In Vitro and in Vivo Drug Metabolism Analysis of BPI-460372, a Covalent TEAD1/3/4 Inhibitor

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Abstract

Background: BPI-460372 is an orally available, covalent, irreversible small molecule inhibitor of the tran Objective: This study aimed to determine the cytochrome P450 (CYP) phenotyping, metabolic stability, in vitro and in vivo metabolic profile of BPI-460372. Methods: The CYP phenotyping and metabolic stability were assessed by measuring the depletion of substrate. The metabolic profile in hepatocytes, rats, and dog plasma was analyzed using ultra-high performance liquid chromatography combined with orbitrap tandem mass spectrometry (UHPLC-Orbitrap-HRMS). Results: BPI-460372 was mainly metabolized by CYP2D6, CYP3A4, and CYP1A2. BPI-460372 exhibited low clearance in human, monkey, and rat hepatocytes, while moderate clearance in dog and mouse hepatocytes. A total of 10 metabolites were identified in five species of hepatocytes, and no human unique metabolite was detected. In rat plasma and dog plasma, the primary metabolites were M407 (BPI-460430) and M423 (BPI-460456), respectively. The two metabolites were quantitatively determined in rat and dog plasma in pharmacokinetic and toxicological studies. The major metabolic sites were 2-fluoro acrylamide, and major metabolic pathways in hepatocytes, rats, and dog plasma involved oxidative defluorination, hydration, glutathione conjugation, hydrolysis, cysteine conjugation and N-acetyl cysteine conjugation. β-lyase pathway contributes to the metabolism of BPI-460372 in rats to a certain degree. Conclusion: This study elucidated the metabolism of BPI-460372 and provided a basis for pharmacokinetic and toxicological species selection, human pharmacokinetics prediction, clinical co-administration limitations, and possible metabolic pathways in humans.

Full Text

Preamble

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Abstract

Background: BPI-460372 is an orally available, covalent, irreversible small molecule inhibitor of the transcriptional enhanced associate domain (TEAD) 1/3/4, currently in clinical development for the treatment of cancers with Hippo pathway alterations.

Objective: This study aimed to determine the cytochrome P450 (CYP) phenotyping, metabolic stability, and in vitro and in vivo metabolic profile of BPI-460372.

Methods: CYP phenotyping and metabolic stability were assessed by measuring substrate depletion. The metabolic profile in hepatocytes, rats, and dog plasma was analyzed using ultra-high performance liquid chromatography combined with orbitrap tandem mass spectrometry (UHPLC-Orbitrap-HRMS).

Results: BPI-460372 was mainly metabolized by CYP2D6, CYP3A4, and CYP1A2. The compound exhibited low clearance in human, monkey, and rat hepatocytes, while showing moderate clearance in dog and mouse hepatocytes. A total of 10 metabolites were identified across five species of hepatocytes, with no human-unique metabolite detected. In rat plasma and dog plasma, the primary metabolites were M407 (BPI-460430) and M423 (BPI-460456), respectively, and these two metabolites were quantitatively determined in pharmacokinetic and toxicological studies. The major metabolic sites were the 2-fluoro acrylamide moiety, and the predominant metabolic pathways in hepatocytes, rats, and dog plasma involved oxidative defluorination, hydration, glutathione conjugation, hydrolysis, cysteine conjugation, and N-acetyl cysteine conjugation.

The β-lyase pathway contributed to BPI-460372 metabolism in rats to a certain degree.

Conclusion: This study elucidated the metabolism of BPI-460372 and provided a basis for pharmacokinetic and toxicological species selection, human pharmacokinetics prediction, clinical co-administration limitations, and possible metabolic pathways in humans.

Keywords: Hippo signaling pathway, TEAD inhibitor, covalent inhibitor, BPI-460372, UHPLC-Orbitrap-HRMS, cysteine S-conjugate β-lyase.

1. INTRODUCTION

The Hippo signaling pathway consists of a series of conserved kinases found in drosophila that are involved in critical biological functions including tissue development, maintenance of tissue homeostasis, and regenerative repair [?]. Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) are two important transcriptional coactivators in the Hippo pathway that regulate target gene transcription by binding to TEAD1-4 to form the YAP/TAZ-TEAD complex [?]. Dysregulation of the Hippo pathway leads to tumor cell development, migration, and drug resistance, with YAP and TAZ hyperactivated in various solid tumors including lung, colorectal, breast, pancreatic, hepatocellular, melanoma, and glioma [?]. Given the critical role of the YAP/TAZ-TEAD complex in tumorigenesis, it has become an attractive target for anticancer drug development. Some molecules target the YAP/TAZ-TEAD complex directly, while others target the palmitoylation pocket of TEAD [?]. For TEAD small molecule inhibitors, no drug has been approved to date, and only four molecules have entered clinical trials: VT3989 (NCT04665206), IAG933 (NCT04857372), BPI-460372 (NCT05789602), and ODM-212 [?].

BPI-460372 is a covalent and irreversible TEAD inhibitor, unlike VT3989 and IAG933 which are non-covalent and reversible TEAD inhibitors [?, ?]. A Phase I clinical trial (NCT05789602) has been conducted in China to assess the safety, tolerability, pharmacokinetics, and preliminary efficacy of BPI-460372 in solid tumor patients. BPI-460372 exhibited a good safety profile, with preliminary efficacy observed in patients with malignant mesothelioma, lung adenocarcinoma, and lung squamous cell carcinoma [?]. The metabolism profile of BPI-460372 was previously unknown and has now been characterized through a series of preclinical in vitro and in vivo studies.

This study aimed to (i) identify the major metabolic enzymes involved in BPI-460372 metabolism, (ii) evaluate the metabolic stability and profile of BPI-460372 in hepatocytes from different species, and (iii) elucidate the metabolic profile and pathway of BPI-460372 in rat and dog plasma. These findings would generate more reliable biotransformation information on BPI-460372 and provide evidence to elucidate potential drug-drug interaction (DDI) mechanisms.

2. MATERIALS AND METHODS

2.1 Materials

BPI-460372, BPI-460430, BPI-460456, and BPI-460608 were provided by Betta Pharmaceuticals Co., Ltd (Hangzhou, China). Phenacetin, diclofenac, terfenadine, tolbutamide, α-naphthoflavone, ticlopidine, quercetin, sulfaphenazolum, ketoconazole, HPLC-grade acetonitrile and methanol, and nicotinamide adenine dinucleotide phosphate (NADPH; reduced form) were purchased from Sigma-Aldrich (Poole, Dorset, UK). Bupropion and midazolam were supplied by the National Institutes for Food and Drug Control (Beijing, China). Paclitaxel and S-Mephenytoin were obtained from J&K Scientific (Beijing, China). Quinidine and formic acid were purchased from Adamas (Basel, Switzerland). Dextromethorphan, 7-ethoxycoumarin (7-EC), and testosterone were purchased from Toronto Research Chemicals Inc. (North York, Canada), Dalian Meilun Biotechnology Co., Ltd (Dalian, China), and Aladdin (Shanghai, China), respectively. Aminooxyacetic acid (AOAA) was purchased from Shanghai Macklin Biochemical Technology Co., Ltd (Shanghai, China). Ultrapure water was prepared using a Milli-Q system (Molsheim, France). Recombinant human CYP isozymes (1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5) were purchased from Cypex Ltd. (Dundee, UK). Pooled human liver microsomes (HLMs) were obtained from BioIVT (Baltimore, MD, USA). Primary hepatocytes from human, Cynomolgus monkey, Beagle dog, Sprague-Dawley (SD) rat, and ICR mouse were provided by XenoTech (Lenexa, KS, USA).

2.2 Animals

SD rats and Beagle dogs were supplied by Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China) and Changzhou Beile Experimental Animal Breeding Co., Ltd (Changzhou, China), respectively.

2.3 Metabolic Stability of BPI-460372 in HLMs with or without Specific CYP Inhibitors

Before starting the experiments, HLMs were thawed gently on ice. Metabolic stability incubations were performed at 37 °C in mixtures containing 100 mM phosphate buffer saline (PBS, pH 7.4), HLMs (1 mg protein $\cdot$ mL^{-1}), DMSO (blank control) or a selective CYP inhibitor, and BPI-460372 or positive control substrates (final incubation concentration, 1 M). Reactions were initiated by adding NADPH (1 mM final concentration) after a 5-minute preincubation period. The chemical inhibitors used were as follows: α-naphthoflavone (2 µM) for CYP1A2, ticlopidine (20 µM) for 2B6, quercetin (20 µM) for CYP2C8, sulfaphenazole (20 µM) for CYP2C9, ticlopidine (200 µM) for CYP2C19, quinidine (20 µM) for CYP2D6, and ketoconazole (20 µM) for CYP3A. Aliquots were removed into acetonitrile containing internal standard (IS) at various time points (0, 5, 10, 20, 30, and 60 min). All incubations were performed in triplicate, and concentrations in the supernatant were determined by LC-MS/MS.

The elimination rate constant k (min^{-1}) was obtained by linear regression from the natural logarithm of the elimination percentage of the parent drug versus incubation time. The inhibition rate was calculated using equation 1 [**?**]:

$$\text{Inhibition rate (\%)} = \frac{k_{\text{blank control group}} - k_{\text{inhibitor group}}}{k_{\text{blank control group}}} \times 100 \tag{1}$$

2.4 Metabolic Stability of BPI-460372 in Recombinant Human CYP Isoenzymes

To identify the specific isoforms participating in BPI-460372 metabolism, 1 µM BPI-460372 or positive control substrates were mixed with recombinant human CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, or 3A4 (100 pmol P450 · mL^{-1}). Reactions were initiated by adding NADPH (1 mM final concentration) after a 5-minute preincubation at 37 °C. Aliquots were removed into acetonitrile containing IS at time points of 0, 5, 10, 20, 30, and 60 min. All incubations were performed in triplicate, and supernatant concentrations were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

A substrate depletion approach was used to determine the intrinsic clearance (CLint (rCYPj)) for each isoform. The contribution of each isoform was calculated using equations 2 and 3, where CYPj abundance represents the abundance of each isoform in human liver microsomes (pmol/mg protein) [**?**].

$$CLint_{CYPj} = CLint_{(rCYPj)} \times CYPj\ abundance \tag{2}$$

$$\%contribution, CYPj = \frac{CLint_{CYPj}}{\sum(CLint_{CYPj})} \times 100 \tag{3}$$

2.5 Metabolic Stability of BPI-460372 in Human, Monkey, Dog, Rat, and Mouse Hepatocytes

BPI-460372 (1 µM) or positive controls (testosterone and 7-EC) were incubated in triplicate with cryopreserved hepatocytes (1 × 10^{6} cells · mL^{-1}) at 37 °C in a 5% CO$_2$ atmosphere. After mixing, 20 L of cell suspension was serially transferred into 200 L of cold acetonitrile containing IS at time points of 0, 5, 15, 30, 60, and 120 min. For negative control samples, inactive hepatocytes (boiled in a water bath at 100 °C for 10 min to eliminate enzymatic activity) were used, with 20 L of cell suspension transferred into 200 L of chilled acetonitrile containing IS at time points of 0 and 120 min. Samples were analyzed by LC-MS/MS and semi-quantified using the peak area ratio of analyte versus IS.

Metabolic stability was assessed by measuring substrate depletion. The half-life ($t_1/_2$), intrinsic clearance (CLint, in vitro, µL · min^{-1} · million cells^{-1}), whole liver intrinsic clearance (CLint, in vivo, mL · min^{-1} · kg^{-1}), and in vivo hepatic clearance (CLH, $\mathrm{mL \cdot min^{-1} \cdot kg^{-1}}$) were calculated following a reported method [?]. In CLH calculations, the fraction of unbound drug (Fub) was set as 1. Hepatic blood flow, liver weight, and hepatocellularity for the five species were used for CLint, in vivo calculations [?, ?]. The hepatic extraction ratio (Eh) represents the ratio of CLH to hepatic blood flow.

2.6 Metabolism and Metabolite Formation in Human, Monkey, Dog, Rat, and Mouse Hepatocytes

BPI-460372 (10 µM) was incubated in hepatocytes from five species (human, cynomolgus monkey, beagle dog, SD rat, and ICR mouse) at 1×10^6 $\mathrm{cells \cdot mL^{-1}}$ and 37 °C for 4 h. 7-EC was included as a positive control and incubated for 0 h and 4 h. For blank controls, hepatocytes were inactivated by adding ice-cold acetonitrile before adding BPI-460372 solution. All samples were prepared in duplicate. Incubations were terminated using an equal volume of cold acetonitrile, tubes were vortexed, and duplicate samples were combined. Metabolite profiling and identification were conducted using UHPLC-Orbitrap-HRMS. Samples containing 7-EC were analyzed by LC-MS/MS and semi-quantified using the peak area ratio of analyte versus IS.

2.7 Metabolite Identification in SD Rats and Dogs Plasma

Plasma samples were obtained from SD rats (n=6, 3 female and 3 male) and beagle dogs (n=6, 3 female and 3 male) after administration of 12 mg/kg and 8 mg/kg BPI-460372, respectively. Rat urine, feces, and bile samples were collected after administration of 6 mg/kg BPI-460372.

Rat plasma samples from 3 female and 3 male rats at pre-dose, 0.25, 0.5, 1, 2, 4, 6, 8, 10, and 24 h post-dose were selected and pooled according to the AUC-pooling principle for individuals [?]. Equal volumes from each rat were combined to obtain one male and one female 0-24 h plasma sample. For urine, equal percentages by volume of individual samples (0-8, 8-24, and 24-48 h) from 3 female and 3 male rats were pooled by time interval across rats, resulting in two pooled urine samples (one male, one female). For feces, equal percentages by weight of individual samples (0-8, 8-24, and 24-48 h) were pooled by time interval across female and male rats, yielding two pooled fecal samples. For bile, equal percentages by volume of individual samples (0-4, 4-8, 8-24, and 24-48 h) were pooled by time interval across female and male rats, resulting in two pooled bile samples.

Dog plasma samples from 3 female and 3 male dogs at pre-dose, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 24, and 48 h post-dose were selected and pooled according to the AUC-pooling principle for individuals [?]. Equal volumes from 3 female and 3 male rats were combined to obtain two 0-48 h plasma samples. For blank plasma, urine, and bile samples, equal volumes were pooled across subjects. For blank feces samples, equal weight was pooled across rats.

Pooled plasma, feces, and bile samples were extracted by adding 3-fold volume of acetonitrile, vortexing (1 min), sonicating (1 min), and centrifuging (7220 g, 5 min). Pooled urine samples were centrifuged directly (7220 g, 5 min). The supernatant was transferred to a clean tube and evaporated to dryness under a nitrogen stream. Residues were redissolved in an appropriate volume of acetonitrile/water (20:80, v:v), and after centrifugation, the redissolved solution was transferred to an HPLC vial for UHPLC-Orbitrap-HRMS analysis (15000 g, 10 min, 4 °C).

2.8 The Effect of Cysteine S-conjugate β-lyase Inhibitor on the Metabolism of BPI-460372

To investigate the role of cysteine S-conjugate β-lyase in BPI-460372 metabolism, SD rats were treated with 20 mg/kg BPI-460372 alone (n=3, male) or 20 mg/kg BPI-460372 co-administered with 100 mg/kg cysteine S-conjugate β-lyase inhibitor AOAA (n=3, male) once daily for 21 consecutive days. On day 21, urine samples were collected from 0-8, 8-24, and 24-48 h, with equal percentage urine samples from each rat combined at each time interval. Samples were analyzed by LC-MS/MS and semi-quantified using the peak area ratio of analyte versus IS.

2.9 LC-MS/MS Detection

For CYP phenotyping studies, BPI-460372 was quantitated using a Sciex ExionLC AD liquid chromatograph coupled with a Triple Quad 6500+ MS (AB Sciex, Singapore). For hepatocyte metabolic stability studies, BPI-460372 was detected using an ACQUITY UPLC I-Class Plus system (Waters, Milford, MA, USA) coupled with a Triple Quad 6500+ MS (AB Sciex, Framingham, MA, USA). For studies on the effect of cysteine S-conjugate β-lyase inhibitor on BPI-460372 metabolism, metabolites were analyzed using a Sciex ExionLC AD liquid chromatograph coupled with a Triple Quad 4500 MS (AB Sciex, Singapore). Data acquisition and processing were performed using Analyst software version 1.7.1 (AB Sciex). Detailed LC-MS/MS and LC/MS methods are provided in the Supplementary Material section.

2.10 UHPLC-Orbitrap-HRMS Detection

UHPLC-Orbitrap-HRMS analysis was performed using a Vanquish UHPLC system (Thermo, Waltham, MA, USA) coupled with a Thermo Q-Exactive Plus Hybrid Quadrupole Orbitrap Mass spectrometer equipped with an ESI interface operated in positive ion mode.

For hepatocyte metabolite identification, BPI-460372 metabolites were detected using an ACQUITY UPLC HSS T3 column (1.8 µm, 100 mm × 2.1 mm), while an ACQUITY UPLC BEH C18 column (1.7 µm, 100 mm × 2.1 mm) was used for rat and dog metabolite identification. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient elution program was as follows: 0.0-1.0 min, 5% B; 1.0-7.0 min, 5%-30% B; 7.0-12.0 min, 30%-80% B; 12.0-16.0 min, 80%-95% B; 16.0-18.0 min, 95% B; 18.0-18.1 min, 95%-5% B. From 18.1 to 20.0 min, 5% B was maintained for equilibration. The flow rate was set to 0.5 mL/min, and the column temperature was maintained at 40 °C. Eluted fractions were monitored by a UV detector at 314 nm.

MS parameters were optimized as follows: spray voltage 3.5 kV; capillary temperature 320 °C; sheath gas flow rate 40 L/h; auxiliary gas flow rate 10 L/h. MS data were acquired in centroid mode within an m/z range of 100-1500 Da (full mass-MS/MS scan). Collision energies were 35, 45, and 60 V. Mass resolutions for full mass scan and MS/MS scan were 35000 and 17500, respectively.

Data acquisition was performed using Xcalibur software (Thermo). Data analysis was conducted using Compound Discoverer software (Thermo). The molecular formula of each metabolite was determined based on accurate molecular mass. Based on the BPI-460372 mass spectral fragmentation pattern, metabolite fragmentation information was compared with that of the parent compound to speculate on possible metabolic sites or ranges. Each metabolite was named using the letter 'M' followed by its molecular weight.

3. RESULTS

3.1 CYP Reaction Phenotyping of BPI-460372

Using human liver microsomes and recombinant CYP enzymes to determine the CYP reaction phenotyping of BPI-460372, results showed that BPI-460372 was mainly metabolized by CYP2D6, CYP3A4, and CYP1A2 (Table 1). Quercetin is not a specific inhibitor of CYP2C8 and can also inhibit CYP2C19, CYP3A4, CYP2D6, and carboxylesterase to a certain extent [?, ?]. Therefore, CYP2C8 reaction phenotyping results for BPI-460372 only referred to data from recombinant CYP enzymes. Data from positive control substrates confirmed that experimental conditions were reliable (Supplemental Table 1).

3.2 Metabolic Stability of BPI-460372 in Human, Monkey, Dog, Rat, and Mouse Hepatocytes

The hepatic extraction ratios of BPI-460372 in human, monkey, dog, rat, and mouse hepatocytes were 11.5%, 25.3%, 44.6%, 20.0%, and 40.6%, respectively (Table 2). In human, monkey, and rat hepatocytes, the hepatic extraction ratio was less than 30%, indicating low clearance. In dog and mouse hepatocytes, the hepatic extraction ratio was between 30% and 70%, indicating moderate clearance. The remaining percentage of BPI-460372 after 120 min incubation in human, monkey, dog, rat, and mouse hepatocytes was 97.3%, 61.7%, 66.6%, 72.4%, and 54.7%, respectively. The $t_1/_2$ values of testosterone and 7-hydroxycoumarin were in the ranges of 5-30 min and 10-50 min, respectively, meeting acceptance criteria (Supplemental Table 2).

3.3 Metabolism and Metabolite Formation in Human, Monkey, Dog, Rat, and Mouse Hepatocytes

A total of 10 metabolites of BPI-460372 were identified across five species of hepatocytes, with extracted ion chromatograms (XICs) providing an overall metabolite profile (Fig 1 [Figure 1: see original paper]). BPI-460372 and metabolites were detected by UV ($\lambda = 314$ nm), with BPI-460372 representing the main drug-related component, accounting for 76.96%, 63.98%, 48.28%, 70.87%, and 32.74% of total drug-related components in human, monkey, dog, rat, and mouse hepatocytes, respectively (Table 3). Data for the 10 detected metabolites, including metabolic pathway, retention time, formula, observed and theoretical m/z, mass error, and fragment ions, are summarized (Table 4), and proposed structures are illustrated (Fig. 2 [Figure 2: see original paper]).

The major metabolic pathways of BPI-460372 in five species hepatocytes involved hydrolysis, hydration, glucose conjugation, oxidation, glucuronidation, glutathione (GSH) conjugation, oxidative defluorination, cysteine conjugation, N-acetylcysteine conjugation, and acetylation. Metabolites identified in human hepatocytes were also detected in animal hepatocytes, with no human-unique metabolite detected.

The metabolite 7-EC-GluA, formed by de-ethylation and glucuronidation of 7-EC, was detected in hepatocytes from all five species (Supplemental Table 3), confirming that phase I and phase II enzymatic activities were qualified in all hepatocyte preparations.

The chromatographic behaviors and MS fragmentation patterns of BPI-460372 and its metabolites BPI-460430, BPI-460456, and BPI-460608 were first assessed to identify other potential metabolites. Fragment ions and tentative fragmentation patterns are illustrated (Supplemental Figure 1-4).

3.4 Metabolite Identification in Rat Plasma, Urine, Feces, and Bile

A total of 17 metabolites were identified in rat plasma, urine, feces, and bile (Table 5). In rat plasma, unchanged BPI-460372 was the most abundant component, accounting for 75.36% of total drug-related components (Table 6). M335 (BPI-460608), M423 (BPI-460456), and M407 (BPI-460430) were the most abundant metabolites, accounting for 2.48%, 2.71%, and 16.39% of total drug-related components, respectively (Table 6).

In rat urine, M570, M423 (BPI-460456), and M453 were the most abundant metabolites, accounting for 34.25%, 17.63%, and 17.25% of total drug-related components, respectively (Table 6). In rat feces, M389, M391, and unchanged BPI-460372 were the most abundant components, accounting for 16.59%, 21.50%, and 16.95% of total drug-related components, respectively (Table 6). In rat bile, downstream products of glutathione-conjugated metabolites M528 and M570 were the most abundant components, accounting for 43.88% and 45.40% of total drug-related components, respectively (Table 6). The major metabolic pathways in rats involved oxidative defluorination, hydration, glutathione conjugation, hydrolysis, cysteine conjugation, and N-acetyl cysteine conjugation, with proposed metabolic pathways presented in Fig. 3 [Figure 3: see original paper].

3.5 Metabolite Identification in Dog Plasma

A total of 9 metabolites were identified in dog plasma (Table 6). Unchanged BPI-460372, M423 (BPI-460456), and M335 (BPI-460608) were the most abundant components, accounting for 30.93%, 40.81%, and 8.45% of total drug-related components, respectively (Table 6). The major metabolic pathways in dog plasma involved oxidative defluorination, hydration, hydrolysis, cysteine conjugation, and reductive defluorination, with proposed metabolic pathways presented in Fig. 4 [Figure 4: see original paper].

3.6 The Effect of Cysteine S-conjugate β-lyase Inhibitor on the Metabolism of BPI-460372

In this study, SD rats were treated with 20 mg/kg BPI-460372 alone or co-administered with 100 mg/kg AOAA once daily for 21 consecutive days. BPI-460372 and metabolites M453, M528, M568, M570, M335, and M423 were detected in rat urine. The area ratios of mercapturate and β-lyase pathway metabolites (M453, M528, M568, and M570) were reduced when BPI-460372 was co-administered with AOAA (Table 7), indicating that S-conjugate β-lyase participated in BPI-460372 metabolism.

4. DISSUSION

The CYP enzyme phenotyping study revealed that BPI-460372 was predominantly metabolized by CYP2D6 (43.6%), followed by CYP3A4 (28.3%), CYP1A2 (13.1%), CYP2C8 (8.8%), and CYP2C9 (6.3%). In the Phase I clinical study, to avoid potential DDI, strong or moderate CYP1A2 and CYP3A4 inducers, and strong or moderate CYP1A2, CYP3A4, and CYP2D6 inhibitors were prohibited for 7 days before and during the clinical study.

The metabolic stability study indicated that BPI-460372 had low clearance in human, monkey, and rat hepatocytes, and moderate clearance in mouse and dog hepatocytes. BPI-460372 exhibited low clearance in rats and monkeys, and moderate clearance in dogs in vivo (data unpublished), showing good in vitro-in vivo correlation. Based on metabolic stability in human hepatocytes, clearance in humans was predicted to be low.

Our study showed that metabolites identified in human hepatocytes were also detected in animal hepatocytes, with no human-unique metabolite detected. Based on these results, rats and dogs were selected as species for pharmacokinetic and toxicological studies. For BPI-460372, the main metabolic pathways included: (1) hydrolysis (M335), followed by glucose conjugation (M497), mono-oxidation and glucuronidation (M527), and acetylation (M377); (2) glutathione conjugation (M714) followed by metabolism to form N-acetylcysteine conjugation (M570) and cysteine conjugation (M528). Oxidative defluorination and hydration also participated in BPI-460372 metabolism. Without NADPH addition, M335 formation after 45 min incubation of BPI-460372 with HLM was comparable to the HLM+NADPH group, suggesting that other non-CYP enzymes such as esterases may be involved in M335 production (unpublished data).

In rat and dog plasma, the primary metabolites were M407 (BPI-460430) and M423 (BPI-460456), respectively. These two metabolites were quantitatively determined in rat and dog plasma in pharmacokinetic and toxicological studies.

BPI-460372 contains a modified electrophilic functionality ("warhead") 2-fluoro acrylamide that can covalently bind to the Cys-368 residue of TEAD1/3/4 without significant off-target covalent binding affinity and inhibit TEAD palmitoylation [**?**]. All metabolites detected in hepatocytes, rats, and dog plasma were more polar than the parent drug. The 2-fluoro acrylamide was the main metabolic site that was inactivated during metabolism, except for M369 which retained the acrylamide moiety. In vitro studies showed that metabolites BPI-460608, BPI-460456, and BPI-460430 are inactive metabolites that do not covalently bind to TEAD proteins or inhibit TEAD palmitoylation.

Adagrasib is a small molecule inhibitor of the KRAS G12C mutant isoform indicated for treatment of adult patients with KRAS G12C-mutated locally advanced or metastatic non-small cell lung cancer (NSCLC). Adagrasib contains 2-fluoro acrylamide and undergoes extensive metabolism in animals and humans [**?**]. Compared with the acrylamide counterpart, the 2-fluoro acrylamide electrophile showed reduced reactivity toward GSH [**?**]. Adagrasib (2-fluoro acrylamide warhead) demonstrated higher stability in mouse, dog, and human whole blood and much reduced GST-mediated GSH conjugation compared to compound 18 (acrylamide warhead) [**?**].

Glutathione is a nucleophile that can form GSH conjugates with target covalent inhibitors (TCIs) and serves as a metabolic route for TCIs in vivo. Glutathione-S-transferase (GST) enzymes can accelerate the addition reaction through catalysis, and GST is expressed in the liver, spleen, and extrahepatic tissues [**?**]. The metabolism of GSH conjugates is called the mercapturic acid pathway [**?**]. GSH conjugates can be further metabolized by γ-glutamyl transpeptidase (GGT) and cysteinylglycine dipeptidase to form cysteine-glycine conjugates and cysteine conjugates, respectively.

Cysteine conjugates can be further acetylated by N-acetyltransferase to form mercapturic acid conjugates (N-acetylcysteine conjugates), primarily occurring in the kidney. Cysteine conjugates can also be cleaved by cysteine S-conjugate β-lyase to release a free thiol. Cysteine S-conjugate β-lyase is present in high concentrations in the kidney [**?**]. The free thiol can be catalyzed by thiomethyltransferase and UDP-glucuronosyltransferases to form methylthio derivatives and glucuronyl S-conjugates, respectively [**?**].

The high reactivity of free thiol can lead to covalent modification of macromolecules, depletion of nonprotein thiols, lipid peroxidation, and carbonylation of susceptible proteins, resulting in toxicity. The renal proximal tubule has a high metabolic rate, and free thiol inactivates key enzymes in the tricarboxylic acid (TCA) cycle, causing mitochondrial toxicity and renal proximal tubule injury [?]. Due to greater specific activities of cysteine S-conjugate β-lyase in rats than humans, rat renal tissue is more susceptible than human tissue to renal toxicity caused by cysteine S-conjugate β-lyase-mediated bioactivation. Relevant examples of species-dependent metabolism leading to nephrotoxicity include efavirenz, hexachlorobutadiene, and sotorasib [?].

In rats, mercapturic acid and β-lyase pathway metabolites accounted for 60.72% and 97.95% of total drug-related material in urine and bile, respectively. These results indicated that mercapturic acid and β-lyase pathways contribute to BPI-460372 metabolism in rats to a certain degree. In the 4-week toxicity study of BPI-460372, the main target organs in rats included the kidney.

Our study indicated that cysteine S-conjugate β-lyase participated in BPI-460372 metabolism, and the relationship between nephrotoxicity observed in rats and cysteine S-conjugate β-lyase-mediated bioactivation requires further investigation.

Acrylamide warheads are widely used in covalent tyrosine kinase inhibitors (TKIs), with 8 covalent TKIs currently approved by the FDA including acalabrutinib, afatinib, dacomitinib, ibrutinib, neratinib, osimertinib, ritlecitinib, and zanubrutinib [?]. For acalabrutinib, dacomitinib, ibrutinib, neratinib, and zanubrutinib, hepatic metabolism via P450 is the major route of elimination [?]. Ritlecitinib is metabolized through multiple CYP and GST pathways with no single route contributing more than 25% of total metabolism [?]. For afatinib and osimertinib, the main clearance pathway is covalent binding to plasma proteins [?, ?]. Various covalent TKIs have been reported to covalently bind to human serum albumin, leading to instability in human plasma [?]. BPI-460372 remained stable when incubated with mouse, rat, dog, monkey, and human plasma at 37 °C for 4 h, indicating it does not covalently bind to plasma proteins.

5. CONCLUSION

BPI-460372 was mainly metabolized by CYP2D6, CYP3A4, and CYP1A2. To avoid potential clinically relevant pharmacokinetic DDI, strong or moderate inducers or inhibitors of these enzymes were restricted in the Phase I clinical study. The good in vitro-in vivo correlation of clearance is favorable for predicting BPI-460372 pharmacokinetics in humans. These in vitro and in vivo studies provide important information on BPI-460372 metabolism, and further clinical development is ongoing.

AUTHORS' CONTRIBUTIONS

XYL and CZT contributed to experimental design and data analysis. All authors were involved in interpretation of results as well as manuscript writing and review.

LIST OF ABBREVIATIONS

NADPH: Nicotinamide Adenine Dinucleotide Phosphate
LC-MS/MS: Liquid Chromatography-tandem Mass Spectrometry
UHPLC-Orbitrap-HRMS: Ultra-high Performance Liquid Chromatography Combined with Orbitrap Tandem Mass Spectrometry
AOAA: Aminooxyacetic Acid
CYP: Cytochrome P450
DDI: Drug-drug Interaction
GGT: γ-glutamyl Transpeptidase
GSH: Glutathione
GST: Glutathione-S-transferase
HLM: Human Liver Microsomes
IS: Internal Standard
TAZ: Transcriptional Coactivator with PDZ-binding Motif
TCA: Tricarboxylic Acid
TCI: Target Covalent Inhibitors
TEAD: Transcriptional Enhanced Associate Domain
TKI: Tyrosine Kinase Inhibitors
$t_1/_2$: Half-life
YAP: Yes Associated Protein
7-EC: 7-ethoxycoumarin

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Chengze Biological Technology Co., LTD before experiments were conducted.

HUMAN AND ANIMAL RIGHTS

Animal studies complied with “The Regulations on the Management of Experimental Animals” of The People’ s Republic of China Ministry of Science and Technology.

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CONFLICT OF INTEREST

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Xiaoyun Liu, Xiaofeng Xu, and Hong Lan are employees of Betta Pharmaceuticals, and Hong Lan may own Betta Pharmaceuticals stock. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher' s website along with the published article.

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Fig. 1. The extracted ion chromatograms of BPI-460372 and its metabolites from human (a), monkey (b), dog (c), rat (d), and mouse (e) hepatocytes. M528 (H,Mk,D,R,Ms) M714 (H,Mk,D,R,Ms) M377 (H,Mk,D,R,Ms) M527 (Mk,Ms) M570 (H,D,R,Ms) BPI-460372 (H,Mk,D,R,Ms) M335 (BPI-460608) (H,Mk,D,R,Ms) M497 (H,Mk,D,R,Ms) M423 (BPI-460456) (H,Mk,D,R,Ms) M407 (BPI-460430) (H,Mk,D,R,Ms) M712 (H,Mk,D,R,Ms) H: human, Mk: monkey, D: Dog, R: rat, Ms: mouse.

Fig. 2. Proposed metabolic pathways of BPI-460372 in human, monkey, dog, rat, and mouse hepatocytes. M335 (BPI-460608) M423 (BPI-460456) Parent (BPI-460372) M407 (BPI-460430) / M407-2

Fig. 3. Proposed metabolic pathways of BPI-460372 in rats. M423 (BPI-460456) Parent (BPI-460372) M335 (BPI-460608) M407 (BPI-460430)

Fig. 4. Proposed metabolic pathways of BPI-460372 in dog plasma.

Table 1. The inhibition rate of metabolism of BPI-460372 in HLM by specific CYP inhibitors and percentage of metabolism of BPI-460372 through recombinant expressed cytochrome isoforms. Inhibition rate (%) Contribution (%) BPI-460372 had no metabolic trend in liver microsomes with CYP2C9 inhibitor, thus the k and inhibition rate were not calculated. BPI-460372 showed no metabolic trend upon incubation with rCYP2B6 and 2C19, thus the k and contribution were not calculated. NC, not calculated.

Table 2. Metabolic stability results of BPI-460372 in human, monkey, dog, rat, and mouse hepatocytes. Species Human Monkey Mouse (min) CLint, in virto (µL$\cdot$min$^{-1}\cdot$million cells^{-1}) CLint, in vivo (mL$\cdot$min$^{-1}\cdot$kg^{-1}) (mL$\cdot$min$^{-1}\cdot$kg^{-1})

Table 3. UV (λ=314 nm) relative abundance of BPI-460372 and its metabolites in human, cynomolgus monkey, beagle dog, SD rat, and ICR mouse hepatocytes after 4 h incubation (%). Retention Time UV (314 nm) peak areas relative abundance (%) BPI-460372 (BPI-460608) (BPI-460456) (BPI-460430) Human Monkey (min) Mouse ND: Not detected by UV and MS; +: The UV signal was too weak to be quantified, but could be detected in MS.

Table 4. Characterization of BPI-460372 metabolites in human, cynomolgus monkey, beagle dog, SD rat and ICR mouse hepatocytes through UHPLC-Orbitrap-HRMS. Metabolic pathway Retention (min) Formula Observed (m/z) Theoretical (m/z) error (ppm) Fragment Ions glucose $C_{21}H_{22}F_3N_5O_6$ Hydrolysis conjugation (BPI-460608) Hydrolysis Hydrolysis, mono-oxidation glucuronidation Oxidative (-F+OH) conjugation defluorination and glutathione

C15H12F3N5O C21H20F3N5O8 C28H31F3N8O9S Glutathione conjugation C28H30F4N8O8S Cysteine conjugation C21H20F4N6O4S (BPI-460456) Oxidative (-F+OH) and hydration defluorination (BPI-460430) Defluorination (-F+H) and hydration C18H16F3N5O4 Fragment Ions 480.1489, 336.1067, 174.0774, 98.0600 462.1284, 307.0801, 307.0801, 97.0396, 56.0495 281.0645, 378.1172, 281.0645, 238.0587, 352.1016, 281.0645, 238.0587 323.0750, 322.0910, 638.1639, 390.1172, 464.0999, 319.0801, 438.0842, 307.0801, 440.0799, 336.1067, 640.1601, 390.1172, 440.0799, 336.1067, 281.0645, 56.0495 406.1122, 307.0801, 281.0645, 56.0495 408.1078, 319.0801, 336.1067, 408.1078, 319.0801, 390.1172, 307.0801, 319.0801, C18H16F3N5O3 364.1016, 307.0801, 281.0645, 56.0495 336.1067, 319.0801, N-acetylcysteine conjugation C23H22F4N6O5S Hydrolysis and acetylation C17H14F3N5O2 BPI-460372 Parent drug C18H13F4N5O2 529.1276, 336.1067, 281.0645, 162.0219 440.0799, 319.0801, 337.0907, 307.0801, 97.0396, 56.0495 336.1067, 281.0645, 319.0801, 238.0587, 97.0396, 73.0084 307.0801, 128.0506, 408.1078, 307.0801, 319.0801, 238.0587, 281.0645, 102.0350, Mass error = (Observed m/z -Theoretical m/z) / Theoretical m/z. 1 ppm = 1 $\times 10^{-6}$.

Table 5. Characterization of BPI-460372 metabolites in rat plasma, urine, feces, bile, and dog plasma through UHPLC-Orbitrap-HRMS. Metabolic pathway BPI-460372 Parent drug Retention (min) Formula Observed (m/z) Theoretical (m/z) Mass error (ppm) Fragment Ions C18H13F4N5O2 319.0801, 238.0587, 97.0396, 73.0084 307.0801, 128.0506, 281.0645, 102.0350, (BPI-460608) Oxidation BPI-460608 (+2O) C15H12F3N5O3 Oxidation hydrogenation BPI-460608 C15H14F3N5O2 Hydrolysis C15H12F3N5O Oxidative defluorination (-F+OH) and glutathione conjugation Oxidative defluorination (-F+OH) cysteine conjugation Oxidative defluorination (-F+OH), hydration, and hydrogenation C28H31F3N8O9S C21H21F3N6O5S C18H18F3N5O4 351.0700, 293.0645, 281.0645, 97.0396 339.0700, 307.0801, 337.0907, 281.0645, 97.0396 319.0801, 307.0801, 307.0801, 97.0396, 56.0495 281.0645, 238.0587, 638.1639, 390.1172, 464.0999, 319.0801, 438.0842, 307.0801, 438.0842, 336.1067, 281.0645, 56.0495 406.1122, 319.0801, 364.1016, 307.0801, 319.0801, 238.0587, 146.0812, 56.0495 307.0801, 281.0645, Cysteine conjugation C21H20F4N6O4S 440.0799, 336.1067, 281.0645, 56.0495 408.1078, 319.0801, 390.1172, 307.0801, (BPI-460456) Oxidative defluorination (-F+OH) and hydration C18H16F3N5O4 M407-2 Isomer of BPI-460430 C18H16F3N5O3 406.1122, 307.0801, 281.0645, 56.0495 336.1067, 319.0801, 364.1016, 307.0801, 281.0645, 56.0495 336.1067, 319.0801, Glutathione conjugation C28H30F4N8O8S Dealkylation (-C3H5NO2) and methylation (+CH2) of M526 Oxidative defluorination (-F+OH), N-acetylcysteine conjugation Defluorination conjugation (-F+H) N-acetylcysteine C19H18F3N5O3S C23H23F3N6O6S C23H23F3N6O5S 640.1601, 390.1172, 440.0799, 336.1067, 408.1078, 319.0801, 390.1172, 307.0801, 110.0600, 84.0444 337.0907, 281.0645, 319.0801, 238.0587, 438.0842, 336.1067, 406.1122, 319.0801, 390.1172, 307.0801, 422.0893, 307.0801, 281.0645 390.1172, 319.0801, (BPI-460430) Defluorination and hydration N-acetylcysteine conjugation (-F+H) C18H16F3N5O3 364.1016, 307.0801, 281.0645, 56.0495 336.1067, 319.0801,

C23H22F4N6O5S 529.1276, 336.1067, 281.0645, 162.0219 440.0799, 319.0801, 408.1078, 307.0801, Defluorination (-F+H) C18H14F3N5O2 319.0801, 307.0801, 281.0645 Defluorination and hydrogenation (-F+H) C18H16F3N5O2 319.0801, 307.0801, 281.0645, Mass error = (Observed m/z -Theoretical m/z) / Theoretical m/z. 1 ppm = 1 × 10^{-6}.

Table 6. Relative abundance of MS peak areas of BPI-460372 and its major metabolites after oral administration of BPI-460372 to rats and dogs. Retention time (min) Rat Plasma 0-24 h Rat Urine 0-48 h Rat Feces 0-48 h Pathway BPI-460372 M335 (BPI-460608) M423 (BPI-460456) M407-2 (+2O) Parent drug Oxidation BPI-460608 Oxidation hydrogenation BPI-460608 Hydrolysis Oxidative defluorination (-F+OH) and glutathione conjugation Oxidative defluorination cysteine (-F+OH) conjugation Oxidative defluorination (-F+OH), hydration, and hydrogenation Cysteine conjugation Oxidative defluorination (-F+OH) and hydration Isomer of BPI-460430 Glutathione conjugation Dealkylation (-C3H5NO2) and methylation (+CH2) of M526 Oxidative defluorination (-F+OH), N-acetylcysteine conjugation Defluorination (-F+H) N-acetylcysteine Rat Bile 0-48 h Dog Plasma 0-48 h M407 (BPI-460430) -, not detected. (-F+H) conjugation Defluorination and hydration N-acetylcysteine conjugation Defluorination (-F+H) Defluorination and hydrogenation (-F+H) Relative abundance(%) The relative abundance values shown in the table were the average of male and female samples. MS peak areas of metabolite Total peak areas of parent drug and metabolites × 100.

Table 7. The area ratio of BPI-460372 and metabolites in rat urine after treatment with 20 mg/kg BPI-460372 alone or co-administration with 100 mg/kg AOAA. Group 20 mg/kg BPI-460372 0.89 (54) 0.51 (58) 0.48 (114) 0.16 (71) 0.28 (112) 1.36 (67) 20 mg/kg BPI-460372 + 100 mg/kg AOAA 1.48 (69) 0.23 (23) 0.12 (37) 0.06 (20) 0.08 (40) 1.41 (48) The data were presented as mean (%CV).

SUPPLEMENTARY MATERIAL

1.1 Metabolic stability of BPI-460372 in HLMs with or without specific CYP inhibitors and recombinant human CYP isoenzyme

BPI-460372 was detected through an XSelect HSS T3 column (2.5 µm, 50 mm × 2.1 mm). The mobile phase contained 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient elution program was as follows: 0.00-0.50 min, 30% B; 0.50-1.80 min, 30%-50% B; 1.80-1.85 min, 50%-95% B; 1.85-2.50 min, 95% B; 2.50-2.55 min, 95%-30% B. From 2.55 to 3.00 min, 30% B was maintained for equilibration. The flow rate was set to 0.6 mL/min, and the column temperature was kept at 40 °C. The mass spectrometer (MS) was operated in positive ion mode using electrospray ionization (ESI). MS parameters were optimized as follows: 5500 V ion spray voltage, 550 °C probe temperature, 60 ms dwell time, 50 psi ion source gas 1, 50 psi ion source gas 2, and 45 psi curtain gas pressure. Multiple reaction monitoring (MRM) mode was used, with ion transitions for BPI-460372 and BPI-460608 (IS) of m/z 407.9$→318.9*and*336.2→$307.2, respectively. The declustering potential (DP) for BPI-460372 and BPI-460608 were 161 V and 96 V, respectively. The collision energies (CE) were 33 V for BPI-460372 and 31 V for BPI-460608.

1.2 Metabolic stability of BPI-460372BBB in human, monkey, dog, rat, and mouse hepatocytes

BPI-460372 was detected using an ACQUITY UPLC BEH C18 column (1.7 µm, 50 mm × 2.1 mm). The mobile phase contained a mixture of 2 mmol/L ammonium acetate with 0.1% formic acid (A) and 0.1% formic acid in acetonitrile (B). The gradient elution program was as follows: 0.00-0.20 min, 30% B; 0.20-1.60 min, 30%-95% B; 1.60-1.90 min, 95% B; 1.90-2.20 min, 95%-30% B. The flow rate was set to 0.5 mL/min, and the column temperature was kept at 40 °C. The MS was operated in positive ion mode using ESI. MS parameters were optimized as follows: 5500 V ion spray voltage, 550 °C probe temperature, 50 ms dwell time, 60 psi ion source gas 1, 60 psi ion source gas 2, and 40 psi curtain gas pressure. MRM mode was used, with ion transitions for BPI-460372 and verapamil (IS) of m/z 408.2 → 319.2 and 455.3 → 165.2, respectively. The DP for BPI-460372 and verapamil were 170 V and 130 V, respectively. The collision energies (CE) were 29 V for BPI-460372 and 40 V for verapamil.

1.3 The effect of cysteine S-conjugate β-lyase inhibitor on the metabolism of BPI-460372

BPI-460372 and metabolites were separated on a Kinetex C18 column (1.7 m, 2.1 mm × 100 mm; Phenomenex Corporation, Torrance, CA, USA). Mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Gradient elution procedures were as follows: 0.00-1.00 min, 5% B; 1.00-2.00 min, 5%-30% B; 2.00-7.00 min, 30%-80% B; 7.00-7.20 min, 80%-95% B; 7.20-8.00 min, 95%B; 8.00-8.10 min, 95%-5% B. From 8.10 to 10.00 min, 5% B was maintained for equilibration. The flow rate was set at 0.40 mL/min. Samples were maintained at 4 °C in an autosampler, and the column temperature was set at 30 °C. The mass spectrometer was operated in positive ion mode using ESI. MS parameters were optimized as follows: ion spray voltage, 5500 V; probe temperature, 500 °C; dwell time, 20 ms; ion source gas 1, 55 psi; ion source gas 2, 55 psi; curtain gas, 20 psi; collision gas, 8 psi; entrance potential, 10 V. Verapamil was used as the IS. MRM mode was used, and ion transitions, DP, CE, and collision cell exit potential (CXP) for these analytes were set as follows:

Analyte	Q1 Mass (Da)	Q3 Mass (Da)	DP (V)	CE (V)	CXP (V)
BPI-460372	408.2	319.2	170	29	15
verapamil	455.3	165.2	130	40	15

Figure S1. The MS/MS product ion spectrum of BPI-460372 (a) and the tentative fragmentation patterns (b).

Figure S2. The MS/MS product ion spectrum of BPI-460608 (a) and the tentative fragmentation patterns (b).

Figure S3. The MS/MS product ion spectrum of BPI-460456 (a) and the tentative fragmentation patterns (b).

Figure S4. The MS/MS product ion spectrum of BPI-460430 (a) and the tentative fragmentation patterns (b).

Table S1. The inhibition rate of metabolism of positive control substrates in HLM by specific CYP inhibitors and the CLint (rCYPj) of positive control substrates through recombinant expressed cytochrome isoforms. Positive control substrates Inhibition rate (%) CLint (rCYPj) (Lmin^{-1}pmol^{-1}) Phenacetin Bupropion Paclitaxel Diclofenac S-Mephenytoin Dextromethorphan Midazolam Dextromethorphan was completely metabolized in recombinant human CYP2D6 in 5 min, thus K and CLint (rCYPj) were not calculated. NC, not calculated.

Table S2. Metabolic stability results of testosterone and 7-hydroxycoumarin in human, monkey, dog, rat, and mouse hepatocytes. Positive control Species (min) CLint, in virto (µL · min^{-1} · million cells^{-1}) CLint, in vivo (mL · min^{-1} · kg^{-1}) (mL · min^{-1} · kg^{-1}) Testosterone 7-hydroxycoumarin Human Monkey Mouse Human Monkey Mouse

Table S3. The metabolism of 7-EC in five species. Species Human Monkey Mouse Area Ratio 7-EC-GluA Area Ratio Proportion of 7-EC remaining in hepatocytes after 4 h incubation (%) 3.36E+07 2.65E+07 2.18E+07 2.64E+07 2.39E+07 7.50E+06 9.51E+04 1.01E+05 6.62E+06 1.46E+05 1.07E+03 6.08E+02 0.00E+00 1.45E+02 0.00E+00 3.85E+06 1.55E+07 1.27E+07 8.08E+05 7.44E+06 T0 h, 0 h time point; T4 h, 4 h time point; 7-EC, 7-ethoxycoumarin; 7-EC-GluA, de-ethylation and glucuronidation metabolite of 7-EC.

Note: Figure translations are in progress. See original paper for figures.

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