Published in final edited form as:

Xenobiotica. 2019 June ; 49(6): 655–670. doi:10.1080/00498254.2018.1489167.

A metabolomic perspective of pazopanib-induced acute hepatotoxicity in mice

Yi-Kun Wang^{a,b,*}, Xiao-Nan Yang^{a,*}, Wei-Qing Liang^{c,*}, Yao Xiao^{a,b}, Qi Zhao^{a,b}, Xue-Rong Xiao^a, Frank J. Gonzalez^d, and Fei Li^{a,*}

^aState Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China

^bUniversity of Chinese Academy of Sciences, Beijing, China

^cCenter for Medicinal Resources Research, Zhejiang Academy of Traditional Chinese Medicine, Hangzhou, China

^dLaboratory of Metabolism, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

Abstract

1. To elucidate the metabolism of pazopanib, a metabolomics approach was performed based on ultra-performance liquid chromatography coupled with electrospray ionization quadrupole mass spectrometry.
2. A total of 22 pazopanib metabolites were identified *in vitro* and *in vivo*. Among these metabolites, 17 were novel, including several cysteine adducts and aldehyde derivatives. By screening using recombinant CYPs, CYP3A4 and CYP1A2 were found to be the main forms involved in the pazopanib hydroxylation. Formation of a cysteine conjugate (**M3**), an aldehyde derivative (**M15**) and two *N*-oxide metabolites (**M18** and **M20**) from pazopanib could induce the oxidative stress that may be responsible in part for pazopanib-induced hepatotoxicity.
3. Morphological observation of the liver suggested that pazopanib (300 mg/kg) could cause liver injury. The aspartate transaminase and alanine aminotransferase in serum significantly increased after pazopanib (150, 300 mg/kg) treatment; this liver injury could be partially reversed by the broad-spectrum CYP inhibitor 1-aminobenzotriazole (ABT). Metabolomics analysis revealed that pazopanib could significantly change the levels of *L*-carnitine, proline and lysophosphatidylcholine 18:1 in liver. Additionally, drug metabolism-related gene expression analysis revealed that hepatic *Cyp2d22* and *Abcb1a* (P-gp) mRNAs were significantly lowered by pazopanib treatment.

Full Terms & Conditions of access and use can be found at <https://www.tandfonline.com/action/journalInformation?journalCode=ixen20>

CONTACT Fei Li, lifeib@mail.kib.ac.cn, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China.

*These authors contributed equally to this work.

Disclosure statement

No potential conflict of interest was reported by the authors.

Supplemental data for this article can be accessed [here](#).

4. In conclusion, this study provides a global view of pazopanib metabolism and clues to its influence on hepatic function.

Keywords

Pazopanib; hepatotoxicity; metabolomics; oxidative stress; ultra-performance liquid chromatography-electrospray ion source-quadrupole/time-of-flight mass spectrometer (UPLC-ESI-QTOFMS)

Introduction

Pazopanib, a tyrosine-kinase inhibitor, was approved by the United States Food and Drug Administration (US FDA) and the European Medicines Agency to treat renal cell carcinoma and prevent its metastasis (Pal et al., 2015). Pazopanib also shows therapeutic efficacy on non-adipocytic advanced soft tissue sarcoma (Endo & Nielsen, 2012; Sedef et al., 2015; Karch et al., 2016), epithelial ovarian cancer (Dinkic et al., 2017) and non-small cell lung cancer (Koinis et al., 2017). However, adverse effects were frequently reported, including increased liver injury, hypertension and lupus erythematosus (Casado-Verrier et al., 2014). For example, meta-analyses of clinical trials reported that pazopanib is associated with increased risk of liver toxicity (Lim et al., 2013; Rickmann et al., 2013; Jain et al., 2015; Vlenterie et al., 2015; Shantakumar et al., 2016; Choudhury et al., 2017). *In vitro* screening of 31 small-molecule kinase inhibitors approved by the US FDA revealed that pazopanib is associated with significant liver toxicity involving mitochondria damage (Zhang et al., 2017). Thus, pazopanib is clinically accompanied by a black box warning on potential hepatotoxicity (Klempner et al., 2012; Shantakumar et al., 2016; Choudhury et al., 2017). However, the mechanism by which pazopanib causes hepatotoxicity and other side-effects remains unclear.

Drug metabolism plays a key role in the safety of drug, and side-effects of drug frequently result from drug metabolism, especially for drug-drug interaction and bioactivation. To date, there are few studies reporting metabolism and disposition of pazopanib. One study revealed that seven pazopanib metabolites were derived from hydroxylation, *N*-demethylation and glucuronidation in humans and pazopanib was primarily excreted as unchanged drug in feces (Deng et al., 2013). Another clinical study found that pazopanib was primarily metabolized by CYP3A4, and its metabolic rate can be markedly attenuated by the CYP3A4 inhibitor ketoconazole (Tan et al., 2013). The objectives of the present study were to determine pazopanib metabolism in mice using a systematic metabolomics approach, identify the drug-metabolizing enzymes involved in pazopanib metabolism and investigate the change of endogenous metabolites in mouse liver following oral treatment with pazopanib. Expression of mRNAs encoding hepatic transporters and drug metabolic enzymes closely related to drug toxicity were also examined. Several novel reactive metabolites of pazopanib including cysteine conjugates, aldehyde derivatives and *N*-oxide metabolites were found in this study, which could induce the oxidative stress. These data contribute to understanding the mechanism of hepatotoxicity associated with pazopanib treatment.

Materials and methods

Chemicals and reagents

Pazopanib was provided by Medchem Express (Shanghai, China). Nicotinamide adenine dinucleotide phosphate (NADPH), formic acid, *L*-carnitine, proline and chlorpropamide were purchased by Sigma-Aldrich (St. Louis, MO). Mouse liver microsomes (MLM) and human liver microsomes (HLM) were obtained from Bioreclamationivt Inc. (Hicksville, NY). Recombinant human CYPs were purchased from Xenotech, LLC (Kansas City, KS). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Superoxide dismutase (SOD) and malondialdehyde (MDA) kits were purchased from Suzhou Comin Biotechnology Co. Ltd (Suzhou, China).

Animal experiment and biological samples preparation

Mice are a classical model to investigate metabolism-mediated toxicity of drugs, including acetaminophen (Chen et al., 2008) and procainamide (Li et al., 2012). In the present study, C57BL/6J mice (male, 20–22 g, 6- to 7-week old) were obtained from Shanghai laboratory animal central (Shanghai, China) and used to investigate the metabolism-mediated hepatotoxicity of pazopanib. All mouse studies were conducted strictly in accordance with study protocols approved by the Institutional Animal Care and Use Committee of the Kunming Institute of Botany, Chinese Academy of Sciences. The animals were maintained under a standard light/dark cycle of 12/12 h at constant temperature 23 ± 1 °C and humidity 50–60%, with water and chow provided *ad libitum*. The clinical oral dose of pazopanib is up to 800 mg once daily for 50 kg patients (Deng et al., 2013), which is equivalent to about 150 mg/kg in mice. Here, onefold and twofold equivalent doses in mice (150 and 300 mg/kg) were used to investigate pazopanib-related hepatotoxicity. Twelve mice were randomly divided into three groups, a high dose group (300 mg/kg), low dose group (150 mg/kg) and control group (vehicle) and acclimated in metabolic cages for 24 h prior to the administration of pazopanib. The experimental group was intragastrically administered pazopanib suspended in 0.5% carboxymethylcellulose sodium (CMC-Na), and the control group administered an equal volume of 0.5% CMC-Na. The mice were housed separately in metabolic cages for 24h after treatments. Blood samples were collected from the orbital venous sinus 3 and 24 h after pazopanib treatment, respectively. The mice were killed by CO₂ asphyxiation at 24 h, and the liver tissue was harvested. Plasma samples were obtained from blood samples by centrifugation at $2000 \times g$ for 5 min. Urinary and feces samples were collected for a period of 0–24h. A portion of liver was preserved in 4% paraformaldehyde for histopathological examination. All biological samples were stored at -80 °C before further analysis.

Samples processing was performed according to previous studies (Li et al., 2013; Zhao et al., 2018). Plasma samples were prepared by adding the 10 μ L plasma into 190 μ L of 67% aqueous acetonitrile containing 5 μ M chlorpropamide. Urinary samples were prepared by mixing 20 μ L of urine with 180 μ L of 50% aqueous acetonitrile containing 5 μ M of chlorpropamide as the internal standard. The plasma and urine samples were vortexed for 1 min, and then centrifuged at $18,000 \times g$ for 20 min at 4 °C. For feces samples, 20 mg weight

of feces were added into 200 μ L of 50% aqueous acetonitrile and homogenized by shaking for 20min. The feces samples were centrifuged at $18,000 \times g$ for 20 min, subsequently, 100 μ L of each supernatant from feces homogenate was transferred to a clean centrifuge tube and mixed with 200 μ L of 50% aqueous acetonitrile containing 5 μ M chlorpropamide. Twenty milligram of liver was homogenized in 200 μ L 50% aqueous acetonitrile containing 5 μ M chlorpropamide. The homogenate was centrifuged at $18,000 \times g$ for 20 min, and 100 μ L of supernatant was transferred to a new centrifuge tube and mixed with 100 μ L of 50% aqueous acetonitrile. All diluted solutions were centrifuged at $18,000 \times g$ for 20min to obtain aliquot supernatant until injection. A 5 μ L aliquot of each urine, feces and plasma samples was injected for UPLC-ESI-QTOFMS system to detect the potential metabolites.

Pre-treatment of ABT

The C57BL/6 mice were intraperitoneally injected with ABT (100 mg/kg, dissolved in physiological saline solution) 1 h prior to the administration of pazopanib (300 mg/kg, suspended in 0.5% CMC-Na). Injection with the saline solution and pazopanib was used as a control group. The samples were collected in the same way as above.

In vitro metabolism of pazopanib

Incubations of pazopanib with MLM, HLM or each c-DNA-expressed recombinant CYPs *in vitro* were carried out in 96 wells plates. The metabolic reaction was performed in phosphate buffer solution (pH = 7.4). 200 μ L of final incubation system volume contained 20 μ M of pazopanib, 0.5g/mL of MLM, HLM or 2 pmol/mL of each c-DNA-expressed CYPs (control protein, CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP3A4, CYP3A5 and CYP4A11). After preincubation at 37 $^{\circ}$ C for 5 min, the reactions were initiated by adding 20 μ L of freshly prepared NADPH (10 mM) into the system, and then incubation continued for 30 min with gentle shaking. The reactions were quenched by adding 200 μ L of ice cold acetonitrile. The absence of NADPH in the system was incubated separately as a control. The incubations were conducted in triplicates. After centrifugation at $18,000 \times g$ for 20 min at 4 $^{\circ}$ C, a 5 μ L aliquot of the supernatant was injected into UPLC-ESI-QTOFMS system for metabolite analysis.

UPLC-ESI-QTOFMS analysis

Chromatographic analysis of metabolites was conducted on an Agilent 1290 infinity UPLC system (Agilent Technologies, Santa Clara, CA) equipped with an XDB-C18 column (2.1 $\times$ 100mm, 1.8 μ M, Agilent) following a previous report with minor modification (Zhao et al., 2017b). The column temperature was maintained at 45 $^{\circ}$ C. The flow rate of mobile phase was 0.3mL/min with a gradient elution ranging from 2% to 98% acetonitrile containing 0.01% formic acid in 16 min run. The Q-TOFMS (Agilent 6500, Santa Clara, CA) was operated positive mode with electrospray ionization. Nitrogen was applied as cone gas and drying gas which was set at 350 $^{\circ}$ C. The nebulizer pressure was set at 35 psi, and capillary voltage was set at 3.5 kV. The MS/MS of pazopanib metabolites was performed in the targeted mode by collision energy of 20 eV. Relative quantitation of pazopanib metabolites was determined according to the abundance of peak area normalized by the internal standard. The quantitation of metabolites in urine, serum and feces was not precise without the standards which are not available.

Raw data extraction and processing

MassHunter profiler version B.06.00 software (Agilent, Santa Clara, CA) was adopted to process the molecular feature normalization and alignment of raw data to form data matrices. The parameters of normalization and alignment were set as follows: absolute peaks height above 1000 counts; centroid format range, 100 to 1000; adducts species in positive mode: +H, +Na, +K; compound ion count threshold, 2 or more ions; alignment RT window, 0.15 min intercept and 0.2% slope; alignment mass window, 20 ppm; integrator algorithm, agile. TOF spectra was excluded if above 60% of saturation in the m/z ranges; centroiding was carried out with maximum spike width 2 and required valley 0.70; mass spectral data format: centroid when available, otherwise profile. The normalized features were filtered again (absolute height 300 counts), and masses appearing in at least one of three sample groups was accepted, because the exogenous metabolites appeared only in the pazopanib-treated group. Background noise was eliminated through subtracting mass found in blank runs from filtered mass. The processed neutral mass, retention time and abundance of each feature were exported to a data matrix (.csv file) for orthogonal projection to latent structures-discriminant analysis (OPLS-DA) in SIMCA-P+13.0.

Multivariate data analysis

After the abundance of data matrix was calibrated by the abundance of chlorpropamide (internal standard normalization), OPLS-DA was used to determine the pazopanib metabolites and the endogenous metabolites. The quality of the model was evaluated by the cross-validation parameter Q^2 , R^2 Y. Potential metabolites of pazopanib were identified by analyzing the ions contributing to the separation from control group in the S-plot of the OPLS-DA. The metabolites with a VIP value >1.0 were regarded as potential candidates. Key criterion of screening potential drug metabolites is that the chromatographic peak of candidates was not detected in blank control for exclusion of endogenous interference. A one-way ANOVA was adopted to the differences of endogenous metabolites between the pazopanib treated group and the control group, and $p < 0.05$ was considered to be statistically significant.

Identification of pazopanib metabolites and altered endogenous metabolites

MassHunter Workstation data Acquisition software (Agilent, Santa Clara, CA) was applied to qualitative and quantitative analysis of potential metabolite, including molecular feature extraction and manual integration of peak area. The extracted ion chromatogram (EIC) of each metabolite was extracted with ± 20 ppm single ion expansion using MassHunter software. The molecular formulae for all parent ions of the target peaks were calculated from the accurate mass using a formula predictor by setting the related parameters as follows: C [0–40], H [0–50], N [0–10], O [0–20], S [0–5] and ring double bond (RDB) equivalent value [0–20]. The structural elucidation of pazopanib metabolites and endogenous metabolites were conducted according to previous approaches (Zhao et al., 2017b). Based on the formula, mass error (ppm) = $10^6 \times (\text{experimental molecular weight} - \text{theoretical molecular weight}) / \text{experimental molecular weight}$, the mass error of metabolites was accurately calculated. After the potential metabolites *in vitro* and *in vivo* were characterized by OPLS-DA analysis, tandem MS of selected metabolites was operated in the

targeted mode with a default isolation width of 0.01 m/z acquired by ramping collision energies 20V. The chemical structures of pazopanib metabolites were elucidated by interpretation of their MS/MS fragmentation patterns. The Human Metabolome Database (HMDB, <http://www.hmdb.ca>) was searched to match the changed endogenous metabolites. To confirm metabolite identities, the retention times and MS/MS fragmentation patterns of the potential endogenous metabolites were compared with that of authentic compounds.

Histological and biochemical assessment

The activities of aspartate amino transferase (AST) and alanine amino transferase (ALT) in serum were measured following the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Oxidative stress-related superoxide dismutase (SOD), and malondialdehyde (MDA) contents in hepatic tissue were detected by a colorimetric method using kits according to the manufacturer's protocols. Liver tissues were fixed in formaldehyde, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) for histological examination.

QPCR analysis of liver gene expression

Total RNA was extracted from approximately 50 mg of liver using TRIzol reagent (Lifetechnologies, Carlsbad, CA). Specific mRNA expression was determined using SYBR green PCR master mix (TaKaRa, Dalian, China) in a CFX Connect Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA). Primer sequences and the abbreviation of genes are shown in Supplementary Table 1. The relative amount of each mRNA was calculated after normalizing to 18S mRNA, and the results were expressed as fold change comparing to the control group.

Statistical analysis

All experimental data were expressed as mean $\pm$ SEM. Statistical analysis was carried out using Prism v. 6 (Graph Pad Software, San Diego, CA). Statistical significance between two groups was performed using one-way ANOVA.

Results

Metabolic profiling of pazopanib metabolites in mice

Metabolism of pazopanib in the serum, urine and feces of mice treated with pazopanib were investigated by mass spectrometry-based metabolomics. OPLS-DA model was used to distinguish the pazopanib group (300 mg/kg) from the control group (Figure 1(A)). Pazopanib metabolites were identified from the top-ranking ions in the S-plot (Figure 1(B)), and the signals representing pazopanib metabolites were only detected in the treatment group from the trend plot (Figure 1(C,D)). Overall 22 metabolites were identified by metabolomics analysis of feces, urine and plasma, including five previously reported metabolites and 17 novel metabolites (Table 1). Among these metabolites were two glucose-conjugated products, two glucuronic acid-conjugated products and three cysteine-conjugated adducts generated from pazopanib.

Pazopanib and its metabolites were excreted in both feces and urine (Figure 2(A,B)). Ten phase I metabolites of pazopanib were observed in urine, including **M1-7**, **M10-11** and **M15**, and seven phase II metabolites of pazopanib (**M17-23**) (Figure 2(B)). Fifteen phase I metabolites (**M1-11** and **M14-16**) and three phase II metabolites of pazopanib (**M18-20**) were found in feces (Figure 2(A)). Only the parent compound and five metabolites (**M1-2**, **M4-5** and **M13**) were detected in plasma (Figure 2(C)). The observed m/z , retention times, mass errors, putative identities and the source of the metabolites are summarized in Table 1. Hydroxylation, demethylation and hydroformylation were identified as the major phase I metabolic reactions. Moreover, the finding of three *N*-oxides indicated a novel metabolic reaction for pazopanib. The percentage of the observed metabolites of pazopanib in mouse feces, urine and serum is shown in Figure 2(A–C).

Identification of phase I metabolites

Since pazopanib is an alkaline nitrogenous organic compound, the mass spectrum signal of pazopanib and its metabolites exhibit a strong signal in the MS positive mode. The chemical structures of pazopanib metabolites were determined by high-resolution mass spectrometry and tandem MS fragmentography by comparing with the mass fragmentography of pazopanib (Figure 3(A,B)).

Metabolites **M1**, **M2** and **M3** eluted at 5.07, 5.30 and 5.70 min (Figure 3(C)), and exhibited similar molecular ions at m/z 454.167⁺, m/z 454.165⁺ and m/z 454.164⁺, respectively. These metabolites were well matched with the same formula C₂₁H₂₄N₇O₃S, suggesting that these three metabolites were isomeric compounds. **M1**, **M2** and **M3** were 16 Da (O) higher than that of pazopanib, indicating the formation of hydroxylated pazopanib or *N*-oxidized pazopanib. Both the molecular ions of metabolites **M1** and **M2** yielded two major fragmentation ions of m/z 436⁺ and m/z 373⁺, which were indicative of loss of H₂O and HSO₂NH₂ moiety, respectively. The formation of ion m/z 343⁺ was generated from m/z 373⁺ due to neutral losses of aldehyde moiety. Furthermore, the MS/MS spectrum of **M1** provided a signature ion at m/z 265⁺, revealing that the hydroxylated reaction site occurred in the moiety of indazol's methyl group. In contrast to **M1**, the characteristic ions of **M2** at m/z 279⁺ and m/z 170⁺ suggested that the hydroxylated reaction site was the moiety of benzolsulfonamide methyl group. The high-resolution mass spectrum of **M3** showed the fragment ions at m/z 438⁺, m/z 436⁺ and m/z 424⁺ corresponding to elimination of 16 Da (O), 18 Da (H₂O) and 30 Da (formaldehyde), respectively. The neutral cleavage of *O*-moiety suggested the formation of pazopanib *N*-oxide. The cleavage of formaldehyde, which was associated with a Meisenheimer rearrangement reaction (the methyl transfect from nitrogen atom into oxygen atom, N-CH₃ → O-CH₃) to generate the ion at m/z 424⁺, indicating that the *N*-oxide reaction formed in the terminal nitrogen atom linked with the methyl group (Patel et al., 2015). The patterns of MS/MS fragmentation of **M1**, **M2** and **M3** are provided in Figure 3(D–F). Metabolites **M11** and **M12** displayed a similar molecular ion at m/z 470.164⁺ and m/z 470.158⁺, respectively, which yielded the same molecular formula C₂₁H₂₄N₇O₄S. The [M + H]⁺ of **M11** and **M12** were higher by 16 Da (O) than **M1** and **M2**, respectively. The major product ions of **M11** contained m/z 434, m/z 371⁺, m/z 344⁺ and m/z 262⁺, suggesting that **M11** was bi-hydroxylated metabolic product of pazopanib. Similar to the fragmentation pattern of **M3**, the enamine cation product ion of **M12** showed m/z

453⁺, m/z 435⁺ and m/z 371⁺, suggesting that **M12** was the hydroxylated product of **M3**. In addition, **M9** was deduced as C₂₁H₂₆N₇O₄S based on the accurate molecule [M + H]⁺ ion at m/z 472.1753⁺, higher by 18 Da (2H + 2O) than parent, suggesting that **M9** underwent both reduction and bi-hydroxylation of parent. **M13** showed the ion [M + H]⁺ ion at m/z 439.157⁺, and was higher by only 1 Da than pazopanib, which gave a good match for C₂₁H₂₁N₆O₃S, suggesting **M13** was hydrolysis metabolic product of parent on the site of sulfanilamide.

Metabolites **M4** and **M5** were deduced as C₂₀H₂₂N₇O₂S based on the protonated molecule [M + H]⁺ ion at m/z 424.152⁺ and m/z 424.154⁺ and was 14 Da (CH₂) lower than the parent ion. Therefore, **M4** and **M5** were assumed to be demethylated metabolites of pazopanib. The product ions m/z 343⁺ of **M4** was lost 81 Da (HSO₂NH₂) from pazopanib, and the ion m/z 328⁺ was formed from the ion m/z 343⁺ due to elimination of methyl group. Moreover, the diagnostic ion at m/z 145⁺ of **M4** was identified as the protonated 2,3-dimethyl-2H-indazol, indicating that **M4** was demethylated metabolite on indazol's methylamino. The diagnostic ions at m/z 184⁺, m/z 89⁺ of **M5** suggested that the ring of 2,3-dimethyl-indazol was the demethylated site. Metabolites **M7** and **M8** were calculated as C₂₀H₂₂N₇O₃S based on their excimer ions ([M + H]⁺ at m/z 440.153⁺ and m/z 440.150⁺), higher by 16 Da (O) than **M4** and **M5**, indicating that they were the hydroxylation products of **M4** and **M5**. The product ions m/z 329⁺ and m/z 182⁺ of **M7** indicated the location of hydroxylation site in indazol's ring. In contrast to **M7**, the characteristic ion m/z 407⁺ suggested that the hydroxylated site of **M8** occurred in methyl-benzolsulfonamide. In addition, metabolite **M10** exhibited the molecular ion at m/z 410.138⁺ and was 14 Da (CH₂) lower than **M4** and **M5**, which gave a match for C₁₉H₂₀N₇O₂S, suggesting **M10** was bi-demethylated metabolic product of pazopanib (Supplementary Table 2).

Metabolites **M14** and **M15** were elucidated as two novel aldehydes. The fragment ions at m/z 198⁺ and m/z 120⁺ of **M14** was identified as 4-(pyrimidin-2-ylamino) cyclo-hexa-1,3-dien-5-yne-1-carbaldehyde and 4-aminocyclohexa-1,3-dien-5-yne-1-carbaldehyde, respectively, suggesting that the hydroformylation occurred in the ring of methylbenzol-sulfonamide (Figure 4(A)). The characteristic product ion at m/z 146⁺ generated from **M15** was confirmed as 2H-indazole-3-carbaldehyde, revealing the hydroformylated site of **M15** occurred in phenyl ring (Figure 4(A)). **M6**, the protonated molecule [M + H]⁺ at m/z 468.150⁺, was higher by 16 Da (O) than **M15**, which matched the molecular formula as C₂₁H₂₂N₇O₄S. Elimination of the neutral molecule of 46 (HCOOH) obtained from m/z 468⁺ to m/z 422⁺, suggested the existence of a carboxyl group in metabolite **M6**. Based on these data, it determined that **M6** was the aldehyde oxidation product of **M15** (Supplementary Table 2).

Identification of cysteine conjugates and other phase II metabolites

Among these pazopanib metabolites, cysteine-, *N*-acetylcysteine- and glycylcysteine-conjugated metabolites were found in the feces following treatment with pazopanib. The cysteine adduct (**M18**) showed a protonated molecule [M + H]⁺ at m/z 557.178⁺ and was 119 Da higher than that of pazopanib. The ion m/z 438⁺ was formed from the quasi-molecular ion m/z 557⁺ due to neutral elimination of cysteine (Figure 4(B)). The *N*-

acetylcysteine adduct (**M19**) had a protonated molecule at m/z 599.182⁺ and was 161 Da higher than that of pazopanib. The major product ions of **M19**, m/z 438⁺ and m/z 357⁺, were similar to those of pazopanib. The product ion m/z 438⁺ resulted from a loss of an *N*-acetylcysteine moiety from m/z 599⁺, indicating that **M19** was an *N*-acetylcysteine adduct of pazopanib (Figure 4(C)). Since the fragmentation pattern of **M20** was similar to **M18** and **M19**, **M20** was determined to be a glycylcysteine adduct based on its $[M + H]^+$ at m/z 614.193⁺ (Figure 4(D)).

Metabolite **M16** showed an accurate mass of $[M + H]^+$ ion (m/z 600.224⁺) and was 162 Da ($C_6H_8O_5$) higher than pazopanib. The product ion at m/z 438⁺ of **M16** was identified as the protonated pazopanib, and ion at m/z 357⁺ was confirmed to be protonated pazopanib without sulfanilamide, indicating that **M16** was the glucose-conjugated product of pazopanib. Due to neutral loss of **M16** Da, **M21** was determined to be a glucose-conjugated metabolite of **M1**, based on the $[M + H]^+$ at m/z 630.200⁺. The $[M + H]^+$ and fragmentation patterns of **M17** and **M22** were in consistent with previous report (Deng et al., 2013).

Pazopanib metabolites formed by liver microsomes and CYPs

Pazopanib metabolism in MLM and HLM incubation systems was investigated in order to determine the mechanism of metabolism. The OPLS-DA model displayed two different clusters corresponding to the pazopanib-treated group and control group. The top-ranking ions contributing to group separation in *S*-plot were identified as pazopanib metabolites. Fifteen metabolites (**M1-M15**) were identified in MLM or HLM incubation system (Table 1). Among these metabolites, **M1**, **M2** and **M5** were previously reported (Deng et al., 2013). The structures of the other 12 phase I novel metabolites (**M3-M4** and **M6-M15**) are reported in the present study. The metabolites of pazopanib in the HLM incubation system were similar to that in MLM incubation system (Table 1). The percentage of whole observed metabolites of pazopanib is shown in Figure 2(D). The percentage of unchanged pazopanib was 47% in MLM and 75% in HLM individually, revealing that MLM exhibited higher metabolic activity than HLM.

Metabolic analysis of the incubation of pazopanib in MLM demonstrated that the monohydroxylated metabolites of parent (**M1** and **M2**) were the major metabolites that accounted for 43% of whole identified metabolites, and the demethylated metabolites (**M4** and **M5**) that accounted for 4.9% of the collective group of identified metabolites. The highest proportion of metabolites were the hydroxylated metabolites of pazopanib among all identified metabolites (Figure 2(D)). This result revealed that hydroxylation was the primary metabolic reaction in the phase I metabolism of pazopanib.

In order to systematically determine the CYPs enzymes involving in its metabolism, pazopanib was incubated with 96-well plate of recombinant human P450s including CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP3A4, CYP3A5 and CYP4A11, respectively. Among these enzymes, CYP3A4 and CYP1A2 were identified as primary enzymes contributing to the metabolism of pazopanib. Nearly all recombined human CYPs participated in the formation of **M1-M2**, **M4-M8** and **M13** but the *N*-oxidation and hydroformylation reactions were not catalyzed by

CYPs. CYP3A4 contributed to the formation of **M1-M2**, **M7-M9** and **M11** through hydroxylation. Additionally, **M13** is the hydrolysis product of the parent compound primarily mediated by CYP1A1. The roles of CYPs in the formation of pazopanib metabolites are summarized in Table 2.

Influence of hepatic function by pazopanib exposure in mice

To determine whether the levels of endogenous metabolites in liver were affected by pazopanib, the OPLS-DA model was used to analyze the 300 mg/kg pazopanib treatment group and control group. Significant differences in several metabolites were uncovered between the two groups (Figure 5(A)). The signal of unchanged pazopanib could still be detected in liver at 24 h following treatment (Figure 5(B)), and the level of pazopanib in livers of mice treated with the high dose (300 mg/kg) was higher than that of mice treated with the low dose (150 mg/kg) (Figure 5(C)). However, there were no significant differences for the level of unchanged pazopanib in serum between high dose and low dose at 24 h (Figure 6(A)). The *S*-plot indicated that the top changed endogenous metabolites included *L*-carnitine ($C_7H_{15}NO_3$, MW = 161.104Da, observed $[M + H]^+ = 162.113$, RT = 0.86 min), proline ($C_5H_9NO_2$, MW = 115.063 Da, observed $[M + H]^+ = 116.070$, RT = 0.90min) and LPC 18:1 ($C_{26}H_{52}NO_7P$, MW = 521.34 Da, observed $[M + H]^+ = 522.345$, RT = 5.79 min) (Figure 5(B)), which were identified by comparing the chromatographic behaviors and MS/MS fragmentation patterns with a reference standard. As shown in Figure 5(D), the level of *L*-carnitine was increased by pazopanib at the high dose, while the levels of proline and LPC 18:1 were decreased by high dose pazopanib exposure. *L*-carnitine was shown to protect against valproate-induced hepatotoxicity (DeVivo, 2002), and acetaminophen-induced hepatotoxicity (Yapar et al., 2007). The elevated content of *L*-carnitine in liver of pazopanib-treated mice may be associated with a liver protective feedback loop. Several studies demonstrated that chemically-induced liver injury results in the change of proline metabolism and lysophosphatidylcholine metabolism (Chen et al., 2016; Zhao et al., 2017a). Biochemical analysis revealed that the two doses of pazopanib could significantly elevate the serum ALT and AST, compared to the control group (Figure 5(E,F)). Liver histology revealed inflammatory cell infiltration after high dose treatment (Figure 5(G)). The levels of oxidative stress related SOD and GSH in liver were significantly decreased by high dose pazopanib exposure (Figure 6(B,C)), while the level of MDA was slightly elevated (Figure 6(D)), suggesting that pazopanib administration resulted in increased hepatic oxidative stress. Further analysis revealed that the abundance of the N-oxide metabolite (**M3**), aldehyde derivative (**M15**) and cysteine adducts (**M18** and **M20**) in urine were positively correlated with serum ALT and AST activities (Figure 7(A–D), Table 3) ($p < .05$). More importantly, the elevated serum ALT and AST activities could be reversed by the CYP inhibitor ABT (Figure 7(E,F)). Compared to the pazopanib group, excretion of the novel metabolites (**M3**, **M15**, **M18** and **M19**) in urine was markedly reduced by ABT pretreatment (Figure 7(G)). These data demonstrate that pazopanib metabolism is responsible for its hepatotoxicity, which may be due in part to increased oxidative stress.

Pazopanib down-regulated the expression of drug transporters and drug metabolizing enzymes

To examine the influence of pazopanib on liver functions, mRNA levels of the main drug metabolic enzymes and drug transporters were determined, including *Cyp1a2*, *Cyp3a11* (the murine homolog corresponding to human *Cyp3a4*), *Cyp2d22* (the murine homolog corresponding to human *CYP2D6*), *Abcb1a* (P-gp), *Abcg1*, *Slc10a1* and *Slco1a2*. Treatment with pazopanib significantly decreased *Cyp2d22* and *Abcb1a* mRNAs expression. However, there were no significant changes in the expression of *Cyp3a11*, *Cyp1a2*, *Abcg1*, *Slc10a1* and *Slco1a2* mRNAs, which encoded the enzymes CYP3A11, CYP1A2 and transporters breast cancer resistance protein (BCRP1), Na⁺-taurocholate cotransporting polypeptide (NTCP1), organic anion transporting protein 1A2 (OATP1A2), respectively (Figure 6(E)).

Discussion

The present study systematically determined the metabolic map of pazopanib using a metabolomics approach, and explored the potential metabolites related to its side-effects. The data suggest that the formation of cysteine adducts, aldehyde derivatives and *N*-oxide metabolites could contribute to understand the hepatotoxicity of pazopanib in the clinic. In agreement with the finding of electrophilic metabolites, pazopanib treatment causes liver damage, as revealed by increased serum ALT and AST, and affects the levels of several hepatic endogenous metabolites related to the physiological function of liver, including *L*-carnitine, proline and LPC 18:1. A summary of the metabolism of pazopanib is shown in Figure 8.

Metabolomics is a powerful tool for the comprehensive profiling of drug metabolites (Fang et al., 2012; Lv et al., 2017; Yang et al., 2017b) and identification of endogenous metabolic pathways associated with liver injury (Li et al., 2011; Liu et al., 2015a; Xie et al., 2018). By using this strategy, a series of hydroxylated, demethylated, hydroformylated and glucuronidated metabolites of pazopanib were found in this study. A total of 17 metabolites (**M1-7**, **M10-11** and **M15-22**) were identified in mouse urine, 17 metabolites (**M1-11**, **M14-16** and **M18-20**) were observed in feces and 5 metabolites (**M1-2**, **M4-5** and **M13**) were found in serum. Additionally, the unchanged pazopanib was detected in serum, feces and urine, and based on abundance of metabolites and parent compound suggests that the drug is primarily excreted in the feces. The chemical structures of these metabolites were determined and characterized by MS/MS fragmentography. The primary metabolic pathway *in vivo* was hydroxylation followed by demethylation. Among these metabolites, 9 were phase II metabolites, including the conjugates with cysteine, glycylcysteine, *N*-acetylcysteine, glucose and glucuronic acid. Although glucose- and glucuronic acid-conjugates may increase drug polarity and hydrophilicity and contribute to the excretion of parent drugs. Pazopanib still can be detected in liver at 24 h following treatment at a dose of 150 mg/kg which corresponds to the clinical dose. These data indicate that the repetitive dosing of pazopanib for long periods may result in the accumulation of pazopanib in liver.

Metabolism of a drug can generate metabolites with totally different pharmacological properties from the parent compound, some of which might be associated with side-effects. By using a metabolomics strategy, three types of important metabolites were found and

identified in the current study. Three cysteine, *N*-acetylcysteine and glycylcysteine adducts (**M18**, **M19** and **M20**) were detected in the feces following exposure to pazopanib. Cysteine and its analogs are important endogenous antioxidants that can neutralize free radicals and reduce hepatic oxidative damage (McBean, 2017). It is well-known that cysteine and acetylcysteine act as the precursors for the synthesis of glutathione (GSH). The level of hepatic GSH was slightly depleted by pazopanib. Thus, it is not surprising that some cysteine and *N*-acetylcysteine metabolites are thought to be correlated with hepatotoxicity of drugs (Fang et al., 2015; Liu et al., 2016). Another observation from this study was that two aldehyde metabolites (**M14** and **M16**) were generated from pazopanib. As the electrophilic agents, these aldehyde metabolites can attack macromolecules such as nucleic acid and lipids through covalent binding (Amin & Witz, 2001). It was reported that many genes encoding anti-oxidative stress enzymes are induced as a result of an increase in cellular electrophiles (Itoh et al., 2004), including glutathione S-transferases (GSTs) and NAD(P)H-quinone oxidoreductase 1 (NQO1) via the electrophile responsive element (EpRE) (Friling et al., 1990; Rushmore et al., 1991). In agreement with this study, the aldehyde metabolites of another tyrosine kinase inhibitor gefitinib were demonstrated to be responsible for gefitinib-induced liver injury (Liu et al., 2015b). The activity of hepatic SOD was significantly decreased by high dose pazopanib. An additional observation was that two *N*-oxide metabolites (**M3** and **M12**) were found in feces, which can be formed by both MLM and HLM. It was reported that *N*-oxidation of drugs is responsible for liver injury induced by agents such as clozapine (Pirmohamed et al., 1995) and cocaine (Lloyd et al., 1993). It was demonstrated that the quaternary ammoniums can result in O_2^- production, contributing to oxidative stress and apoptosis in conjunctival cells (Debbasch et al., 2001). The content of hepatic MDA was increased by high dose pazopanib. A recent study revealed that a pyrrolizidine alkaloid *N*-oxide also can induce hepatotoxicity in humans and rodents (Yang et al., 2017a). Formation of all these reactive metabolites can induce hepatic oxidative stress. Consistent with the present observations, others reported that pazopanib-induced hepatotoxicity is associated with generation of reactive oxygen species and induction of oxidative stress (Choudhury et al., 2017). A recent study on the toxicity of six tyrosine kinase inhibitors revealed that the mechanism of pazopanib toxicity was due to increased oxidative stress in mitochondria (Mingard et al., 2018). Further analysis revealed that the production of these four metabolites (**M3**, **M15**, **M18** and **M20**) was positively correlated with the levels of serum transaminase ALT and AST (Table 3). Taken together, the molecular mechanism of pazopanib-induced hepatotoxicity may be due to oxidative stress that results from the formation of these reactive metabolites.

It was demonstrated in this study that CYP3A4 and CYP1A2 were the major enzyme contributing to the metabolism of pazopanib. The elevated AST and ALT in serum significantly increased after pazopanib (150, 300 mg/kg) treatment, which also could be reversed partly by the CYP broadspectrum inhibitor ABT. These results suggested that CYPs mediating metabolic activation played pivotal role in pazopanib-induced hepatotoxicity. Considering that CYP3A4 involved in the formation of hydroxylated and dimethylated metabolites of pazopanib, drug-drug interactions should be monitored when co-administering pazopanib with CYP3A4 substrate or inhibitor or an activator of pregnane X receptor (PXR) that regulates CYP3A4 expression in liver and intestine (Cheng et al., 2011).

It is suggested that the dose of pazopanib should be reduced when pazopanib is administered with a drug that is a CYP3A4 substrate or inhibitor (Tan et al., 2013). Most tyrosine kinase inhibitors are predominantly metabolized by CYP3A4, including imatinib (Filppula et al., 2012), gefitinib (Fujita et al., 2005) and dasatinib (Zakharyants et al., 2017). Screening of CYPs revealed that both CYP3A4 and CYP1A2 played major roles in the formation of **M1** and **M2**, which were then further transformed into reactive aldehyde metabolites. In addition, CYP1A2 and CYP3A4 inducers through the aryl-hydrocarbon receptor and PXR, respectively, may accelerate the formation of these aldehyde metabolites, which can increase pazopanib-related toxicity. Consequently, the present data suggests that pazopanib should be used with caution in patients receiving other CYP3A4 and CYP1A2 substrates or modulators.

Hepatic mRNA levels of *Abcb1a* (P-gp) and *Cyp2d22* (CYP2D6) were significantly inhibited by pazopanib at the high dose. P-glycoprotein (P-gp) is member of the ATP binding cassette superfamily of transporter proteins expressed in hepatocytes, that couples hydrolysis of ATP to the transport of drugs out of the hepatocyte and lowers the intracellular concentrations of many drugs (Corsini & Bortolini, 2013). A previous study reported that inhibition of P-gp and BCRP1 could enhance brain accumulation of pazopanib in mice, suggesting that decrease of P-gp expression lead to its excessive accumulation (Minocha et al., 2012). Consistent with that observation, the accumulation of unchanged pazopanib was increased 2.3-fold at the high dose (300 mg/kg) compared to that at the low dose (150 mg/kg). Moreover, CYP2D6 (the human homolog to murine *Cyp2d22*) can metabolize many drugs, including antidepressants, neuroleptics, beta-blockers, anti-arrhythmics and anti-cancer agents (Yu et al., 2004). The reduced function of CYP2D6 partly accounted for gefitinib-induced hepatotoxicity (Takimoto et al., 2013). Here, *Cyp2d22* mRNA was decreased by high dose pazopanib and CYP2D6 was involved in the metabolism of pazopanib as revealed in HLM, suggesting that the decrease of *Cyp2d22* also contributed to pazopanib-induced hepatotoxicity. Therefore, clinical inhibition of P-gp and CYP2D6 or the CYP2D6 genetic deficiency would lead to pazopanib accumulation, resulting in a greater risk of hepatotoxicity.

In summary, the present study provided a metabolomic perspective of pazopanib-induced adverse effects in mice. The data here suggested that pazopanib hepatotoxicity may result from the formation of cysteine conjugates, aldehyde derivatives and *N*-oxide metabolites, which could induce the oxidative stress. CYP3A4 and CYP1A2 were demonstrated to be the major enzymes contributing to the metabolism of pazopanib. The use of pazopanib should be monitored for patients receiving CYP3A4 and CYP1A2 modulators.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

This work was supported by the Thousand Young Talents Program of China, the National Key Research and Development Program of China [2017YFC1700906, 2017YFC0906903], CAS “Light of West China” Program [Y72E8211W1], Kunming Institute of Botany [Y76E1211K1, Y4662211K1] and State Key Laboratory of Phytochemistry and Plant Resources in West China [52Y67A9211Z1].

References

- Amin RP, Witz G. (2001). DNA-protein crosslink and DNA strand break formation in HL-60 cells treated with trans,trans-muconaldehyde, hydroquinone and their mixtures. *Int J Toxicol* 20:69–80. [PubMed: 11354468]
- Casado-Verrier B, Pérez-Santos S, Delgado-Mucientes C, Beato-Merino M. (2014). Subacute cutaneous lupus erythematosus induced by the new multikinase inhibitor pazopanib. *Br J Dermatol* 171:1559–61. [PubMed: 24909204]
- Chen C, Krausz KW, Idle JR, Gonzalez FJ. (2008). Identification of novel toxicity-associated metabolites by metabolomics and mass isotopomer analysis of acetaminophen metabolism in wild-type and Cyp2e1-null mice. *J Biol Chem* 283:4543–59. [PubMed: 18093979]
- Chen Z, Zhu Y, Zhao Y, et al. (2016). Serum metabolomic profiling in a rat model reveals protective function of paeoniflorin against ANIT induced cholestasis. *Phytother Res* 30:654–62. [PubMed: 26806614]
- Cheng J, Ma XC, Gonzalez FJ. (2011). Pregnane X receptor- and CYP3A4-humanized mouse models and their applications. *Br J Pharmacol* 163:461–8. [PubMed: 21091656]
- Choudhury Y, Toh YC, Xing J, et al. (2017). Patient-specific hepatocyte-like cells derived from induced pluripotent stem cells model pazopanib-mediated hepatotoxicity. *Sci Rep* 7:41238. [PubMed: 28120901]
- Corsini A, Bortolini M. (2013). Drug-induced liver injury: the role of drug metabolism and transport. *J Clin Pharmacol* 53:463–74. [PubMed: 23436293]
- Debbasch C, Brignole F, Pisella PJ, et al. (2001). Quaternary ammoniums and other preservatives' contribution in oxidative stress and apoptosis on Chang conjunctival cells. *Invest Ophthalmol Vis Sci* 42:642–52. [PubMed: 11222522]
- Deng Y, Sychterz C, Suttle AB, et al. (2013). Bioavailability, metabolism and disposition of oral pazopanib in patients with advanced cancer. *Xenobiotica* 43:443–53. [PubMed: 23548165]
- DeVivo DC. (2002). Effect of L-carnitine treatment for valproate-induced hepatotoxicity. *Neurology* 58:507. [PubMed: 11839873]
- Dinkic C, Eichbaum M, Schmidt M, et al. (2017). Pazopanib (GW786034) and cyclophosphamide in patients with platinum-resistant, recurrent, pre-treated ovarian cancer - results of the PACOVAR-trial. *Gynecol Oncol* 146:279–84. [PubMed: 28528917]
- Endo M, Nielsen TO. (2012). Pazopanib for metastatic soft-tissue sarcoma. *Lancet* 380:801. [PubMed: 22939674]
- Fang ZZ, Krausz KW, Li F, et al. (2012). Metabolic map and bioactivation of the anti-tumour drug nescapine. *Br J Pharmacol* 167:1271–86. [PubMed: 22671862]
- Fang ZZ, Tosh DK, Tanaka N, et al. (2015). Metabolic mapping of A3 adenosine receptor agonist MRS5980. *Biochem Pharmacol* 97:215–23. [PubMed: 26212548]
- Filppula AM, Laitila J, Neuvonen PJ, Backman JT. (2012). Potent mechanism-based inhibition of CYP3A4 by imatinib explains its liability to interact with CYP3A4 substrates. *Br J Pharmacol* 165:2787–98. [PubMed: 22014153]
- Friling RS, Bensimon A, Tichauer Y, Daniel V. (1990). Xenobiotic-inducible expression of murine glutathione S-transferase Ya subunit gene is controlled by an electrophile-responsive element. *Proc Natl Acad Sci USA* 87:6258–62. [PubMed: 2166952]
- Fujita K, Ando Y, Narabayashi M, et al. (2005). Gefitinib (Iressa) inhibits the CYP3A4-mediated formation of 7-ethyl-10-(4-amino-1-piperidino)-carbonyloxycamptothecin but activates that of 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin from irinotecan. *Drug Metab Dispos* 33:1785–90. [PubMed: 16123050]

- Itoh K, Tong KI, Yamamoto M. (2004). Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles. *Free Radic Biol Med* 36:1208–13. [PubMed: 15110385]
- Jain A, Canipari C, Haque F, et al. (2015). Liver toxicity associated with the use of pazopanib in renal cancer patients: UK teaching hospital experience. *J Clin Oncol* 33:S513.
- Karch A, Koch A, Grunwald V. (2016). A phase II trial comparing pazopanib with doxorubicin as first-line treatment in elderly patients with metastatic or advanced soft tissue sarcoma (EPAZ): study protocol for a randomized controlled trial. *Trials* 17:312. [PubMed: 27387325]
- Klempner SJ, Choueiri TK, Yee E, et al. (2012). Severe pazopanib-induced hepatotoxicity: clinical and histologic course in two patients. *J Clin Oncol* 30:e264–8. [PubMed: 22802316]
- Koinis F, Agelaki S, Karavassilis V, et al. (2017). Second-line pazopanib in patients with relapsed and refractory small-cell lung cancer: a multicentre phase II study of the Hellenic Oncology Research Group. *Br J Cancer* 117:8–14. [PubMed: 28510571]
- Li F, Jiang C, Krausz KW, et al. (2013). Microbiome remodelling leads to inhibition of intestinal farnesoid X receptor signalling and decreased obesity. *Nat Commun* 4:2384. [PubMed: 24064762]
- Li F, Patterson AD, Hofer CC, et al. (2011). A comprehensive understanding of thioTEPA metabolism in the mouse using UPLC-ESI-QTOFMS-based metabolomics. *Biochem Pharmacol* 81:1043–53. [PubMed: 21300029]
- Li F, Patterson AD, Krausz KW, et al. (2012). Metabolomics reveals the metabolic map of procainamide in humans and mice. *Biochem Pharmacol* 83:1435–44. [PubMed: 22387617]
- Lim L, Mathias J, Lampron M, et al. (2013). Liver toxicity in patients with metastatic renal cell carcinoma treated with pazopanib therapy. *Eur J Cancer* 49:S660.
- Liu K, Yan J, Sachar M, et al. (2015a). A metabolomic perspective of griseofulvin-induced liver injury in mice. *Biochem Pharmacol* 98:493–501. [PubMed: 26343413]
- Liu X, Lu Y, Guan X, et al. (2015b). Metabolomics reveals the formation of aldehydes and iminium in gefitinib metabolism. *Biochem Pharmacol* 97:111–21. [PubMed: 26212543]
- Liu X, Lu YF, Guan X, et al. (2016). Characterizing novel metabolic pathways of melatonin receptor agonist agomelatine using metabolomic approaches. *Biochem Pharmacol* 109:70–82. [PubMed: 27021842]
- Lloyd RV, Shuster L, Mason RP. (1993). Reexamination of the microsomal transformation of N-hydroxynorcocaine to norcocaine nitroxide. *Mol Pharmacol* 43:645–8. [PubMed: 8386313]
- Lv QQ, Yang XN, Yan DM, et al. (2017). Metabolic profiling of dehydrodii-soeugenol using xenobiotic metabolomics. *J Pharmaceut Biomed* 145:725–33.
- McBean GJ. (2017). Cysteine, glutathione, and thiol redox balance in astrocytes. *Antioxidants* 6:62.
- Mingard C, Paech F, Bouitbir J, Krähenbühl S. (2018). Mechanisms of toxicity associated with six tyrosine kinase inhibitors in human hepatocyte cell lines. *J Appl Toxicol* 38:418–31. [PubMed: 29072336]
- Minocha M, Khurana V, Qin B, et al. (2012). Enhanced brain accumulation of pazopanib by modulating P-gp and Bcrp1 mediated efflux with canertinib or erlotinib. *Int J Pharm* 436:127–34. [PubMed: 22688250]
- Pal SK, Hossain DMS, Zhang QF, et al. (2015). Pazopanib as third line therapy for metastatic renal cell carcinoma: clinical efficacy and temporal analysis of cytokine profile. *J Urol* 193:1114–21. [PubMed: 25286010]
- Patel PN, Kalariya PD, Sharma M, et al. (2015). Characterization of forced degradation products of pazopanib hydrochloride by UHPLC-Q-TOF/MS and *in silico* toxicity prediction. *J Mass Spectrom* 50:918–28. [PubMed: 26349647]
- Pirmohamed M, Williams D, Madden S, et al. (1995). Metabolism and bioactivation of clozapine by human liver *in vitro*. *J Pharmacol Exp Ther* 272:984–90. [PubMed: 7891353]
- Rickmann M, Seidel C, Reuter C, et al. (2013). Treatment-associated liver-toxicity in patients with advanced metastatic renal cell carcinoma (mRCC) treated with Pazopanib. *Onkologie* 36:76–7.
- Rushmore TH, Morton MR, Pickett CB. (1991). The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J Biol Chem* 266:11632–9. [PubMed: 1646813]

- Sedef AM, Köse F, Doğan Ö, et al. (2015). Targeted treatment with pazopanib in metastatic soft tissue sarcoma: nearly complete response in two cases. *Mol Clin Oncol* 3:400–2. [PubMed: 25798275]
- Shantakumar S, Nordstrom BL, Djousse L, et al. (2016). Occurrence of hepatotoxicity with pazopanib and other anti-VEGF treatments for renal cell carcinoma: an observational study utilizing a distributed database network. *Cancer Chemother Pharmacol* 78:559–66. [PubMed: 27438066]
- Takimoto T, Kijima T, Otani Y, et al. (2013). Polymorphisms of CYP2D6 gene and gefitinib-induced hepatotoxicity. *Clin Lung Cancer* 14:502–7. [PubMed: 23664723]
- Tan AR, Gibbon DG, Stein MN, et al. (2013). Effects of ketoconazole and esomeprazole on the pharmacokinetics of pazopanib in patients with solid tumors. *Cancer Chemother Pharmacol* 71:1635–43. [PubMed: 23636448]
- Vlenterie M, Van Erp NP, Van der GWT. (2015). Promising management of pazopanib-induced liver toxicity. *Acta Oncol* 54:1064–6. [PubMed: 25734660]
- Xie C, Gao X, Sun D, et al. (2018). Metabolic profiling of the novel hypoxia-inducible factor 2alpha inhibitor PT2385 *in vivo* and *in vitro*. *Drug Metab Dispos* 46:336–45. [PubMed: 29363499]
- Yang M, Ruan J, Gao H, et al. (2017a). First evidence of pyrrolizidine alkaloid N-oxide-induced hepatic sinusoidal obstruction syndrome in humans. *Arch Toxicol* 91:3912–25.
- Yang XN, Lv QQ, Zhao Q, et al. (2017b). Metabolic profiling of myrislignan by UPLC-ESI-QTOFMS-based metabolomics. *RSC Adv* 7:40131–40.
- Yapar K, Kart A, Karapehlivan M, et al. (2007). Hepatoprotective effect of *L*-carnitine against acute acetaminophen toxicity in mice. *Exp Toxicol Pathol* 59:121–8. [PubMed: 17716880]
- Yu AM, Idle JR, Gonzalez FJ. (2004). Polymorphic cytochrome P450 2D6: humanized mouse model and endogenous substrates. *Drug Metab Rev* 36:243–77. [PubMed: 15237854]
- Zakharyants AA, Burmistrova OA, Poloznikov AA. (2017). The use of human liver cell model and cytochrome P450 substrate-inhibitor panel for studies of dasatinib and warfarin interactions. *Bull Exp Biol Med* 162:515–9. [PubMed: 28243904]
- Zhang J, Salminen A, Yang X, et al. (2017). Effects of 31 FDA approved small-molecule kinase inhibitors on isolated rat liver mitochondria. *Arch Toxicol* 91:2921–38. [PubMed: 28032146]
- Zhao Q, Yang R, Liu F, et al. (2017a). Metabolomics reveals that PPARα activation protects against lithocholic acid-induced liver injury. *RSC Adv* 7:49849–57.
- Zhao Q, Yang R, Wang J, et al. (2017b). PPARα activation protects against cholestatic liver injury. *Sci Rep* 7:9967. [PubMed: 28855630]
- Zhao Q, Li XM, Liu HN, et al. (2018). Metabolic map of osthole and its effect on lipids. *Xenobiotica* 48:285–99. [PubMed: 28287022]

Figure 1.

Metabolomic analysis of pazopanib metabolites in mouse feces. (A) Scores plot of an OPLS-DA model from pazopanib treatment without drug (control) and with 300 mg/kg pazopanib. (•, control-treated pazopanib; ▲, pazopanib-treated pazopanib) (B) S-plot obtained from OPLS-DA analysis. (C) Trend plot of **M2** in feces of mice treated with vehicle and pazopanib. (D) Trend plot of **M7** in feces of mice treated with vehicle and pazopanib. Metabolite codes correspond to those in Table 1.

Figure 2.

Abundance of pazopanib and its metabolites in mouse feces, urine, serum, MLM and HLM after treatment with pazopanib or vehicle. (A) Percentage of whole metabolites (%) in feces. (B) Percentage of whole metabolites (%) in urine. (C) Percentage of whole metabolites (%) in serum. (D) Percentage of whole metabolites (%) in MLM and HLM.

Figure 3.

Tandem MS spectra and fragmentation pattern of pazopanib and its three isomeric metabolites. (A) Chemical structure of pazopanib. (B) MS/MS pattern of pazopanib. (C) Chromatogram of **M1**, **M2** and **M3**. (D) MS/MS fragments and chemical structures of **M1**. (E) MS/MS fragments and chemical structure of **M2**. (F) MS/MS fragments and chemical structure of **M3**.

Figure 4.

Tandem MS spectra and fragmentation pattern of aldehyde metabolites and cysteine adducts of pazopanib. (A) MS/MS fragments and chemical structure of **M14**. (B) MS/MS fragments and proposed structure of **M18**. (C) MS/MS fragments and proposed structure of **M19**. (D) MS/MS fragments and proposed structure of **M20**.

Figure 5. Examination of hepatic function after exposure to pazopanib. (A) Scores plot of an OPLS-DA model from pazopanib treatment without drug (control) and with 300 mg/kg pazopanib. (●, control-treated pazopanib; ▲, pazopanib-treated pazopanib) (B) S-plot obtained from OPLS-DA analysis. (C) The relative content of unchanged pazopanib in liver after administration of 150 and 300 mg/kg. (D) The change of representative endogenous metabolites in mouse liver by pazopanib. (E) The change of serum ALT by pazopanib. (F) The change of serum AST by pazopanib. (G) Histological examination of liver from control

and 150 mg/kg, 300 mg/kg pazopanib using H&E staining. Arrow represents inflammatory cell infiltration. Statistical analysis was performed using one-way ANOVA ($n = 4$ in each group). * $p < 0.05$, compared with control group.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6.

Unchanged pazopanib and GSH in serum and liver. (A) The content of unchanged pazopanib in serum at 3 and 24 h after administration of 150 and 300 mg/kg. (B) The level of GSH in liver. (C) The activity of SOD in liver. (D) The content of MDA in liver. (E) Effects of pazopanib administration on the expression levels of drug metabolic enzymes and transporters. Statistical analysis was performed using one-way ANOVA ($n = 4$ in each group). * $p < 0.05$, compared with control group.

Figure 7.

The relationship between reactive metabolites with pazopanib-induced hepatotoxicity. (A) The correlation between the abundance of **M3** in urine with ALT activity. (B) The correlation between the abundance of **M3** in urine with AST activity. (C) The correlation between the abundance of **M18** in urine with ALT activity. (D) The correlation between the abundance of **M18** in urine with AST activity. (E) The change of serum ALT by pazopanib (300 mg/kg) in pre-administration with ABT (100 mg/kg). (F) The change of serum ALT by pazopanib (300 mg/kg) in pre-administration with ABT. (G) The changes of reactive

metabolites following the ABT pretreatment. Correlation factor (r) and p value were calculated with Pearson's correlation analysis. Statistical analysis was performed using two-tailed Student's t -test ($n = 4$ in each group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with control group. # $p < 0.05$, compared with pazopanib group.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 8.

Summary of the major in vivo metabolism pathway of pazopanib. Pazopanib (**M0**) can be conjugated with the group of cysteine, *N*-acetylcysteine and glycylcysteine to generate three conjugates **M18**, **M19** and **M20**. Pazopanib also can be transformed into three aldehyde derivatives **M14** and **M15** by hydroformylation. Additionally, pazopanib can be converted two *N*-oxides **M3** and **M12** under the effect of oxidation.

Table 1.

Summary of pazopanib metabolites produced *in vivo* and *in vitro* metabolism.

Metabolites (ID)	Rt (min)	Observed m/z [$M + H$] ⁺	Formula	Mass error (ppm)	Reaction	Source
M0	5.76	438.168	$C_{21}H_{23}N_7O_2S[H^+]$	-7.30	–	MLM, HLM, Plasma, Feces, Urine
M1	5.07	454.167	$C_{21}H_{23}N_7O_3S[H^+]$	1.98	Hydroxylation	MLM, HLM, Feces, Plasma, Urine
M2	5.30	454.165	$C_{21}H_{23}N_7O_3S[H^+]$	-2.42	Hydroxylation	MLM, HLM, Feces, Plasma, Urine
^aM3	5.70	454.164	$C_{21}H_{23}N_7O_3S[H^+]$	-4.62	N-oxidation	MLM, HLM, Feces, Urine
^aM4	5.45	424.152	$C_{20}H_{21}N_7O_2S[H^+]$	-8.49	N-demethylation	MLM, HLM, Plasma, Feces, Urine
M5	5.71	424.154	$C_{20}H_{21}N_7O_2S[H^+]$	-3.77	N-demethylation	MLM, HLM, Plasma, Feces, Urine
^aM6	5.32	468.150	$C_{21}H_{21}N_7O_4S[H^+]$	9.83	Oxidation	MLM, HLM, Plasma, Feces, Urine
^aM7	4.74	440.153	$C_{20}H_{21}N_7O_3S[H^+]$	5.68	Hydroxylation + N-demethylation	MLM, HLM, Feces, Urine
^aM8	4.86	440.150	$C_{20}H_{21}N_7O_3S[H^+]$	-1.14	Hydroxylation + N-demethylation	MLM, HLM, Feces
^aM9	5.25	472.175	$C_{21}H_{25}N_7O_4S[H^+]$	-3.58	Reduction + bi-hydroxylation	MLM, HLM, Feces
^aM10	5.33	410.138	$C_{10}H_{19}N_7O_2S[H^+]$	-4.63	Bi-N-demethylation	MLM, Urine, Feces
^aM11	4.58	470.164	$C_{21}H_{23}N_7O_4S[H^+]$	6.38	Bi-hydroxylation	MLM, HLM, Feces, Urine
^aM12	4.63	470.158	$C_{21}H_{23}N_7O_4S[H^+]$	-6.38	N-oxidation + hydroxylation	MLM, HLM
^aM13	4.97	439.157	$C_{21}H_{22}N_6O_3S[H^+]$	4.04	Hydrolysis	MLM, HLM, Plasma
^aM14	5.29	452.152	$C_{21}H_{21}N_7O_3S[H^+]$	3.32	Hydroxylation + hydroformylation	HLM, Feces
^aM15	5.07	452.154	$C_{21}H_{21}N_7O_3S[H^+]$	7.74	Hydroxylation + hydroformylation	HLM, Feces, Urine
^aM16	5.30	600.224	$C_{27}H_{33}N_7O_7S[H^+]$	0.00	Glucosylation	Feces
M17	5.13	614.206	$C_{27}H_{31}N_7O_8S[H^+]$	4.40	Glucuronidification	Urine
^aM18	4.63	557.178	$C_{24}H_{28}N_8O_4S_2[H^+]$	4.85	Cysteine conjugate	Feces, Urine
^aM19	4.80	599.182	$C_{26}H_{30}N_8C_5S_2[H^+]$	-6.51	N-Acetyl(Cysteine conjugate	Feces, Urine
^aM20	5.14	614.193	$C_{26}H_{31}N_9O_5S_2[H^+]$	-6.19	Glycylcysteine conjugate	Feces, Urine
^aM21	4.82	616.212	$C_{27}H_{33}N_7O_8S[H^+]$	-11.20	Hydroxylation + glucosylation	Urine

Metabolites (ID)	Rt (min)	Observed m/z [$M + H$] ⁺	Formula	Mass error (ppm)	Reaction	Source
M22	4.69	630.200	C ₂₇ H ₃₁ N ₇ O ₉ S ⁺ [H ⁺]	2.86	Hydroxylation + glucuronidification	Urine

^g Novel metabolites found in this study.

Table 2.

Roles of CYPs in the formation of pazopanib metabolites.

Enzyme	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CYP1A1	6.57	2.45	0.00	8.54	7.64	7.91	8.61	8.84	0.00	0.00	0.00	0.00	48.4	0.00	0.00
CYP1A2	15.0	29.4	0.00	13.2	8.68	8.76	5.99	6.07	10.9	0.00	36.9	0.00	3.35	0.00	0.00
CYP1B1	2.85	1.40	0.00	6.22	7.95	9.99	6.51	6.59	0.00	0.00	0.00	0.00	3.63	0.00	0.00
CYP2A6	1.20	0.62	0.00	5.28	7.68	2.76	6.51	6.65	0.00	0.00	0.00	0.00	3.59	0.00	0.00
CYP2B6	1.38	1.31	0.00	5.61	7.79	13.7	8.97	8.96	0.00	0.00	0.00	0.00	5.11	0.00	0.00
CYP2C19	2.03	0.70	0.00	5.23	7.45	4.45	5.00	5.01	0.00	0.00	0.00	0.00	2.79	0.00	0.00
CYP2C8	3.91	5.57	0.00	6.75	6.82	6.93	8.29	7.48	3.21	0.00	0.00	0.00	4.71	0.00	0.00
CYP2C9	3.03	1.40	0.00	7.09	7.25	8.82	9.82	9.91	0.00	0.00	0.00	0.00	5.60	0.00	0.00
CYP2D6	12.1	2.09	0.00	11.6	7.51	0.98	8.11	8.26	15.7	0.00	0.00	0.00	4.64	0.00	0.00
CYP2E1	1.03	0.83	0.00	5.10	7.65	3.73	7.95	7.98	0.00	0.00	0.00	0.00	4.48	0.00	0.00
CYP3A4	36.5	40.0	0.00	8.41	7.93	16.1	10.0	10.0	70.1	0.00	42.4	0.00	5.70	0.00	0.00
CYP3A5	12.56	13.6	0.00	11.5	7.68	7.88	6.64	6.58	0.00	0.00	20.7	0.00	3.74	0.00	0.00
CYP4A11	1.71	0.65	0.00	5.64	7.98	7.96	7.57	7.63	0.00	0.00	0.00	0.00	4.29	0.00	0.00

cDNA-expressed CYP450 isoenzymes (control, CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C19, CYP2C8, CYP2C9B, CYP2D6, CYP2E1, CYP3A4, CYP3A5B and CYP4A11) were used to determine the role of individual CYPs in pazopanib metabolism.

All samples were analyzed by UPLC-ESI-QTOFMS. The total peak area of the metabolite from all the CYPs was set as 100%. All data are expressed as mean ($n = 2$).

Table 3.

The relation between the abundance of reactive metabolites with the levels of transaminases.

Metabolites	ALT		AST	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
M3	0.89	<i>p</i> < 0.01	0.85	<i>p</i> < 0.01
M15	0.76	<i>p</i> < 0.05	0.75	<i>p</i> < 0.05
M18	0.80	<i>p</i> < 0.05	0.77	<i>p</i> < 0.05
M19	0.59	<i>p</i> = 0.12	0.18	<i>p</i> = 0.13
M20	0.79	<i>p</i> < 0.05	0.79	<i>p</i> < 0.05

Correlation factor (*r*) and *p* value were calculated with Pearson's correlation analysis