.

On the contrary, co-administering a CYP3A4 inducer, such as rifampicin, with LPV may increase LPV bioactivation and augment LPV side effects. Previous clinical studies have demonstrated that rifampicin decreases the plasma concentration of LPV (la Porte et al., 2004; Ren et al., 2008) and increases the incidence of LPV/r hepatotoxicity (Nijland et al., 2008; L'Homme R et al., 2009). Further studies are suggested to determine the role of rifampicin-mediated CYP3A4 induction in LPV/r bioactivation and hepatotoxicity.

In addition to CYP3A4, we noted that CYP2D6 and 1A2 also contribute to LPV bioactivation. CYP2D6 is involved in the formation of adducts I, II, XI, and XII (Figure 8). Although CYP2D6 plays a minor role in these pathways of LPV bioactivation, CYP2D6 cannot be neglected because of the extremely high polymorphism of this enzyme (Ingelman-Sundberg, 2005). DMP is a metabolite of LPV that can be further metabolized to form quinone and/or quinone methide intermediates that are known to be toxic mediators in drug-induced toxicity (Bolton et al., 2000; Evans et al., 2004). We trapped these intermediates by using GSH. CYP1A2 is determined to be the primary enzyme contributing to DMP bioactivation (Figures 8B). CYP1A2 expression is highly inducible by a number of dietary components and some polycyclic aromatic hydrocarbons, some of which are found in cigarette smoke (Kalow and Tang, 1991;

Sachse et al., 2003). Therefore, smoking and CYP1A2 inducers should be avoided in patients treated with LPV/r, especially in the condition of CYP3A4 induction.

In summary, LPV bioactivation was investigated using a metabolomic approach. Twelve GSH-trapped and three semicarbazide-trapped reactive metabolites of LPV were identified. RTV effectively suppressed LPV bioactivation via CYP3A4 inhibition. Our data together with previous reports suggest that LPV/r is an ideal combination because RTV not only boosts LPV plasma concentration, but decreases LPV bioactivation.

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Authorship contributions

Participated in research design: Feng Li, Xiaochao Ma

Conducted experiments: Feng Li, Jie Lu

Contributed the new reagents or analytic tools: Feng Li

Performed data analysis: Feng Li, Xiaochao Ma

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

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Footnote

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FIGURE LEGENDS

Figure 1. Metabolomic analysis of control groups and GSH trapped group of LPV in the incubations with HLM. The incubation conditions of LPV were detailed in experimental procedures. All samples were analyzed by UPLC-TOFMS. (A) Separation of each incubation group in OPLS-DA score plot. The $t[1]P$ and $t[2]O$ values represent the score 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. The top ranking ions are labeled. *, in-source fragment.

Figure 2. Identification of GSH conjugated LPV adducts I and II. LPV was incubated in HLM containing GSH and analyzed by UPLC-TOFMS. Major daughter ions from fragmentation were interpreted in the inlaid structural diagrams. (A) Extracted ion chromatograms of LPV adducts I and II. (B) MS/MS of LPV. (C) MS/MS of LPV adduct I. (D) MS/MS of LPV adduct II.

Figure 3. The extracted ion chromatograms (A) and MS/MS spectra of LPV_hydrazone_1 (B), 2 (C), and 3 (D). Three LPV hydrazones (LPV_hydrazone_1, 2 and 3) were detected in the incubation of LPV in HLM containing semicarbazide. All samples were analyzed by UPLC-TOFMS. Major daughter ions from fragmentation were interpreted in the inlaid structural diagrams.

Figure 4. Proposed mechanism of the formation of aldehydes and DMP in LPV metabolism, and the strategies to trap these metabolites.

Figure 5. Identification of GSH conjugated DMP adducts XI and XII. The incubation conditions of LPV were detailed in experimental procedures. All samples were analyzed by UPLC-TOFMS. Major daughter ions from fragmentation were interpreted in the inlaid structural diagrams. (A) Extracted ion chromatograms of adducts XI and XII. (B) MS/MS of adduct XI. (C) MS/MS of adduct XII.

Figure 6. Effect of RTV on the formation of GSH conjugated LPV adducts. Quadruplicate incubations were conducted in PBS (pH 7.4) containing LPV (30 μ M), GSH (2.5 mM), NADPH (1.0 mM), HLM (1.0 mg/ml). RTV (2.0 μ M) was used in the inhibitory test. All samples were analyzed by UPLC-TOFMS. The abundance of each adduct was relatively quantified using peak areas and presented as mean \pm SD. ND, not detected.

Figure 7. Effect of RTV on the formation of semicarbazide conjugated LPV adducts. Duplicate incubations were conducted in PBS (pH 7.4) containing LPV (30 μ M), semicarbazide (2.5 mM), NADPH (1.0 mM), HLM (1.0 mg/ml). RTV (2.0 μ M) was used in the inhibitory test. All samples were analyzed by UPLC-TOFMS. The abundance of each adduct was relatively quantified using peak areas and presented as mean. ND, not detected.

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Figure 8. Role of CYP450s in LPV bioactivation. cDNA-expressed CYP450s were used to determine the role of individual CYP450 in LPV bioactivation. The incubation conditions of LPV were detailed in experimental procedures. All samples were analyzed by UPLC-TOFMS. (A) Enzymes contributing to the formation of LPV adducts I and II. The substrate was LPV. The peak area of each adduct from the incubation with CYP3A4 was set as 100%. (B) Enzymes contributing to the formation of adducts XI and XII. The substrate was DMP. The peak area of each adduct from the incubation with CYP1A2 was set as 100%. All data are expressed as mean (n=2). ND, not detected.

Table 1. Summary of trapped reactive metabolites of LPV*.

Mass	Predicted formula	RT (min)	Mass error (ppm)	Identification	Code
934.4430	C ₄₇ H ₆₄ N ₇ O ₁₁ S	5.73	4.8	LPV_GSH	I
934.4443	C ₄₇ H ₆₄ N ₇ O ₁₁ S	5.68	6.2	LPV_GSH	II
950.4378	C ₄₇ H ₆₄ N ₇ O ₁₂ S	5.81	4.6	O_LPV_GSH	III
950.4404	C ₄₇ H ₆₄ N ₇ O ₁₂ S	5.65	7.4	O_LPV_GSH	IV
950.4346	C ₄₇ H ₆₄ N ₇ O ₁₂ S	5.11	1.3	O_LPV_GSH	V
950.4398	C ₄₇ H ₆₄ N ₇ O ₁₂ S	5.00	5.2	O_LPV_GSH	VI
964.4189	C ₄₇ H ₆₂ N ₇ O ₁₃ S	4.66	6.5	2O_LPV_GSH (-2H)	VII
966.4221	C ₄₇ H ₆₄ N ₇ O ₁₃ S	5.03	-6.3	2O_LPV_GSH	VIII
980.4088	C ₄₇ H ₆₂ N ₇ O ₁₄ S	4.70	1.3	3O_LPV_GSH (-2H)	IX
980.4104	C ₄₇ H ₆₂ N ₇ O ₁₄ S	4.78	3.0	3O_LPV_GSH (-2H)	X
444.1468	C ₁₈ H ₂₆ N ₃ O ₈ S	3.22	6.1	O_DMP_GSH	XI
444.1460	C ₁₈ H ₂₆ N ₃ O ₈ S	2.98	4.3	O_DMP_GSH	XII
580.3259	C ₃₀ H ₄₂ N ₇ O ₅	4.99	2.1	LPV_aldehyde_semicarbazide	LPV_hydrazone_1

578.3099	$C_{30}H_{40}N_7O_5$	4.58	1.4	LPV_aldehyde_semicarbazide (-2H)	LPV_hydrazone_2
594.3040	$C_{30}H_4N_7O_6$	4.74	0.5	O_LPV_aldehyde_semicarbazide (-2H)	LPV_hydrazone_3

*The incubation conditions of LPV were detailed in experimental procedures. All samples were analyzed by UPLC-TOFMS. RT, retention time; O, monohydroxylated; 2O, dihydroxylated; 3O, trihydroxylated; -2H, dehydrogenated.

Figure 1

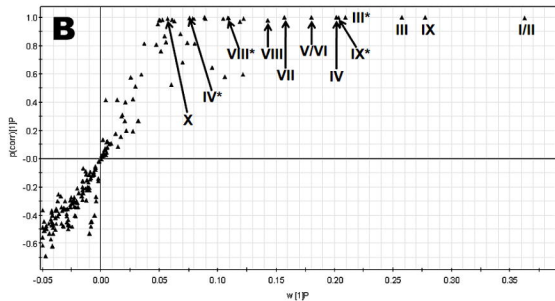
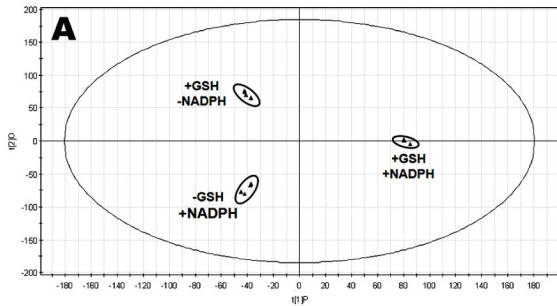


Figure 2

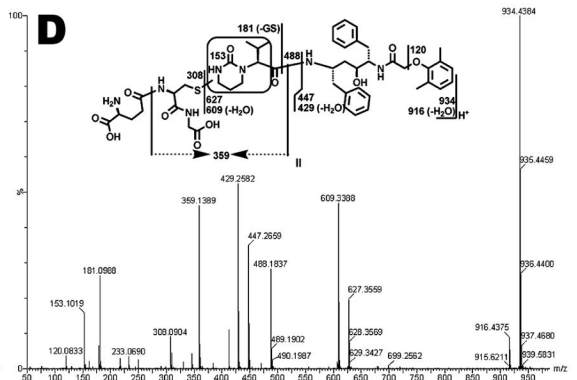
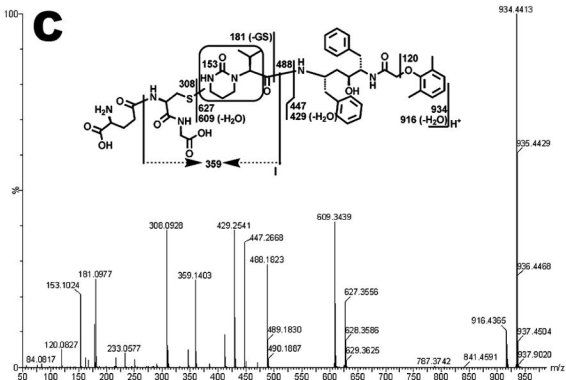
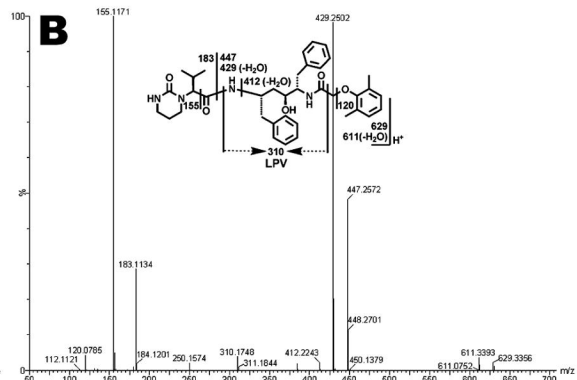
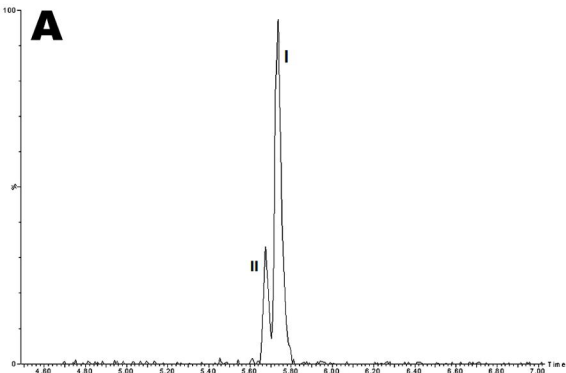


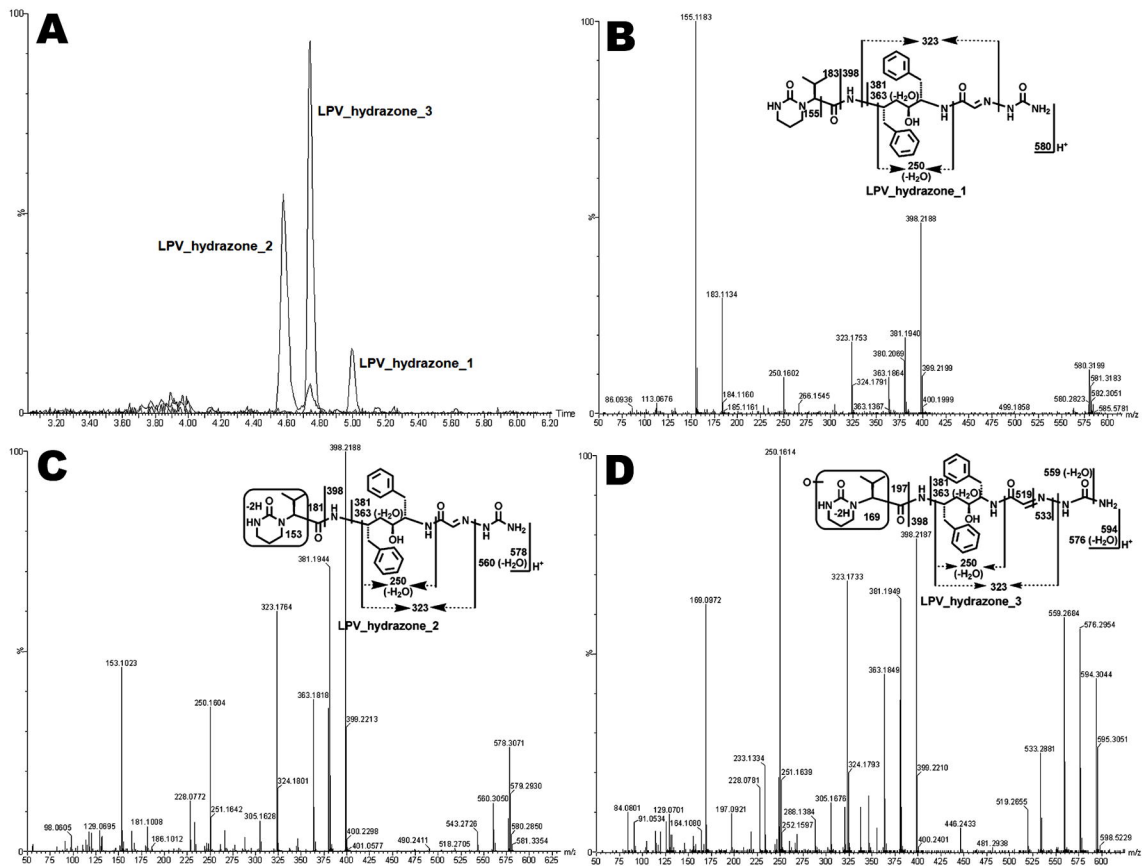
Figure 3

Figure 4

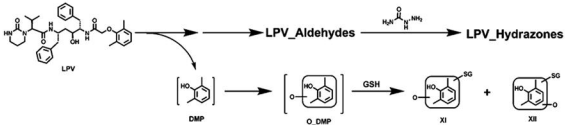


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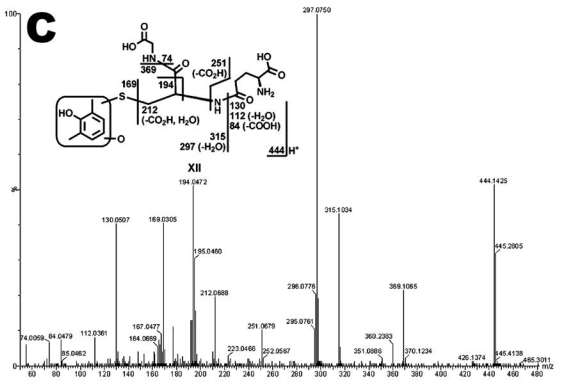
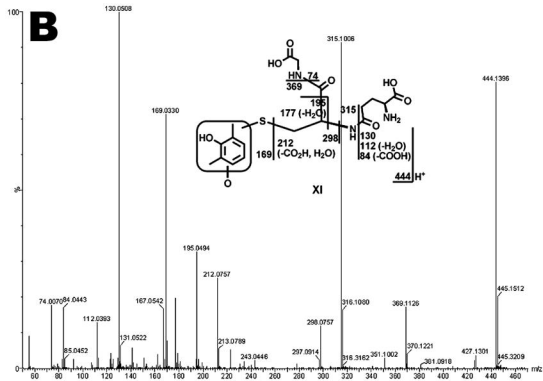
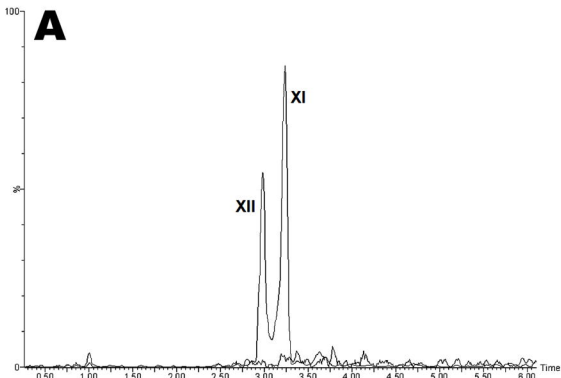


Figure 6

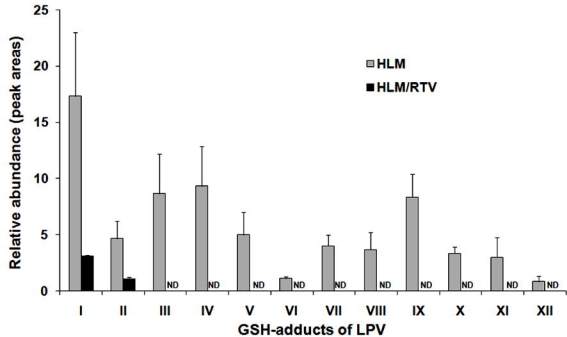


Figure 7

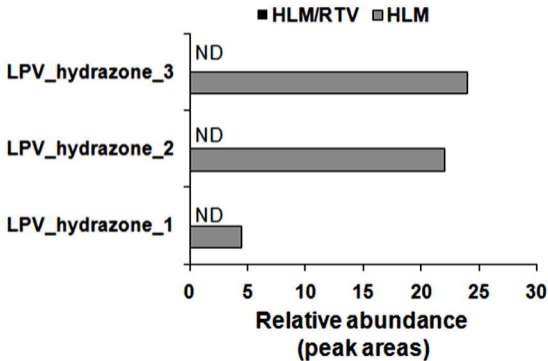
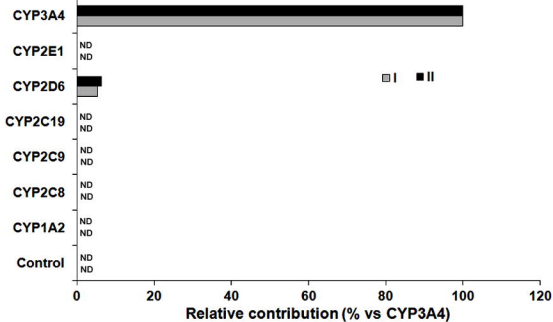


Figure 8

A



B

