In vitro metabolism of 2-methoxy-N-[3-[4-[3-methyl-4-[(6-methyl-3-pyridinyl)oxy]anilino]-6-quinazolinyl]prop-2-enyl]acetamide (CP-724,714) by aldehyde oxidase and predicting its percent contribution relative to CYP-mediated metabolism

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Nonstandard Abbreviations

AO, aldehyde oxidase; 1-ABT, 1-aminobenzotriazole; CHRM, cryopreserved hepatocyte recovery medium; NADPH, nicotinamide adenine dinucleotide phosphate; CP-724,714, 2-methoxy-N-[3-[4-[3-methyl-4-[(6-methyl-3-pyridinyl)oxy]anilino]-6-quinazolinyl]prop-2-enyl]acetamide.
Abstract

2-methoxy-N-[3-[4-[3-methyl-4-[(6-methyl-3-pyridinyl)oxy]anilino]-6-quinazolinyl]prop-2-enyl]acetamide (CP-724,714) is an anticancer drug that was discontinued owing to hepatotoxicity found in clinical studies. Metabolite analysis of CP-724,714 was conducted using human hepatocytes, where twelve oxidative metabolites and one hydrolyzed metabolite were formed. Among the three mono-oxidative metabolites, the formation of two was inhibited by adding 1-aminobenzotriazole, a pan-CYP inhibitor. In contrast, the remaining one was not affected by this inhibitor but partially inhibited by hydralazine, indicating that aldehyde oxidase (AO) was involved in metabolizing CP-724,714, which contains a quinazoline substructure, a heterocyclic aromatic quinazoline ring, known to be preferably metabolized by AO. One of the oxidative metabolites of CP-724,714 observed in human hepatocytes was also generated in recombinant human AO. Although CP-724,714 is metabolized by both CYPs and AO in human hepatocytes, the contribution level of AO could not be evaluated using its specific inhibitors because of low AO activity in in vitro human materials. Here, we present a metabolic pathway for CP-724,714 in human hepatocytes and the involvement of AO in CP-724,714 metabolism. We showed here a plausible workflow for predicting AO contribution to the metabolism of CP-724,714 based on DMPK screening data.

Significance Statement

CP-724,714 was identified as a substrate of aldehyde oxidase (AO) rather than xanthine oxidase. Since CP-724,714 is also metabolized by CYPs, the contribution levels of AO and CYPs in the metabolism of CP-724,714 were estimated simultaneously based on in vitro drug metabolism screening data.
**Introduction**

CP-724,714, developed as an anticancer drug candidate, showed unanticipated hepatotoxicity, possibly due to transporter inhibition, thereby causing hepatocellular injury and hepatobiliary cholestasis, resulting in the discontinuation of its development for use in humans (Feng et al., 2009). Aldehyde oxidase (AO), a molybdenum enzyme, preferentially oxidizes aldehydes and azaheterocyclic substructures such as quinazoline derivatives (Pryde et al., 2010; Hutzler et al., 2013). AO enzyme transfers oxygen from a water molecule to the electrophilic carbon of heteroaromatic compounds. Due to the presence of highly distinct species and inter-individual variability, the role of AO in humans is of concern at the clinical stage. In humans, AOX1 is the predominant AO isoform that contributes to the metabolism of endogenous and exogenous substrates. Species-dependent distribution of AOX1 has been observed with high activity in monkeys and humans, relatively low activity in rodents such as rats and mice, and no activity in dogs (Beedham et al., 1995; Kitamura et al., 2006). The contribution of AO could result in undesirable pharmacokinetic (PK) and toxicity profiles even at the clinical stage because AO contribution is not accurately evaluated in preclinical species such as rodents and dogs due to species-dependent activity. One compound, FK3453, showed an unpredictably low plasma concentration after administration to humans based on dosage projected from PK profiles observed during preclinical studies (Akabane et al., 2011). In other cases, SGX523 was metabolized by AO to generate a quinolinone derivative, resulting in species-specific renal toxicity due to the crystallization of the quinolinone metabolite in monkeys and humans (Diamond et al., 2010). Following the development of *in vitro* DMPK screening, drug candidates have been extensively optimized using NADPH-fortified human liver microsomes to avoid cytochrome P450 (CYP)-mediated metabolism and prevent clinical failure dependent on DMPK...
issues (Kola and Landis, 2004). Consequently, drug candidates have less potential for CYP-mediated metabolism; however, the role of non-CYP metabolisms, such as oxidation by AO, has tended to increase (Cerny, 2016; Saravanakumar et al., 2019).

Based on the structural optimization needed for drugs to escape CYP metabolism, the contribution of unpredictable non-CYP metabolism can be increased, resulting in AO contribution to the intrinsic metabolic fate of drug candidates during the drug discovery stage (Cerny, 2016). In addition, drug-drug interactions related to non-CYP metabolism are also beginning to demand attention in drug development because of their increasing influence on drug metabolism. For example, drug-drug interactions related to AO inhibition have been reported for zaleplon, a well-investigated AO substrate (Kawashima et al., 1999), in the combination therapy with cimetidine known as an AO inhibitor. Cimetidine co-administered with zaleplon elevates the maximum plasma concentration and AUC of zaleplon compared to that during the administration of zaleplon alone (Renwick et al., 2002).

CP-724,714 has a quinazoline substructure that has the propensity to be metabolized by AO. To identify the contribution of AO to CP-724,714 metabolism, we examined the susceptibility of CP-724,714 to AO-mediated metabolism using human liver cytosol and human hepatocytes. To confirm the contribution of AO to CP-724,714 metabolism, we employed recombinant human AO and a mechanism-based inhibitor of AO. Because in vitro AO activity tends to be lower than that in vivo, estimation of the contribution of AO to the metabolism of drug candidates at the preclinical stage could be challenging. In silico structure-based approaches have been developed to identify the labile moieties attached to the drug structure; however, it would still be difficult to predict the potential AO contribution at the early stage of drug development. Herein, to estimate the proportion of metabolism mediated by AO in humans, we aimed to predict the contribution
of AO based on *in vitro* human liver microsomal and cytosolic stability assays for CP-724,714 using an empirical scaling factor set for typical AO substrates.
Materials and Methods

CP-724,714 was purchased from Selleck Chemicals (Houston, TX). Human liver microsomes (mixed gender, pool of 200 individuals) and human liver cytosol with high AO/xanthine oxidase (XO) activity (mixed gender, pool of 20 individuals) were purchased from Sekisui XenoTech LLC (Kansas City, KS, USA). Supersome ultra-human aldehyde oxidase (AOX1) was purchased from Corning Inc. (Corning, NY, USA). Cryopreserved human hepatocytes (lot. HJT, a pool of 50 individuals) were obtained from BioIVT (Baltimore, MD, USA). Cryopreserved hepatocyte recovery medium (CHRM) was purchased from Invitrogen, Inc. (Waltham, MA). The NADPH regeneration system used in this study consisted of 3.3 mmol/L \( \beta \)-NADP\(^+ \), 80 mmol/L glucose 6-phosphate, 60 mmol/L MgCl\(_2\), and 1 unit/mL glucose 6-phosphate dehydrogenase. \( \beta \)-NADPH was purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan). Hydralazine, glutamine, O6-benzylguanidine, William’s medium E, and 1-aminobenzotriazole (1-ABT) were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). Zoniporide hydrochloride and zaleplon were obtained from Tocris Biosciences (Bristol, UK). 6-Deoxypenciclovir was purchased from the United States Biological Company (Salem, MA, USA). Acridine carboxamide (DACA) was synthesized by TCG Life Sciences (West Bengal, India). Febuxostat was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Acetonitrile, distilled water, and formic acid were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). All other materials used in this study were of reagent grade.

Incubation conditions for metabolite analysis of CP-724,714

For metabolite analysis, CP-724,714 (10 \( \mu \)M) was incubated in reaction solutions consisting of 0.5 mg/mL pooled human liver microsomes fortified with 5 mM \( \beta \)-NADPH in 100 mM
potassium phosphate buffer (pH 7.4) for 2 h. Recombinant human AOX1 (0.5 mg/mL) was incubated with CP-724,714 (10 μM) in 100 mM potassium phosphate buffer (pH 7.4) for 1 h. Cryopreserved human hepatocytes (one million cells/mL) were incubated with CP-724,714 (10 μM) for 4 h in a CO₂ incubator (5% CO₂/95% air humidified atmosphere; MCO-170AICUV-PJ, Panasonic Corporation, Osaka, Japan) with or without 1-ABT (1 mM) or hydralazine (10 μM) as inhibitors for CYPs and AO, respectively. In the inhibition study, preincubation with the inhibitor (1-ABT or hydralazine) was conducted for 30 min at 37°C in the CO₂ incubator prior to the initiation of metabolic reaction with CP-724,714. The metabolic reaction was terminated by adding double the volume of acetonitrile. After centrifugation (13,200 g, 10 min, 4°C, Kubota 3520; Kubota Corporation, Tokyo, Japan), the supernatants were subjected to liquid chromatography with high resolution mass spectrometry (LC/HRMS) for metabolite analysis.

**Incubation conditions and data analysis for metabolic clearance**

The metabolic clearance of CP-724,714 (0.1, 1, and 10 μM) was evaluated in cryopreserved human hepatocytes (one million cells/mL) incubated for 0, 15, 30, 60, 120, and 240 min in the CO₂ incubator (5% CO₂/95% air humidified atmosphere) maintained at 37°C with or without 1-ABT (1 mM) or hydralazine (25 μM) as inhibitors for CYPs and AO, respectively, in duplicate. In the inhibition study, preincubation with the inhibitor (1-ABT or hydralazine) was conducted for 30 min at 37°C in the CO₂ incubator prior to the initiation of metabolic reaction with CP-724,714. The metabolic reaction was terminated by adding a 7.5-fold volume of acetonitrile. After centrifugation (1,310 × g, 10 min, 4°C, Kubota 5930), the supernatant was subjected to liquid chromatography-mass spectrometry (LC-MS) analysis. The CLₘₚ value in human hepatocytes was obtained based on depletion CP-724,714, as mentioned in a previous report.
In this study, more than 15% depletion of the substrates observed in the incubation period was considered to calculate metabolic clearance.

Concentration of CP-724,714 in each assay system = $C_{\text{time} \ 0} \times e^{-k\Delta t}$

$CL_{\text{int,hep}} \ (\text{mL/min/million cells}) = k^{\Delta t} \times \frac{\text{incubation volume} \ (\text{mL})}{\text{hepatocytes cell number} \ (\text{million cells})}$

Metabolic clearance of CP-724,714 and midazolam (0.1 μM) in human liver microsomes (0.2 mg/mL) fortified with the NADPH regeneration system incubated for 0 and 15 minutes at 37°C was evaluated in duplicate. Metabolic clearance of typical AO substrates, acridine carboxamide (DACA), zaleplon, 6-deoxypenciclovir, zoniporide, and O6-benzylguanine, in human liver microsomes (0.5 mg/mL) fortified with the NADPH regeneration system incubated for 0 and 15 minutes at 37°C was evaluated in duplicate. The metabolic reaction was terminated by adding an equal volume of acetonitrile:methanol in a 7:3 ratio (by volume) to an internal standard (IS). After centrifugation (1,310 × g, 10 min, 4°C, CF7D2, Eppendorf Himac Technologies Co., Ltd, Ibaraki, Japan), the supernatant was subjected to LC-MS analysis. The intrinsic clearance ($CL_{\text{int}}$) values in human liver microsomes were calculated based on the depletion of compounds, as reported previously (Sohlenius-Sternbeck et al., 2010). The free fractions of CP-724,714 and AO substrates in human liver microsomal samples were predicted in accordance with a previous report (Hallifax and Houston, 2006).

$$fu_{\text{mic}} \ (\text{predicted}) = \frac{1}{1+C \cdot 10^{0.072 \cdot \log P/D+0.067 \cdot \log P/D-1.126}}$$

$CL_{\text{int,u,HLM}} \ (\text{mL/min/mg protein}) = k^{\Delta t} \times \frac{\text{incubation volume} \ (\text{mL})}{\text{liver microsomal protein} \ (\text{mg})/fu_{\text{mic}} \ (\text{predicted})}$
The metabolic clearance of CP-724,714 and midazolam (0.1 μM) in human liver cytosol with high AO/XO activity (1.0 mg/mL), incubated for 0 and 60 minutes at 37°C, was evaluated in duplicate. The metabolic reaction was terminated by adding an equal volume of acetonitrile:methanol mixture in a 7:3 ratio (by volume) to the IS. To investigate the contribution of XO, CP-724,714 was incubated in human liver cytosol with high AO/XO activity (1.0 mg/mL) in the presence of hydralazine (100 μM) or febuxostat (5 μM) as an AO and XO inhibitor, respectively. After centrifugation (1,310 × g, 10 min, 4°C, CF7D2, Eppendorf Himac Technologies), the supernatants were subjected to LC-MS analysis. The CL_{int} values in human liver cytosol were calculated based on the depletion of compounds, as reported previously (Sohlenius-Sternbeck et al., 2010). Because estimating the free fraction of CP-724,714 in the human liver cytosol was difficult, the fu value was used as 1.

\[
\text{CL}_{\text{int, u,HLC}} (\text{mL/min/mg protein}) = \frac{k \Delta t \times \frac{\text{incubation volume (mL)}}{\text{liver cytosolic protein (mg)}}}{\text{fu}}
\]

For in vitro/in vivo correlation analysis using the AO substrates, DACA, zaleplon, 6-deoxypenciclovir, zoniporide, and O6-benzylguanine, the metabolic clearance values of these compounds (0.1 μM at final concentration) in human liver cytosol with high AO/XO activity (1.0 mg/mL) incubated for 0 and 60 min at 37°C was evaluated in duplicate, and the experiments were repeated thrice. The metabolic reaction was terminated by adding an equal volume of acetonitrile:methanol mixture in a 7:3 ratio (by volume) to the IS. After centrifugation (1,310 × g, 10 min, 4°C, CF7D2, Eppendorf Himac Technologies), the supernatants were subjected to LC-MS analysis. The CL_{int} values of these AO substrates in the human liver cytosol were calculated based on the depletion of these compounds, as described in a previous report (Sohlenius-Sternbeck et al., 2010) using a fu value of 1 as described above.
Regarding the comparison of *in vitro* and *in vivo* clearance mediated by AO, it has been reported that *in vitro* clearance values of these AO substrates resulted in the underestimation of *in vivo* clearance (Zientek et al., 2010). A discrepancy between *in vitro* clearance using high AO/XO activity lot in this study and *in vivo* clearance values of these AO substrates was observed; therefore, an empirical scaling factor (SF) for CL_{int,u,HLC} of these AO substrates was used to project their *in vivo* clearance values. The SF value was calculated as 8.2, determined by minimizing the sum of the squared errors between common logarithm values of CL_{int,in vivo} and the scaled CL_{int,u,HLC} using the generalized gradient method through the MS® Excel solver (Gabriele and Ragsdell, 1977). The contribution of AO to the metabolism of the typical AO substrates and CP-724,714 was estimated using both metabolic clearance values in NADPH-fortified human liver microsomes and in human liver cytosol with the empirical SF. The estimated percentage of contribution of AO (%) using metabolic clearance values obtained is calculated as follows:

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\text{CL}_{\text{CYP}} \text{ (mL/min/kg): } \text{CL}_{\text{int,u,HLM}} \times 40 \text{ (mg/g liver)} \times 25 \text{ (g liver/kg)}
\]

\[
\text{CL}_{\text{AO}} \text{ (mL/min/kg): } \text{CL}_{\text{int,u,HLC}} \times 8.2 \text{ (SF)} \times 80.7 \text{ (mg/g liver)} \times 25 \text{ (g liver/kg)}
\]

Estimated contribution of AO (%): \(100 \times \frac{\text{CL}_{\text{AO}}}{(\text{CL}_{\text{CYP}} + \text{CL}_{\text{AO}})}\)

**LC-MS/MS analysis**

For metabolite analysis of CP-724,714, Orbitrap Fusion coupled with a Vanquish LC system was operated by Xcalibur™ software version 4.3 (Thermo Fisher Scientific, Waltham, MA, USA). The prepared samples were injected onto an ACQUITY UPLC BEH C18 1.7 μm, 2.1 × 100 mm analytical column (Waters Corporation, Milford, MA, USA) maintained at 40°C with a flow rate of 0.5 mL/min. The initial mobile phases were kept for 0.5 min with 90% solvent A (0.1% formic acid in water)/10% solvent B (0.1% formic acid in acetonitrile), and solvent B was
increased from 10 to 95% over 5.5 min. Subsequently, 95% of solvent B was maintained for 1 min, followed by equilibration with the initial mobile phases for 3 min for the next injection. Mass was measured in the positive ion mode with data-dependent MS$^2$ acquisition by HCD. The settings for Orbitrap Fusion were as follows: MS detection with resolution, 60,000; scan range, 100–1,000; ion source type, H-ESI; spray voltage, static 3,500 V; sheath gas, 50 Arb; aux gas, 15 Arb; sweep gas, 2 Arb; ion transfer temperature, 350°C; vaporizer temperature, 400°C; data-dependent MS$^2$ analysis with resolution, 15,000; activation type, HCD; collision energy mode, stepped; HCD collision energies (%), 27, 30, 33. The obtained data were processed using Compound Discoverer™ software (ver. 4.0; Thermo Fisher Scientific) to identify the metabolites of CP-724,714. For the enzymatic kinetics of CP-724,714 in human liver cytosol, Triple Quad 6500 (SCIEX) coupled with Nexera UHPLC (Shimadzu, Kyoto, Japan) operated by Analyst software version 1.7 (SCIEX) was used. The prepared samples were injected into the L-column ODS2 1.7 μm, 2.1 × 150 mm column (Chemicals Evaluation and Research Institute, Kyoto, Japan) maintained at 40°C with a flow rate of 0.5 mL/min. The initial mobile phase was 98% solvent A (0.02% formic acid in water)/2% solvent B (0.02% formic acid in acetonitrile) maintained for 0.5 min after sample injection, and solvent B was linearly increased from 2% to 99% over 5.5 min. Subsequently, 99% solvent B was maintained for 1 min, followed by equilibration with the initial mobile phases for 1 min for the next injection. The MRM transitions for quantification of CP-724,714 in positive ion mode were as follows: CP-724,714: Q1, 470.2/Q3, 381.0; Dwell time, 150 ms; DP, 80; CE, 40; CPX, 15; niflumic acid (IS): Q1, 283.0/Q3, 245.0; Dwell time, 150 ms; DP, 40; CE, 40; CPX, 15.

To assess metabolic clearance in hepatocytes, the Xevo-TQ-XS/ACQUITY UPLC system operated using MassLynx software version 4.2 (Waters Corporation) was employed.
prepared samples were injected onto an L-column ODS, 5 μm, 2.1 × 150 mm analytical column (Chemicals Evaluation and Research Institute), maintained at 40°C with a flow rate of 0.4 mL/min. The initial mobile phases were maintained for 0.5 min with 95% solvent A (0.1% formic acid in water) and 5% solvent B (0.1% formic acid in acetonitrile), and solvent B was linearly increased from 5% to 95% over 3 min. Subsequently, 95% solvent B was maintained for 1 min, followed by equilibration with the initial mobile phases for 2 min for the next injection. The MRM transitions for quantification of CP-724,714 in positive ion mode were as follows: CP-724,714: Q1; 470.06/Q3; 380.74, Dwell time; 80 ms, Cone voltage; 30 V, CE; 30 eV, niflumic acid (IS); Q1; 283.25/Q3; 216.99, Dwell time: 80 ms, Cone voltage: 30 V, CE: 30 eV.

For LC-MS analysis of AO substrates after incubation in human liver cytosol, a TQ detector coupled with an ACQUITY UPLC system operated using Masslynx software version 4.2 (Waters Corporation) was used. The prepared samples were injected onto Atlantis T3, 5 μm, 2.1 mm× 30 mm (Waters Corporation), maintained at 30°C with a flow rate of 0.6 mL/min. The initial mobile phases were maintained for 0.35 min with 95% solvent A (0.1% formic acid in water) and 5% solvent B (0.1% formic acid in acetonitrile), and solvent B was linearly increased from 5% to 99% over 0.45 min. Subsequently, 99% of solvent B was maintained for 0.7 min, followed by equilibration with the initial mobile phases for 0.3 min for the next injection. MRM transitions for quantification of AO substrates in positive ion mode were as follows: DACA: Q1, 294.19/Q3, 249.02; Dwell time, 0.161 sec; Cone voltage, 30 V; CE, 20 eV; zaleplon: Q1, 306.15/Q3, 236.19; Dwell time, 0.161 sec; Cone voltage, 50 V; CE, 30 eV; 6-deoxypenciclovir: Q1, 238.11/Q3, 136.08; Dwell time, 0.161 sec; Cone voltage, 40 V; CE, 20 eV; zoniporide: Q1, 321.15/Q3, 262.13; Dwell time, 0.161 sec; Cone voltage, 30 V; CE, 20 eV; O6-benzylguanine:
Q1, 242.09/Q3, 90.85; Dwell time, 0.161 sec; Cone voltage, 40 V; CE, 20 eV; propranolol (IS): Q1, 260.11/Q3, 116.07; Dwell time, 0.161 sec; Cone voltage, 40 V; CE, 20 eV.

To assess metabolic clearance in human liver microsomes and human liver cytosol, LCMS-8060 coupled with LC-VP UHPLC system operated by LabSolutions software version 5.99.2, LabSolutions Connect MRM version 2.11.009.3540, and Reifycs MS Quant Manager version 2.2.2.2 (Shimadzu) was employed. The prepared samples were injected into CAPCELL PAK ADME-HR, 3 µm, 2.1 mm ID × 35 mm (OSAKA SODA CO., LTD, Osaka, Japan) maintained at 40°C at a flow rate of 0.6 mL/min. The initial mobile phases were maintained for 0.35 min with 95% solvent A (0.1% formic acid in water)/5% solvent B (0.1% formic acid in acetonitrile), and solvent B was linearly increased from 5% to 99% over 0.45 min. Subsequently, 99% of solvent B was maintained for 0.7 min, followed by equilibration with the initial mobile phases for 0.3 min for the next injection. The MRM transitions for quantification of CP-724,714 in positive ion mode were as follows: CP-724,714: Q1, 470.22/Q3, 381.10; Dwell time, 97.0; CE, 25; midazolam: Q1, 326.09/Q3, 291.00; Dwell time, 97.0; CE, 25; and propranolol (IS): Q1, 260.20/Q3, 183.15; Dwell time, 97.0; CE, 15; source temperature, 650°C.
Results

Metabolite analysis of CP-724,714 in human liver microsomes, human hepatocytes, and recombinant human AOX1

The metabolic pathways of CP-724,714 in vitro are shown in Figure 1, and metabolites observed by LC-MS are summarized in Table 1. CP-724,714 was metabolized to thirteen metabolites, including three major oxidative metabolites, with an increase of mass by 16 Da compared to that of the parent compound (Figure 2). A pan-CYP inhibitor, 1-ABT, completely inhibited the formation of two of the three oxidative metabolites (Oxide 1 and Oxide 2), indicating that CYP is the enzyme majorly responsible for the biotransformation of CP-724,714 to these two metabolites. Although Oxide 1 and Oxide 2 were formed from CP-724,714 incubated in β-NADPH-fortified human liver microsomes, Oxide 3 was not generated. The formation of one of the three oxidative metabolites of CP-724,714 (Oxide 3) was not inhibited by 1-ABT but partially restricted by hydralazine, a mechanism-based inhibitor of AO, suggesting that it might have been generated by AO. Recombinant human AOX1 only formed Oxide 3 in human hepatocytes, clearly indicating that AO is the major enzyme contributing to the formation of Oxide 3 in human hepatocytes. In addition to these three oxidative metabolites, CP-724,714 was metabolized to a desmethylated metabolite (m/z 456, decrease by 14 Da), followed by oxidation (m/z 472, +O, m/z 470, +O-2H). Moreover, a hydrolyzed metabolite (m/z 398) and oxidatively deaminated metabolites (m/z 413 and m/z 415) were also formed (Figure 1). As shown in Figure 3, the product ion spectra of the three oxides observed at m/z 397 and 385 with a neutral loss of aliphatic moieties suggested that oxidation would occur around the aromatic scaffold of CP-724,714. Figure 3 (e) shows the MS³ spectrum of the product ion observed at m/z
397, generated from the molecular ion at m/z 486, suggesting that the proposed metabolic site would be the quinazoline moiety of CP-724,714.

**Metabolism of CP-724,714 in human hepatocytes and metabolic phenotyping using specific inhibitors**

To confirm CP-724,714 is a substrate of AO, the effects of hydralazine (AO inhibitor) and febuxostat (XO inhibitor) were tested in human liver cytosol. Inhibitor concentrations were set at 100 µM of hydralazine and 5 µM of febuxostat because these concentrations did not inhibit the metabolic reactions of 6-thioxanthine and O-benzylguanidine, respectively (Supplemental Table 1). The metabolism of CP-724,714 was inhibited in the presence of hydralazine but not febuxostat, indicating that AO could be a major contributor to CP-724,714 metabolism in human liver cytosol with high AO/XO activity (Supplemental Table 1). As CP-724,714 is metabolized by AO in human hepatocytes, the metabolic clearance of CP-724,714 in human hepatocytes with or without 1-ABT or hydralazine (inhibitors of CYPs and AO, respectively) was investigated. The metabolic reaction of CP-724,714 was remarkably inhibited by 1-ABT, and hydralazine (25 µM) inhibited the metabolism of CP-724,714 by approximately 20% at all tested concentrations of CP-724,714, as shown in Table 2 (Strelevitz et al., 2012). The results of the inhibition assay indicated that CP-724,714 could be metabolized mainly by CYPs, with a minor contribution of AO. In metabolite analysis of CP-724,714, as shown in Figure 2 (b), the levels of the AO-dependent metabolite Oxide 3 and the CYP-dependent metabolite Oxide 1 were found to be similar based on ion intensity, indicating that AO would contribute to the metabolism of CP-724,714 with a 20% higher level than that estimated by the inhibition assay described above.
However, quantitative analysis of the AO contribution level in human hepatocytes can be difficult because of the low AO activity observed in cryopreserved human hepatocytes.

*In vitro-in vivo correlation using human liver cytosol*

Representative AO substrates, DACA, zaleplon, 6-deoxypenciclovir, zoniporide, and O6-benzylguanine, were incubated in human liver cytosol with high AO/XO activity to estimate *in vivo* clearance values mainly mediated by AO, in accordance with the previous reports (Zientek et al., 2010; Hutzler et al., 2013). The empirical SF to project *in vivo* clearance of the five typical AO substrates incubated in human liver cytosol with high AO/XO activity lot was calculated to be 8.2, which is a lot-dependent value based on metabolic activity in each lot of human liver cytosol obtained. *In vitro* and *in vivo* values, \( CL_{int \, HLC} \) and \( CL_{int \, in \, vivo} \), respectively, used for the calculation of SF are summarized in Supplemental Figure 1. Because the metabolic reaction mediated by AO proceeds cofactor independently and it was difficult to calculate the free fractions of AO substrates, we used 1 as the free fraction of the AO substrates to calculate the clearance values. Variations of the *in vitro*/*in vivo* ratio of the 5 AO substrates were calculated between 0.05 of DACA and 0.29 of 6-deoxypenciclovir shown in Supplemental Figure 1. Even when taking into account the metabolism of the AO substrates via CYPs based on clearance values in NADPH-fortified human liver microsomes, AO substrates were estimated to exhibit more than 90% contribution of AO to overall metabolism, as shown in Table 3. The ratio of *in vitro* and *in vivo* clearance values of each AO substrate tested was summarized in Supplemental Table 2.

*Estimation of contribution of aldehyde oxidase and CYP-related oxidation*
Based on metabolite analysis, as shown in Figure 2, AO formed Oxide 3 at the same level as Oxide 1, which forms via CYPs, although a high CYP-mediated contribution in human hepatocytes was estimated when 1-ABT was used as an inhibitor. As the human CL_{int, in vivo} value of CP-724,714 was not reported, the contribution of AO to the metabolism of CP-724,714 could not be estimated based on the CL_{int, hep} values obtained in human hepatocytes. Although 20% of AO contribution to the metabolism of CP-724,714 in human hepatocytes was observed using hydralazine (25 μM), Oxide 3 was found with almost the same intensity as the major oxidative metabolite (Oxide 1) generated by CYPs (Figure 2 (b)). At the early stage of drug discovery, an in vitro stability assay using human liver microsomes was used to estimate the liability of CYP-mediated instability in the human body. Thus, a combination of data that originated from human liver cytosol stability assay with those of conventional human liver microsomal stability assay would be useful to estimate the AO contribution level in metabolism mediated by CYPs as well. Because in vitro-based AO activity is too low to estimate in vivo AO contribution of drug candidates, setting an empirical SF could be necessary for human liver cytosol stability assay. The calculated contribution of AO to the metabolism of CP-724,714 was 45%, as summarized in Table 4. Estimating the risk of AO susceptibility would be more practical than in vitro-in vivo correlation using clearance values individually obtained from human liver microsomal stability assays and human liver cytosol stability assays.
Discussion

Oxidative metabolites were the major metabolites of CP-724,714 observed in human hepatocytes, mainly mediated by CYPs and AO. A hydrolyzed metabolite and some sequential metabolites from the primary metabolites were also seen as minor fractions based on LC/HRMS analysis. In human hepatocytes, three mono-oxidative metabolites, Oxide 1, Oxide 2, and Oxide 3, were detected, out of which, Oxide 1 and Oxide 2 were generated via metabolism by CYPs, and Oxide 3 was formed by AO metabolism, as confirmed by \textit{in vitro} experiments. Based on the mass intensity, as shown in Figure 2 (b), the major oxidative metabolites, Oxide 1 and Oxide 3, showed almost the same mass detection intensity, implying that AO would be one of the major contributors to the metabolism of CP-724,714 in human hepatocytes; however, metabolic clearance of CP-724,714, with or without CYP or AO inhibitors, suggested that CYP would be a major contributor to the metabolism of CP-724,714 (Table 2). Therefore, we anticipated that the estimation of AO contribution based on inhibition assays using specific inhibitors in human hepatocytes was still difficult due to the low activity of AO in \textit{in vitro} human origin materials such as cryopreserved hepatocytes; however, this contribution of AO to the metabolism of drug candidates should be approximated to prevent undesired drug attrition due to AO involvement at the clinical stage. To estimate AO and CYP contributions to the metabolism of CP-724,714, the drug was incubated both in NADPH-fortified human liver microsomes and in human liver cytosol for estimation of CYP and AO involvement, respectively, and the resulting clearance values were utilized to determine the ratio of metabolism mediated by CYPs and AO. As AO activity can be low in human liver cytosol as well, the empirical SF calculated as 8.2 using typical AO substrates in human liver cytosol was used to extrapolate \textit{in vivo} AO-mediated clearance value even when using commercially available high AO/XO activity human liver
cytosol lot. In the case of standard AO substrates, as shown in Table 3, the AO contribution level to their metabolism was estimated as significant, despite considering human liver microsomal clearance values. Because CP-724,714 is metabolized by CYPs in human liver microsomes and has a structurally labile quinazoline moiety, the combinatorial use of data obtained from human liver microsomes and human liver cytosol stability assays would be promising. In the estimation of AO contribution based on human liver cytosol stability, calculations of the free fraction of AO substrates in human liver cytosol were problematic because of the interference of the residual AO activity affecting the remaining concentrations of the test compounds. The unbound fraction of CP-724,714 in human liver microsomes was predicted in accordance with a previous report \( f_{u_{\text{mic}}}^{\text{predicted}} \) as shown in Table 4, and that in human liver cytosol was used as 1 because it was difficult to determine the free fraction of CP-724,714 in human liver cytosol due to the remaining AO activity during the experiment. As a result, the contribution of AO to the metabolism of CP-724,714 was estimated to be 45%, and that of CYPs was calculated as 55%. These results give useful information for determining the fraction of AO metabolism at the preclinical stage employed by commonly conducted DMPK screening studies in cases where drug candidates show susceptibility to metabolism mediated not only by CYPs but also AO.

In *in vitro* human-origin constituents, such as human liver cytosol and hepatocytes, AO-mediated clearance values of typical AO substrates tend to be much lower than those observed *in vivo*, as summarized in Table 3. AO can be a problematic non-CYP enzyme, giving us unexpectedly high clearance at the clinical stage, causing undesired drug attrition (Kaye et al., 1984; Dittrich et al., 2002; Diamond et al., 2010; Akabane et al., 2011; Dalvie et al., 2013; Infante et al., 2013; Lolkema et al., 2015; Jensen et al., 2017). Setting an empirical scaling factor when extrapolating AO susceptibility to estimate *in vivo* liability more precisely at the drug
discovery stage can be necessary when human liver cytosolic or human hepatocyte clearance values are used (Zientek et al., 2010; Hutzler et al., 2012). Since ruling out the contribution of XO was important, using the XO inhibitor febuxostat, we confirmed that CP-724,714 was not a substrate of XO in human liver cytosol with high AO/XO activity (Choughule et al., 2014). In an attempt to escape from the CYP-mediated metabolism of drug candidates using a human liver microsomal stability assay as a DMPK screening portfolio, drug candidates can be optimized without CYP metabolism, but the propensity of structural features based on preferably introduced azaheterocyclic structures might cause susceptibility to non-CYP metabolism. Because AO is known to be the principal cause for some drug attritions, several in silico structural alert systems to indicate the presence of AO susceptibility based on the structures and physicochemical properties calculated for drug candidates have been developed to promote structure optimization at the early stage of drug discovery and development as possible (Beedham, 2020; Zhao et al., 2020; Soltani et al., 2021). Because *in silico* predictions are powerful tools to accelerate the drug optimization process, we must confirm the extent to which AO can contribute to the metabolism of drug candidates once AO liability is suggested based on the experimental procedures as quickly as possible for further optimization of the desired structure. Therefore, combinatorial assessment of data using human liver microsomal and human liver cytosol can be promising for estimating not only the response for CYP but also AO, as we demonstrated for CP-724,714.

Here, we conclude that CP-724,714 can act as an AO substrate based on its metabolite profiles in human hepatocytes and human recombinant AOX1 enzyme. Based on mass spectral data, the quinazoline moiety of CP-724,714 could be a metabolic spot for AO. In addition, as estimating plausible AO contribution to drug metabolism was difficult when drug candidates showed
susceptibility to both CYPs and AO, we demonstrated that combinatorial assessment of clearance values of CP-724,714 in human liver microsomes and human liver cytosol with an empirical SF setting for typical AO substrates could be a useful way to estimate the liability. Because human liver cytosol stability assay needs a relatively high empirical SF to estimate in vivo situation of the AO substrates investigated because of low AO activity in in vitro materials, it should be important to understand the contribution enzymes for the metabolism of drug candidates other than AO, more precisely, based on metabolite analysis. We recognize more data should be collected to overcome the limitation of this approach for optimizing the ion of drug candidates without the potential for AO susceptibility. Based on the high donor-to-donor variability of AO activity, we should consider the range of AO susceptibility of drug candidates to estimate a more precise clinical situation in the future to deliver effective drugs to patients. As precisely estimating the level of AO contribution to drug metabolism at the clinical stage is still challenging due to low AO activity in human constituents such as human hepatocytes and liver cytosol, we believe that by combining in silico predictions, we will be able to understand the metabolic propensity of drug candidates more deeply based on experimental data obtained at the early stage of drug development.

Authorship Contributions

Participated in research design: Inoue, K, Kikuchi, K, Takahashi, K, and Hitaoka, S.

Calculation of potential contribution ratio: Inoue, K, Kikuchi, K, and Hitaoka, S.

Wrote or contributed to the writing of the manuscript: Inoue K, Kikuchi K, Takahashi K, Hitaoka, S, Kusano, K, and Komori, T.
References


Metabolism: Investigated by Use of 14C-microdose, Humanized Mice, Monkey Pharmacokinetics, and In Vitro Methods. *Drug Metab Dispos* **45:**68-75.


Footnotes

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Conflict of Interest

No author has an actual or perceived conflict of interest with the contents of this article.
Figure Captions

**Figure 1.** Proposed metabolic pathway of CP-724,714 in human hepatocytes.

**Figure 2.** Extracted mass chromatograms of CP-724,714 at m/z 486 in human liver microsomes supplemented with NADPH, human hepatocytes, and recombinant human AOX1.

**Figure 3.** Product ion spectra of CP-724,714 and its oxidative metabolites
<table>
<thead>
<tr>
<th>RT&lt;sup&gt;a&lt;/sup&gt; [min]</th>
<th>Name</th>
<th>m/z&lt;sup&gt;b&lt;/sup&gt; (obs)</th>
<th>m/z&lt;sup&gt;c&lt;/sup&gt; (theo)</th>
<th>Error&lt;sup&gt;d&lt;/sup&gt; (ppm)</th>
<th>HLM&lt;sup&gt;e&lt;/sup&gt;</th>
<th>HH&lt;sup&gt;f&lt;/sup&gt;</th>
<th>HH&lt;sup&gt;+1-ABT&lt;/sup&gt;</th>
<th>HH&lt;sup&gt;+Hydralazine&lt;/sup&gt;</th>
<th>AOX1&lt;sup&gt;g&lt;/sup&gt;</th>
<th>% of ratio of interest</th>
</tr>
</thead>
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<tr>
<td>2.60</td>
<td>COOH derivative +O (1)</td>
<td>429.1554</td>
<td>429.1557</td>
<td>-0.7</td>
<td>-</td>
<td>1.8</td>
<td>-</td>
<td>1.7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.75</td>
<td>COOH derivative +O (2)</td>
<td>429.1556</td>
<td>429.1557</td>
<td>-0.2</td>
<td>-</td>
<td>2.9</td>
<td>-</td>
<td>1.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.90</td>
<td>Dioxidation-2H</td>
<td>500.1931</td>
<td>500.1928</td>
<td>0.6</td>
<td>0.6</td>
<td>11.2</td>
<td>0.01</td>
<td>10.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3.06</td>
<td>Desmethylation+O (1)</td>
<td>472.1981</td>
<td>472.1979</td>
<td>0.4</td>
<td>-</td>
<td>1.3</td>
<td>-</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3.19</td>
<td>Oxidative dealkylation +O</td>
<td>415.1767</td>
<td>415.1765</td>
<td>0.5</td>
<td>-</td>
<td>2.4</td>
<td>-</td>
<td>2.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3.24</td>
<td>COOH derivative of m/z 415</td>
<td>413.1607</td>
<td>413.1608</td>
<td>-0.2</td>
<td>-</td>
<td>10.8</td>
<td>-</td>
<td>11.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3.26</td>
<td>Desmethylation+O (2)</td>
<td>472.1982</td>
<td>472.1979</td>
<td>0.6</td>
<td>0.6</td>
<td>2.0</td>
<td>1.7</td>
<td>1.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3.35</td>
<td>Desmethylation+O-2H</td>
<td>470.1823</td>
<td>470.1823</td>
<td>0.0</td>
<td>0.0</td>
<td>1.4</td>
<td>0.02</td>
<td>1.4</td>
<td>-</td>
<td>-</td>
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<tr>
<td>3.36</td>
<td>Mono-oxidation (CYP)</td>
<td>486.2136</td>
<td>486.2136</td>
<td>0.0</td>
<td>-</td>
<td>23.1</td>
<td>11.7</td>
<td>11.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3.41</td>
<td>Dealkylation</td>
<td>398.1975</td>
<td>398.1975</td>
<td>0.0</td>
<td>-</td>
<td>0.3</td>
<td>0.3</td>
<td>0.4</td>
<td>-</td>
<td>-</td>
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<tr>
<td>3.52</td>
<td>Mono-oxidation (CYP)</td>
<td>486.2138</td>
<td>486.2136</td>
<td>0.4</td>
<td>0.4</td>
<td>24.1</td>
<td>1.1</td>
<td>-</td>
<td>1.1</td>
<td>-</td>
</tr>
<tr>
<td>3.59</td>
<td>Mono-oxidation (AO)</td>
<td>486.2132</td>
<td>486.2136</td>
<td>-0.8</td>
<td>-</td>
<td>9.3</td>
<td>49.5</td>
<td>3.8</td>
<td>7.6</td>
<td>-</td>
</tr>
<tr>
<td>3.79</td>
<td>Desmethylation</td>
<td>456.2028</td>
<td>456.2030</td>
<td>-0.4</td>
<td>0.4</td>
<td>5.3</td>
<td>4.3</td>
<td>0.2</td>
<td>4.2</td>
<td>-</td>
</tr>
<tr>
<td>4.14</td>
<td>CP-724,714</td>
<td>470.2186</td>
<td>470.2187</td>
<td>-0.2</td>
<td>47.5&lt;sup&gt;h&lt;/sup&gt;</td>
<td>39.5&lt;sup&gt;h&lt;/sup&gt;</td>
<td>48.4&lt;sup&gt;h&lt;/sup&gt;</td>
<td>48.1&lt;sup&gt;h&lt;/sup&gt;</td>
<td>92.4&lt;sup&gt;h&lt;/sup&gt;</td>
<td>2.0&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> represents retention time (min)

<sup>b</sup> represents observed molecular ion

<sup>c</sup> represents theoretical molecular ion calculated from the predicted formula

<sup>d</sup> calculated as \((\text{observed } m/z - \text{theoretical } m/z) \times 10^6 / \text{theoretical } m/z\)

<sup>e</sup> represents human liver microsomes

<sup>f</sup> represents human hepatocytes

<sup>g</sup> represents recombinant human aldehyde oxidase 1

<sup>h</sup> represents percentage of remaining CP-724,714.
Table 2 Effect of inhibitors on CYPs and AO in human hepatocytes and possible fractions of metabolism incubated at 0.1, 1, and 10 μM of CP-724,714

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>0.1 μM</th>
<th>1 μM</th>
<th>10 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP-724,714</td>
<td>CL_int,hep (mL/min/million cells)</td>
<td>1-ABT</td>
<td>hydralazine</td>
</tr>
<tr>
<td>(-)</td>
<td>0.052</td>
<td>0.0051</td>
<td>0.0446</td>
</tr>
<tr>
<td>1-ABT hydralazine</td>
<td>2</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Remaining activity(^a)</td>
<td>1.00</td>
<td>0.10</td>
<td>0.85</td>
</tr>
</tbody>
</table>

\(^a\) The remaining activity was calculated as follows: slope (no inhibitor) - slope (with CYPs or AO inhibitor) / slope (no inhibitor).
Table 3 Metabolic clearance of representative AO substrates in human liver microsomes and human liver cytosol and estimated contribution level of AO in their metabolism

<table>
<thead>
<tr>
<th>Compound</th>
<th>fm, AO&lt;sup&gt;a&lt;/sup&gt; (mL/min/kg)</th>
<th>CL&lt;sub&gt;int,in vivo&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt; (predicted) (μL/min/mg)</th>
<th>CL&lt;sub&gt;int,u,HLC&lt;/sub&gt; (μL/min/mg)</th>
<th>CL&lt;sub&gt;int,u,HLM&lt;/sub&gt; (μL/min/mg)</th>
<th>CL&lt;sub&gt;AO&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt; (mL/min/kg)</th>
<th>CL&lt;sub&gt;CYP&lt;/sub&gt; (mL/min/kg)</th>
<th>Estimated contribution of AO (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O6-benzylguanine</td>
<td>100</td>
<td>360</td>
<td>0.929</td>
<td>14.3</td>
<td>20.8</td>
<td>236.2</td>
<td>20.8</td>
</tr>
<tr>
<td>DACA</td>
<td>100</td>
<td>3600</td>
<td>0.854</td>
<td>83.3</td>
<td>118.3</td>
<td>1378.6</td>
<td>118.3</td>
</tr>
<tr>
<td>zonisporide</td>
<td>79</td>
<td>180</td>
<td>0.951</td>
<td>16.8</td>
<td>3.1</td>
<td>277.8</td>
<td>3.1</td>
</tr>
<tr>
<td>zaleplon</td>
<td>70</td>
<td>65</td>
<td>0.935</td>
<td>4.2</td>
<td>7.2</td>
<td>68.7</td>
<td>7.2</td>
</tr>
<tr>
<td>6-deoxyxenciclovir</td>
<td>N.D.</td>
<td>140</td>
<td>0.960</td>
<td>20.0</td>
<td>7.0</td>
<td>331.7</td>
<td>7.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Hutzler et al. (2013)

<sup>b</sup> Zientek et al. (2010)

<sup>c</sup> CL<sub>AO</sub> value was calculated based on CL<sub>int,u,HLC</sub> multiplied by the empirical scaling factor set as 8.2. fu<sub>cytosol</sub> was used as 1.

N.D. represents not determined.
Table 4 Predicted contribution of AO on the metabolism of CP-724,714

<table>
<thead>
<tr>
<th>Compound name</th>
<th>fu_{mic, predicted}</th>
<th>HLM (μL/min/mg)</th>
<th>HLC (μL/min/mg)</th>
<th>CL_{CYP} (mL/min/kg)</th>
<th>CL_{AO} (mL/min/kg)</th>
<th>Impact AO (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP-724,714</td>
<td>0.782</td>
<td>380</td>
<td>19.1</td>
<td>380</td>
<td>316</td>
<td>45</td>
</tr>
<tr>
<td>midazolam</td>
<td>0.871</td>
<td>239</td>
<td>0</td>
<td>868</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

HLM and HLC represent human liver microsomes and human liver cytosol, respectively.

CL_{int,u,in vivo} represents CL_{CYP} and CL_{AO} calculated from metabolic clearance in human liver microsomes and human liver cytosol multiplied with SF, respectively.

- CL_{int,u,obs} represents CL_{int} value of HLM and HLC divided by fu values (fu_{mic,predicted} for HLM and 1 for HLC, respectively).
- CL_{CYP} (mL/min/kg) calculated from CL_{int,u,HLM} × 40 (mg/g liver) × 25 (g liver/kg), and that of human liver cytosol represents CL_{AO} calculated from CL_{int,u,HLC} × 8.2 (SF) × 80.7 (mg/g liver) × 25 (g liver/kg).
Figure 1

Chemical structures and mass-to-charge ratios (m/z) for various metabolites and N-demethylation products of CP-724,714. CYP and AO represent cytochrome P450 and aldehyde oxidase, respectively. m/z 470 is the parent compound, and m/z 472, 486, 486 (2 isomers), 429, 500, and 415 correspond to different metabolites and oxidative products.
Figure 2

(a) Human liver microsomes with NADPH

(b) Human hepatocytes

(c) Human hepatocytes + 1-ABT

(d) Human hepatocytes + Hydralazine

(e) Human AOX1
Figure 3

(a) CP-724714 (MS$^2$ at m/z 470)

(b) Oxide 1 (MS$^2$ at m/z 486)

(c) Oxide 2 (MS$^2$ at m/z 486)

(d) Oxide 3 (MS$^2$ at m/z 486)

(e) Oxide 3 (MS$^3$ at m/z 397)