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Mass Balance and Absorption, Distribution, Metabolism, and Excretion Properties of Balcinrenone following Oral Administration in Combination with Intravenous Microtracer in Healthy Subjects[S]

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ABSTRACT

An absorption, distribution, metabolism, and excretion study was performed to determine the basic pharmacokinetic parameters, mass balance, and metabolite profiles of balcinrenone, a mineralocorticoid receptor modulator, in humans. This open-label, single-center, nonrandomized study had a two-period design. In period 1, eight healthy male subjects were dosed with a microtracer intravenous infusion of [^{14}C]balcinrenone shortly after receiving an oral dose of unlabeled balcinrenone in a capsule. Following a 7-day washout, the same group of subjects subsequently received an oral dose of [^{14}C]balcinrenone as a suspension in period 2. Clearance and absolute bioavailability of balcinrenone were determined to be 14.2 l/h and 52%, respectively. Renal clearance was determined to be 5.4 l/h (>fu • glomerular filtration rate), indicating elimination via active tubular secretion, which was potentially mediated by P-glycoprotein 1 and/or organic anion transporter 3, according to in vitro transporter data. In total, 94.1% of the oral dose was recovered: 45.2% in the urine and 48.9% in the feces. Balcinrenone was primarily metabolized via oxidation, and in vitro data suggest that cytochrome P450 3A4 was the main enzyme responsible. Intact [^{14}C]balcinrenone accounted for 55% of drug-related material in the plasma; four metabolites were identified, each representing <6% of the total plasma radioactivity. In conclusion, this two-period study has determined the basic pharmacokinetic parameters of balcinrenone in humans, including absolute bioavailability and disposition. No metabolites warranted further evaluation on account of their low representation, and any contribution to the pharmacodynamic response or potential drug-drug interactions was deemed negligible.

SIGNIFICANCE STATEMENT

This study provides a detailed understanding of the pharmacokinetics, disposition, and metabolism of balcinrenone following oral and microtracer intravenous administration in humans. In vitro phenotyping and transporter data granted mechanistic insights into the absorption, distribution, metabolism, and excretion properties of balcinrenone. This knowledge will guide future nonclinical and clinical studies evaluating drug-drug interactions, organ dysfunction, and safety of metabolites.

Introduction

Balcinrenone is a nonsteroidal, selective mineralocorticoid receptor (MR) modulator under development in combination with the sodium-glucose cotransporter-2 inhibitor dapagliflozin for the treatment of heart failure and comorbid chronic kidney disease. Dapagliflozin is a marketed product that has demonstrated renal and/or cardiovascular benefits in patients with type 2 diabetes mellitus (Neuen et al., 2019; Wiviott et al., 2019), heart failure (Kosiborod et al., 2017; McMurray et al., 2019), and chronic kidney disease (Heerspink et al., 2020), whereas balcinrenone is a new chemical entity. The marketed MR antagonists eplerenone and spironolactone have been shown to reduce mortality and hospitalization in patients with congestive heart failure (Pitt et al., 1999; Pitt et al., 2003; Zannad et al., 2011); however, their use is limited by the compound class-inherent risk of hyperkalemia in patients with declining kidney function (Juurlink et al., 2004; Lazich and Bakris, 2014).

Unlike other MR antagonists, balcinrenone is only a partial antagonist of MR due to its unique interaction with the receptor (Bamberg et al., 2018; Granberg et al., 2019). In preclinical studies, it has been

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demonstrated that the differentiated mode of action of balcinrenone delivers organ-protective effects that can be separated from acute effects on urinary electrolyte levels (Bamberg et al., 2018). This suggests that balcinrenone has the potential to mitigate the risk of hyperkalemia in patients who are at high risk, such as those receiving treatment of heart failure and comorbid chronic kidney disease.

In phase I studies in healthy male subjects, single doses up to 1200 mg and multiple ascending doses up to 300 mg twice daily of balcinrenone in suspension have been shown to be well tolerated, with no safety concerns (Erlandsson et al., 2018; Whittaker et al., 2020). In the multiple ascending doses study, balcinrenone was rapidly absorbed, with C_{max} reached in a median time (T_{max}) of <1 hour and a terminal half-life of 4–10 hours; exposure [area under the curve (AUC) and C_{max}] increased in a dose-proportional manner up to doses of 200 mg, and target engagement was confirmed by a robust dose-dependent rise in mean serum aldosterone levels while serum electrolyte levels remained stable (Whittaker et al., 2020). These observations in humans are therefore consistent with data from preclinical studies and highlight the therapeutic potential of MR blockade with balcinrenone, coupled with a low risk of hyperkalemia.

Human absorption, distribution, metabolism, and excretion (ADME) studies are central among the clinical pharmacology studies performed during drug development for a new chemical entity. They provide quantitative information about absorption, routes and rates of elimination, and circulating as well as excreted metabolites. Results from human ADME studies can provide valuable insights and help to guide further studies if these are warranted, such as studies in organ-impaired subjects, drug-drug interaction studies, and preclinical safety studies (Coppola et al., 2019). Even though balcinrenone is not being developed as a monotherapy, a separate human ADME study is still required according to the European Medicines Agency's "guideline on clinical development of fixed combination medicinal products" (www.ema.europa.eu/en/documents/scientific-guideline/guideline-clinical-development-fixed-combination-medicinal-products-revision-2_en.pdf).

In this study, the mass balance, pharmacokinetic (PK) parameters, and metabolic fate of balcinrenone were evaluated in healthy male subjects. Basic PK parameters, including absolute bioavailability, were evaluated following an intravenous microtracer of [^{14}C]balcinrenone administered shortly after an oral dose of unlabeled balcinrenone. In vitro phenotyping data were generated to quantify the routes of balcinrenone elimination mediated by individual cytochrome P450 (P450) enzymes, and in vitro transporter data were generated to help obtain a mechanistic understanding of the renal elimination process.

Materials and Methods

[^{14}C]Balcinrenone was manufactured by Eurofins Selcia (Essex, UK) on behalf of AstraZeneca. Capsules containing pellets of balcinrenone were manufactured by AstraZeneca (Gothenburg, Sweden). Quotient Sciences (Nottingham, UK) formulated the [^{14}C]balcinrenone microtracer intravenous solution and the oral suspension of [^{14}C]balcinrenone. Synthetic standards of six balcinrenone metabolites (M2 and M5–9) were supplied by AstraZeneca (Gothenburg, Sweden).

Ultima Gold LSC-cocktail was obtained from PerkinElmer (Waltham, MA). Specific chemicals used for analysis by accelerator mass spectrometry (AMS), conducted by TNO (Metabolic Health Research, Leiden, Netherlands), included ANU sucrose-8542 with a certified $^{14}C/^{12}C$ ratio purchased from the National Institute of Standards and Technology (Gaithersburg, MD), and acetanilide and paracetamol purchased from Sigma-Aldrich (Saint Louis, MO). All other solvents and reagents were of analytical or higher grade and acquired from commercial suppliers.

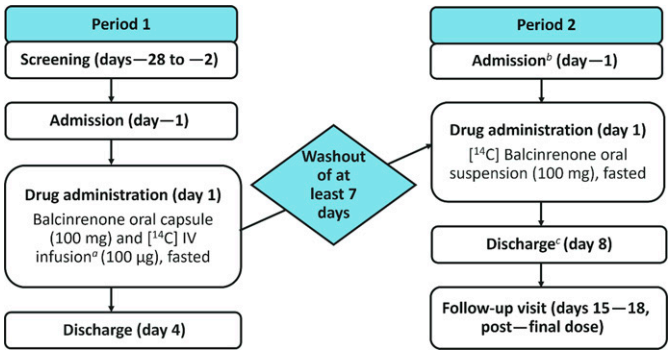

Fig. 1. Schematic of study design. Fifteen-minute intravenous infusion of [^{14}C]balcinrenone solution was given 2 hours and 15 minutes after the oral dose in a capsule. Subjects were asked to collect a fecal sample at home on the day of admission to period 2 (if a subject did not collect a sample at home, they provided a sample between admission and predose). It was planned that subjects would be released as a group when all subjects had achieved a mass balance cumulative recovery of >90% or if <1% of the dose administered had been collected in urine and feces within two separate, consecutive 24-hour periods (if mass balance criteria were not met by day 8, then extended residency/home collections were to be arranged).

Clinical Study Design

This was an open-label, single-center, 2-period, nonrandomized study of healthy subjects conducted at Quotient Sciences between December 21, 2020, and February 4, 2021 (NCT04686591) (Fig. 1). The study adhered to the Declaration of Helsinki and was approved by the London-Surrey Borders Research Ethics Committee. Each subject provided written informed consent prior to commencing any of the study procedures.

In period 1, the absolute bioavailability of a single oral dose of balcinrenone and the PK parameters of [^{14}C]balcinrenone after intravenous infusion were assessed. Following an overnight fast (minimum 10 hours), subjects received a single oral dose of 100 mg balcinrenone in a capsule; 2 hours and 15 minutes later, subjects then received 100 µg [^{14}C]balcinrenone, containing 30.7 kBq (0.8 µCi) [^{14}C]balcinrenone as a continuous 15-minute intravenous infusion. The end of the infusion coincided with the expected oral T_{max} of balcinrenone. Subjects remained resident in the clinical unit until up to 72 hours after the oral dose (up to day 4). In period 2, the ADME properties of oral [^{14}C]balcinrenone were assessed. Following an overnight fast (minimum 10 hours), subjects received a single oral dose of 100 mg [^{14}C]balcinrenone, containing 8 MBq [^{14}C] (216 µCi) as an oral suspension. Subjects remained resident in the clinical unit up to 168 hours postdose (up to day 8) and were discharged as a group after meeting all discharge criteria. The same subjects took part in both study periods. There was a minimum washout period of 7 days between dosing in period 1 and period 2.

Rationale for Dose

A single oral dose of 100 mg was evaluated in the current study, which is within the dose range evaluated in early clinical development and ongoing studies (Erlandsson et al., 2018; Whittaker et al., 2020). Dose levels up to 1200 mg were evaluated during the early clinical studies of balcinrenone, with no tolerability or safety concerns noted and dose-linear pharmacokinetics observed up to 200 mg (Erlandsson et al., 2018; Whittaker et al., 2020). To achieve the primary objectives of the study, the oral radioactive dose of [^{14}C]balcinrenone was

ABBREVIATIONS: ADME, absorption, distribution, metabolism, and excretion; AMS, accelerator mass spectrometry; AUC, area under the curve; BCRP, breast cancer resistance protein; CL_R, renal clearance; F, bioavailability; f_{abs}, fraction absorbed; HRMS, high-resolution mass spectrometry; ICH, International Council for Harmonization; LC, liquid chromatography; LSC, liquid scintillation counting; MATE, multidrug and toxin extrusion; MR, mineralocorticoid receptor; MS, mass spectrometry; NCE, new chemical entity; OAT, organic anion transporter; OCT2, organic cation transporter 2; P450, cytochrome P450; Papp, apparent permeability; P-gp, P-glycoprotein 1; PK, pharmacokinetic; RAD, radioactivity detection; SLC, renal solute carrier; T_{max}, time of C_{max}; UPLC, ultraperformance liquid chromatography.

proposed to be as low as possible but still sufficient enough to allow an adequate assessment of its metabolite profile by radioactivity detection (RAD). The intravenous dose of 100 μg [^{14}C]balcinrenone is in line with the International Council for Harmonization (ICH) M3 definition of a microdose (a dose that is ≤1/100th of the pharmacologically active dose, up to a maximum dose of 100 μg) (www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-m3r2-non-clinical-safety-studies-conduct-human-clinical-trials-marketing-authorisation_en.pdf). Estimates of the expected total radiation exposure following intravenous and oral doses of [^{14}C]balcinrenone resulted in a committed effective dose equivalent of 1.55 mSv, which fell within International Commission on Radiologic Protection risk category IIb (1–10 mSv) for a radioactive dose (ICRP, 1991).

Study Population

Healthy men aged 30–60 years who were nonsmokers and had a body mass index of 18–30 kg/m^2 (body weight, ≥50 kg) were included. Only male subjects were eligible to participate in this study as full reproduction toxicology data in female animals were not available.

Collection of Blood Samples and Excreta

Blood and urine samples (periods 1 and 2) and fecal samples (period 2 only) were collected at regular intervals for PK, safety, and mass balance analysis from day −1 to the follow-up visit. In period 1, blood samples were collected pre–oral dose and 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 8, 12, 24, 30, 36, 48, 60, and 72 hours post–oral dose for analysis of balcinrenone concentrations in plasma. Blood samples were also collected at 2 (predose for intravenous infusion), 2.25, 2.37, 2.5, 2.58, 2.67, 2.83, 3, 3.5, 4, 6, 8, 12, 16, 24, 30, 36, 48, 60, and 72 hours post–oral dose for the analysis of [^{14}C]balcinrenone and total radioactivity in plasma. Urine samples were obtained pre–oral dose and 0–6, 6–12, 12–24, 24–48, and 48–72 hours post–oral dose for the analysis of balcinrenone and [^{14}C]balcinrenone. Samples for safety assessment (hematology and clinical chemistry) were collected at screening, pre–oral dose, and 24 and 72 hours (at time of discharge) post–oral dose. In period 2, whole blood samples were collected predose and 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 8, 12, 24, 36, 48, 60, 72, 96, 120, 144, and 168 hours postdose for analyses of balcinrenone in plasma, total radioactivity in plasma and whole blood, and metabolite profiling and characterization in plasma. Urine samples were collected at admission, predose, and 0–6, 6–12, 12–24, 24–48, 48–72, 72–96, 96–120, 120–144, and 144–168 hours postdose for analyses of balcinrenone, total radioactivity, and for metabolite profiling and characterization. Fecal samples were collected predose (between 24 hours prior to dosing and predose) and 0–24, 24–48, 48–72, 72–96, 96–120, 120–144, and 144–168 hours postdose. Fecal homogenates were analyzed for total radioactivity and metabolite profiling and characterization. Samples for safety assessment were collected predose and 24 and 168 hours postdose.

Bioanalysis of Unlabeled Balcinrenone, [^{14}C]Balcinrenone, and Total Radioactivity

Analysis of Unlabeled Balcinrenone by High-Performance Liquid Chromatography with Tandem Mass Spectrometry. Concentrations of unlabeled balcinrenone in plasma and urine were determined by Covance Laboratories Limited (Harrogate, UK) using high-performance liquid chromatography with tandem mass spectrometry as previously described (Whittaker et al., 2020).

Analysis of [^{14}C]balcinrenone and total ^{14}C by AMS. Following microtracer intravenous administration of [^{14}C]balcinrenone, total ^{14}C radioactivity in plasma was analyzed by TNO (Leiden, Netherlands) using AMS. The concentrations of [^{14}C]balcinrenone in plasma and urine were also determined by AMS after sample fractionation using high-performance liquid chromatography. In brief, plasma samples were added into tin foil cups, dried, and combusted using an elemental analyzer (vario MICRO; Elementar, Langenselbold, Germany), and total ^{14}C was analyzed using 1 MV multielement AMS (model 4110 Bo, High Voltage Engineering, Amersfoort, Netherlands). For the determination of [^{14}C]balcinrenone, plasma samples were extracted using protein precipitation and the supernatant separated and concentrated under a gentle nitrogen stream. Plasma extracts and urine samples were then injected into an ultraperformance liquid chromatography (UPLC) column, and the liquid chromatography (LC) elute of [^{14}C]balcinrenone was fractionally collected in foil cups for AMS analysis. The LC separation of [^{14}C]balcinrenone from the matrices was achieved using an Acquity UPLC HSS C18 SB column (1.8 μm, 2.1 mm × 150 mm; Waters Corp., Milford, Massachusetts) at 45°C. Mobile phases A and B were 0.1% formic acid in water and acetonitrile, respectively. The flow rate was 0.4 ml/min, with a linear gradient from 15% B to 40% B in 17.5 minutes, followed by a ramp to 90% B in 1.4 minutes and hold-up for 4 minutes before decreasing back to 15% B at 23 minutes. A photodiode-array detector was used to monitor the balcinrenone peak. The fraction of [^{14}C]balcinrenone eluate was collected at the retention time between 10.1 and 10.5 minutes. AMS-specific instrument setup, calibration, and data processing for ^{14}C determination were performed as previously described (Bauman et al., 2022) and were applied to the quantification of total ^{14}C in plasma and [^{14}C]balcinrenone fraction in plasma and urine.

Analysis of Total Radioactivity by Liquid Scintillation Counting. Following oral administration of [^{14}C[balcinrenone in Period 2, total ^{14}C radioactivity in plasma, whole blood, urine, and feces was analyzed by Pharmaron (Northamptonshire, UK) using liquid scintillation counting (LSC). Scintillation cocktail was added directly to plasma and urine samples, whereas samples of whole blood were solubilized and then decolored before adding scintillation fluid. Fecal samples were homogenized, and subsamples of each homogenate were dried and combusted before the addition of scintillation cocktail and analysis by LSC. A detailed summary of the procedure used for detection of radioactivity is included in Supplemental Methods. Samples were analyzed in duplicate on a PerkinElmer Tri-Carb 3100 scintillation counter (PerkinElmer Inc., Waltham, MA), with automatic external standard quench correction to determine total ^{14}C radioactivity. The limit of quantification using LSC was taken as twice the background dpm value for samples of the same type.

Metabolite Profiling in Plasma, Urine, and Feces

Metabolite identification and profiling (period 2 only) were conducted at AstraZeneca Gothenburg, Sweden, using LC combined with RAD for quantification and high-resolution mass spectrometry (HRMS) for structural elucidation.

Sample Preparation. Pooled plasma, urine, and fecal homogenates across seven subjects were prepared for metabolite profiling and characterization. Plasma samples were first pooled across subjects at each timepoint between 0 and 24 hours by equivolumetric mixing. Subsequently, one single pool was prepared with the volumes proportional to the sampling timespan to give one $AUC_{0\text{-}24h}$ pool as previously described (Hamilton et al., 1981). For urine and feces, individual samples were pooled across subjects and collection intervals in proportion to the total volume or weight of the excreta collected. The obtained pools of $urine_{0\text{-}24h}$ and $feces_{0\text{-}120h}$ were profiled, which accounted for 95% and 96% of the total excreted radioactivity in urine and feces, respectively, over the entire collection period (0–168 hours).

Plasma. To 1 ml pooled human plasma, 3 ml methanol/acetonitrile (1:1, v/v) was added. The sample was vortex mixed for 2 minutes and centrifuged at 10,000*g*, 4°C, for 10 minutes. The supernatant was transferred to a new tube and concentrated to approximately 100 μl under a gentle nitrogen flow at room temperature. An aliquot of 100 μl of 40% acetonitrile aqueous solution was added to the residue, vortex mixed for 1 minute, and then centrifuged at 10,000*g*, 4°C, for 10 minutes. The supernatants were analyzed by LSC to determine the extraction efficiency. An aliquot sample extract was injected onto the LC column for LC-HRMS analysis and off-line radioactivity measurements.

Urine. To 200 μl pooled human urine, 50 μl methanol/acetonitrile (1:1, v/v) was added. The sample was vortex mixed and centrifuged at 10,000*g*, 4°C, for 10 minutes. The supernatant was analyzed by LSC to determine the extraction recovery, and LC-HRMS and off-line radioactivity detection for metabolite identification and profiling.

Feces. Approximately 0.3 g pooled fecal homogenates were weighed and transferred into Precellys 2-ml reinforced tubes (Bertin Corp., MD) preloaded with six 3-mm diameter ceramic balls in each tube. An extraction mixture of methanol/acetonitrile (1:1, v/v) was added to sample tubes at a ratio of 1:4 of fecal homogenate weight to organic solvent volume. Samples were homogenized and extracted using a Precellys 24 homogenizer (Bertin Corp., MD), 2 × 20 seconds, 5000 rpm, with a 20-second pause between intervals. The mixtures were then centrifuged at 10,000*g*, 4°C, for 10 minutes. An aliquot of 1.5 ml of the supernatant was transferred to a new sample tube and concentrated to near dryness under a gentle nitrogen flow. The sample residue was reconstituted in 200 μl of 40% acetonitrile aqueous solution, vortexed for 1 minute, and then centrifuged at 10,000*g*, 4°C, for 10 minutes. The supernatant was analyzed by LSC and LC-HRMS analysis and off-line radioactivity measurements.

Sample Analysis by LC-HRMS-RAD

For metabolite profiling of [^{14}C]balcinrenone, plasma, urine, and fecal sample extracts were analyzed using a Waters Acquity UPLC system coupled with a Synapt G2-Si Q-TOF mass spectrometer (Waters, UK) and off-line radioactivity measurements. The software for instrument control and data acquisition was MassLynx (version 4.1).

LC System. Reversed-phase chromatography for separation of balcinrenone and its metabolites was performed using an Acquity UPLC HSS C18 SB column (100Å, 1.8 μm, 3 mm × 150 mm; Waters, Milford, MA) at 45°C with mobile phase A: 0.1% formic acid in water and mobile phase B: acetonitrile at a flow rate of 0.5 ml/min. A stepwise gradient was used from 14% to 90% mobile phase B over 54 minutes (14%–18% B from 0 to 35 minutes, 18%–45% B from 35 to 44 minutes, 45%–90% B from 44 to 49 minutes, held at 90% B for 5 minutes, then back to 14% B in 0.1 minute). The system was equilibrated at 14% B for 5 minutes before the injection of the next sample. The LC eluent was split, with approximately 0.4 ml/min transferred for fraction collection and approximately 0.1 ml/min directed for mass spectrometry (MS) detection. The recovery of total radioactivity from the LC column during a gradient run was determined in urine, feces, and plasma samples by comparison of the radioactivity in the eluent pre- and postcolumn following sample injections.

RAD. Plasma, urine, and feces homogenate extracts were injected in triplicate using the UPLC system described above. LC fractions were collected throughout the chromatographic run (every 7.2 seconds) into 96-well plates (LumaPlate; PerkinElmer, Waltham, MA). After drying the collected samples using an EZ2 evaporator vacuum centrifuge (Genevac, UK), the radioactivity (cpm) was determined using a microplate scintillation counter (TopCount NXT; PerkinElmer). The counting results were reconstructed into LC radio-chromatograms using Laura software (version 6, LabLogic, UK) for radioactivity peak integration after background subtraction. The limit of quantification of metabolites in the radiochromatogram was set to a minimum peak area of 7 cpm in plasma and 10 cpm in urine and feces samples. These data and the percent of the recovered dose in the excreta were used to calculate the abundance of balcinrenone and metabolites and expressed as percent of administered dose for the pooled collection intervals in excreta. The abundance of balcinrenone and metabolites in plasma was reported as percent of $AUC_{0\text{-}24h}$.

HRMS Analysis. The eluent from the UPLC column was introduced into a Synapt G2-Si Q-TOF mass spectrometer with an electrospray interface. Data were acquired using TOF MS^E, i.e., two parallel functions where the first, low energy function, generated precursor ion spectra, and the second, high energy function, generated product ion spectra. Mass spectra were acquired in positive and negative electrospray interface modes separately. Product ion MS/MS spectra were acquired on selected metabolites to produce fragment ions, used for structural elucidation. Detailed HRMS instrument settings for metabolite identification are described in Supplemental Methods. The MassLynx software (version 4.1) was used for instrument control and data acquisition. MassLynx and Metabolynx were used for data analysis and processing for metabolite identification.

Metabolite Characterization. Precursor ions ($[M+H]^+$ or $[M-H]^-$) of balcinrenone and metabolites in LC-HRMS chromatograms were identified at retention times that agreed with RAD peaks in the corresponding radio-chromatograms. Observed HRMS full-scan precursor ions were used to propose molecular compositions, and product ion spectra (MS/MS) were used to propose tentative metabolite structures. Some of the metabolites were available as synthesized standards, which were analyzed for unambiguous metabolite identification.

Determination of Blood-Plasma Partitioning

The extent of distribution of total ^{14}C radioactivity into blood cells was evaluated by assessing the total radioactivity in whole blood to plasma ratio in period 2 at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 8, 12, and 24 hours post–100 mg [^{14}C]balcinrenone dose.

PK Analysis

The PK parameters for balcinrenone, [^{14}C]balcinrenone, and total radioactivity were estimated by noncompartmental analysis methods using Phoenix WinNonlin software (v8.0, Certara USA, Inc.). Data are summarized using descriptive statistics.

In Vitro Human Plasma Protein Binding

Equilibrium dialysis was used to assess the fraction unbound in human plasma as previously described (Wernevik et al., 2020). Details of experimental procedures are provided in Supplemental Methods.

In Vitro P450 Phenotyping

The experimental procedures for in vitro P450 phenotyping were performed by incubation of balcinrenone in human recombinant cytochrome P450s and in human hepatocytes in the absence and presence of the potent and selective CYP3A4/5 inhibitor ketoconazole as previously described (Lindmark et al., 2018). Details of experimental procedures are provided in Supplemental Methods.

In Vitro Drug Transporter Studies

The potential of balcinrenone to be a substrate of human efflux transporters P-glycoprotein 1 (P-gp) and breast cancer resistance protein (BCRP) was assessed in polarized Madin-Darby canine kidney (MDCK) cells transfected with multidrug resistance 1 gene (MDR1) and in polarized Caco-2 cell monolayers, respectively. Details of experimental procedures are provided in Supplemental Methods. Efflux ratios were determined for balcinrenone in the absence and presence of reference inhibitors for P-gp and BCRP (Supplemental Methods).

An efflux ratio >2, which is reduced toward unity by at least 50% in the presence of a reference inhibitor [with an accompanying decrease in B–A apparent permeability (P_{app})], indicates whether the test compound is a substrate of the transporter being investigated (https://www.fda.gov/regulatory-information/search-fda-guidance-documents/in-vitro-drug-interaction-studies-cytochrome-p450-enzyme-and-transporter-mediated-drug-interactions).

The potential for balcinrenone to be a substrate of renal solute carrier (SLC) transporters, organic anion transporters (OATs) (OAT1, OAT2, OAT3), organic cation transporter 2 (OCT2), and multidrug and toxin extrusion (MATE) transporters (MATE1 and MATE2-K) was evaluated in human embryonic kidney 293 (HEK293) cells transiently transfected with the drug transporter of interest. Details of experimental procedures are provided in Supplemental Methods. Uptake ratios were determined in the absence and presence of reference inhibitors of the respective transporter. An uptake ratio >2, which is reduced toward unity by at least 50% in the presence of a reference inhibitor, indicates whether the test compound is a substrate of the transporter being investigated at the given conditions (https://www.fda.gov/regulatory-information/search-fda-guidance-documents/in-vitro-drug-interaction-studies-cytochrome-p450-enzyme-and-transporter-mediated-drug-interactions).

Results

Study Population

Eight healthy male subjects were enrolled, with a mean age of 41.6 years and a mean body mass index of 25.5 kg/m^2. All eight subjects were White (Supplemental Table 1). Eight subjects received treatment in period 1, and seven subjects received treatment in period 2 and completed the study. One subject withdrew from the study prior to period 2 due to personal reasons.

Mass Balance and Excretion

Following a single oral dose of [^{14}C]balcinrenone, 94.1% of the radioactivity administered was recovered by the end of the sampling period (168 hours); 45.2% was recovered from the urine, and 48.9% was recovered from the feces. Within the first 24 hours postdose, 42.7% and 4.65% of the total radioactivity was recovered in the urine and feces, respectively (Fig. 2; Supplemental Table 2).

PK Results

Table 1 summarizes the PK parameters following administration of a single oral 100-mg dose of balcinrenone in a capsule, followed by a 100-μg [^{14}C]balcinrenone intravenous infusion of 15 minutes in period 1 and a 100-mg [^{14}C]balcinrenone oral suspension in period 2.

In period 1, the maximum concentration of [^{14}C]balcinrenone was reached at the end of the intravenous microtracer infusion as anticipated.

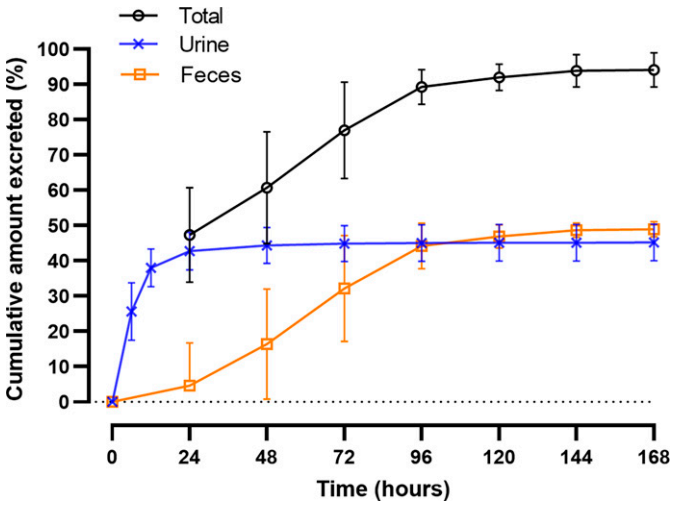

Fig. 2. Arithmetic mean (standard deviation) of cumulative excretion of total radioactivity following a single oral dose of [^{14}C]balcinrenone (n = 7).

After infusion was stopped, concentrations showed a rapid distribution phase followed by an elimination phase with a geometric mean half-life of 4.2 hours. Geometric mean clearance, volume of distribution at steady state, and apparent volume of distribution of [^{14}C]balcinrenone were determined to be 14.2 l/h, 37.8 l, and 86.2 l, respectively. The geometric mean renal clearance (CL_R) of [^{14}C]balcinrenone was estimated to be 5.6 l/h, which corresponds to approximately 40% of the total clearance. The geometric mean absolute bioavailability of the balcinrenone capsule was determined to be 52%. Exposure of total ^{14}C radioactivity was approximately twofold higher compared with exposure of [^{14}C]balcinrenone, with geometric mean $AUC_{(0\text{-}inf)}$ values of 36 and 17.7 nmol·h/l, respectively.

PK profiles of balcinrenone in plasma and total ^{14}C radioactivity in plasma and whole blood following oral administration of [^{14}C]balcinrenone in suspension (period 2) are displayed in Fig. 3. Maximum plasma concentrations of balcinrenone in period 2 were reached 0.5–1 hours postdose, suggesting rapid absorption of balcinrenone when formulated in a suspension. Rate of absorption was somewhat slower with balcinrenone formulated in a capsule (Period 1), with maximum plasma concentrations reached 0.75–2.5 hours postdose. The overall plasma exposures (C_{max}, $AUC_{0\text{-}inf}$) of balcinrenone given either as an oral suspension (geometric mean of 3210 nmol/l, 9060 nmol*h/l) or capsule (geometric mean of 2160 nmol/l, 9180 nmol*h/l) were comparable. The clearance/bioavailability (F) of balcinrenone following oral administration, either in capsule formulation or in a suspension, were similar (geometric mean of 27.3 and 28.6 l/h, respectively). The geometric mean terminal plasma half-life of balcinrenone when administered in a capsule was determined to be 12.3 hours, which was longer than that observed following the intravenous dose (4.2 hours). The geometric mean CL_R of balcinrenone following oral administration in capsule was similar (5.4 l/h) to CL_R estimated after intravenous administration (5.6 l/h). This corresponded to approximately 20% of total oral clearance. In period 2, balcinrenone exposure ($AUC_{0\text{-}inf}$) accounted for 44% of circulating plasma total radioactivity, which is somewhat reduced compared with the ratio observed after intravenous administration of [^{14}C]balcinrenone.

Blood-Plasma Partitioning of ^{14}C Total Radioactivity

Geometric mean whole blood-to-plasma concentration ratios of ^{14}C total radioactivity following the oral dose of [^{14}C]balcinrenone in period 2 ranged from 0.431 to 1.476, with ratios <1 for all time points except at 24 hours postdose.

Metabolite Profiling

After a single oral dose of [^{14}C]balcinrenone suspension, metabolite profiles in the pooled plasma, urine, and fecal samples were established using LC separation and fractionation, followed by HRMS and off-line RAD analysis. The recovery of total radioactivity following sample preparation was determined to be 92% in plasma, 105% in urine, and 94% in feces. Column recovery of total radioactivity was determined as complete (plasma, 101%; urine, 96%; and feces, 99%). Radiochromatograms and percentages of unchanged balcinrenone and metabolites expressed as percent $AUC_{0\text{-}24h}$ in plasma and percent of dose in excreta are shown in Fig. 4 and Table 2, respectively. In the $AUC_{0\text{-}24h}$ pooled plasma, unchanged balcinrenone was identified as the primary circulating species, accounting for approximately 54.8% of the $AUC_{0\text{-}24h}$. The radio-chromatogram indicated the formation of four quantifiable metabolites, M1, M2, M7, and M9, accounting for

TABLE 1

Summary of PK parameters.

PK Parameter, Geometric Mean (% CV)[a]	100 μg [^{14}C]balcinrenone Microtracer Infusion, Period 1		100 mg Balcinrenone Capsule, Period 1	100 mg [^{14}C]balcinrenone Oral Suspension, Period 2		
					Total radioactivity	
	[^{14}C]balcinrenone plasma (n = 8)	Total radioactivity plasma (n = 8)	Balcinrenone plasma (n = 8)	Balcinrenone plasma (n = 7)	Plasma (n = 7)	Whole blood[b] (n = 7)
T_{lag}	–	–	0.25 (0.00–0.27)	0	0	0
T_{max}[c] (h)	0.3 (0.1–0.3)	0.3 (0.3–3.8)	1.3 (0.8–2.5)	0.5 (0.5–1.0)	0.8 (0.5–1.0)	0.8 (0.5–1.0)
C_{max} (nmol/l)	12.5 (23.3)	13.8 (33.7)	2160 (27.7)	3210 (32.2)	3820 (27.8)	2610 (32.3)
$AUC_{0\text{–}48}$	NC	NC	9000 (24.9)	9270 (22.9)	–	–
$AUC_{0\text{-}t}$ (nmol.h/l)	17.6 (35.5)	33.8 (57.5)	9070 (25.9)	9280 (23.0)	19,400 (17.4)	12,600 (26.0)
$AUC_{0\text{-}inf}$ (nmol.h/l)	17.7 (35.5)	36.0 (54.4)	9180 (26.9)	9060 (23.8) [n = 6]	20,400 (17.9)	12,800 (33.2) [n = 5]
$t_{1/2}$ (h)	4.22 (101.6)	36.7 (60.3)	12.3 (90.6)	6.65 (98.6) [n = 6]	6.80 (34.7)	3.34 (52.5) [n = 5]
CL or CL/F (l/h)	14.2 (35.5)	NC	27.3 (26.9)	28.6 (23.8) [n = 6]	NC	NC
CL_R (l/h)	5.61 (40.4)	NC	5.46 (26.7)	5.44 (28.0)	NC	NC
V_z (l) or Vz/F	86.2 (103.0)	NC	485 (84.9)	274 (95.1) [n = 6]	NC	NC
V_{ss} (l)	37.8 (15.8)	NC	–	–	–	–
$MRT_{0\text{-}t}$ (h)	2.56 (37.6)	7.62 (6.7)	5.61 (29.2)	3.82 (24.7)	6.09 (9.8)	5.24 (31.1)
$MRT_{0\text{–}inf}$ (h)	2.67 (40.0)	14.7 (47.7)	6.37 (38.9)	4.06 (28.9) [n = 6]	7.65 (14.1)	5.43 (38.8) [n = 5]
F (%)	–	–	52.0 (24.6)	–	–	–

AUC, area under the curve; CL, clearance; CL/F, oral clearance; CV, coefficient of variation; MRT, mean residence time; NC, not calculated; $t_{1/2}$, half-life; t_{lag}, time prior to first measurable concentration; T_{max}, of C_{max}; V, volume of distribution; V_{ss}, volume of distribution at steady state; V_z, apparent volume of distribution.

[a]Unless specified otherwise (see T_{max}).

[b]Whole blood PK parameters derived based on reduced sampling schedule.

[c]Median (range).

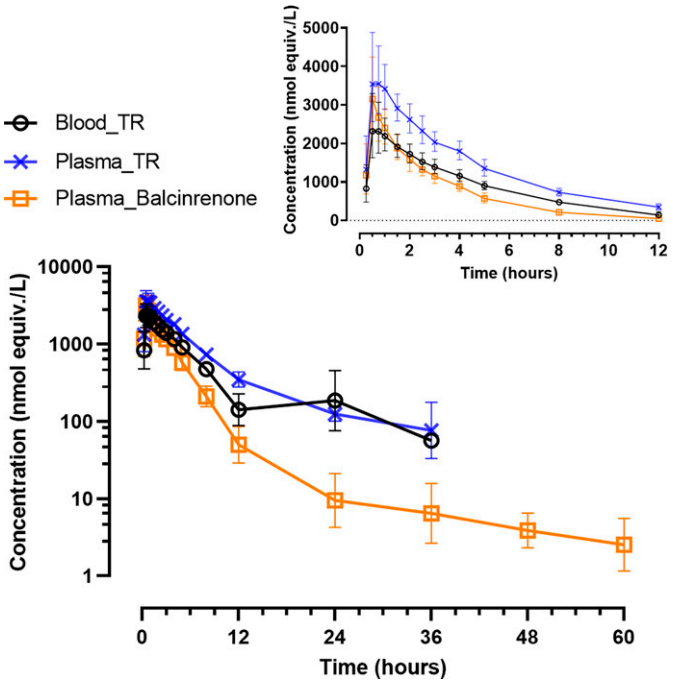

Fig. 3. Mean of total radioactivity in whole blood and plasma, and plasma concentration of balcinrenone over time after a single oral dose of 100 mg [^{14}C]balcinrenone (n = 7). Total radioactivity (TR) concentrations in blood and plasma after 36 hours postdose were below the limit of quantification.

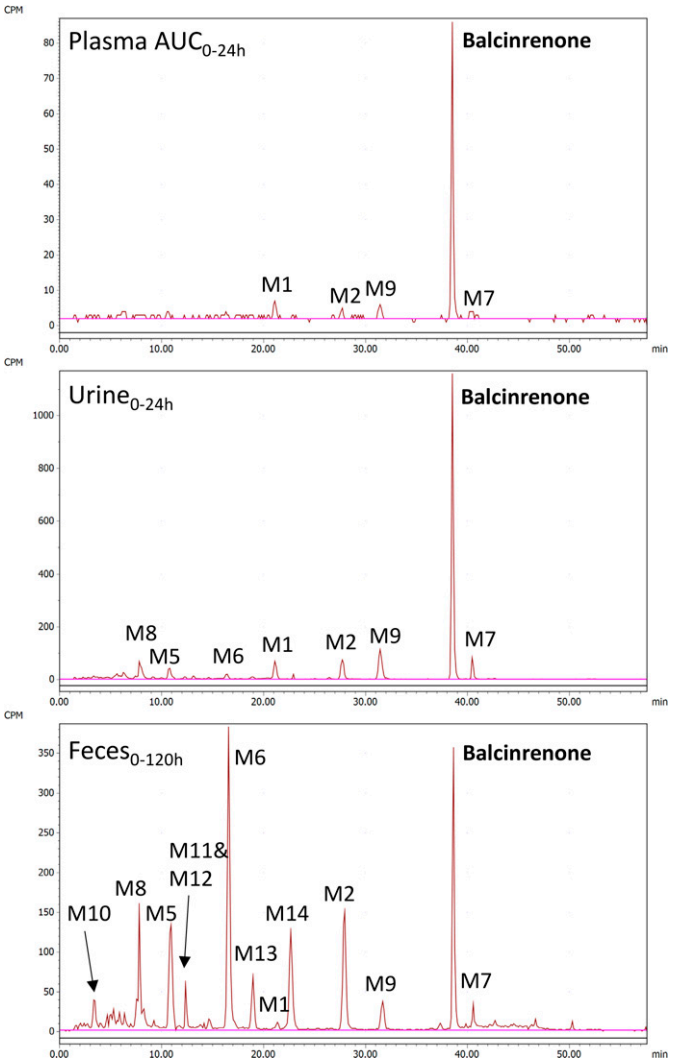

Fig. 4. Metabolite profiles of pooled plasma, urine, and feces following a single oral dose of balcinrenone (n = 7). Feces$_{0\text{-}120h}$, feces collected 0-120h post-dose.

5.1%, 2.4%, 3.7%, and 4.4%, respectively, of the AUC$_{0\text{-}24h}$. The quantified radioactive peaks altogether accounted for 70.4% of total radioactivity in the chromatogram. The remaining radioactivity attributed to a number of lesser radioactive components, each with an area of <7 cpm (i.e., below 2% of total radioactivity or to chromatographic background noise). In the 0–24-hour urine pool, unchanged balcinrenone accounted for 22% of the dose. The radio-chromatograms indicated the formation of seven major metabolite fractions in urine, of which M9 was the most abundant minor metabolite (representing 3.8% of the dose). In the 0–120-hour fecal pool, unchanged balcinrenone accounted for 6.2% of the dose, with M6 being the major drug-related material (accounting for 8.5% of the dose). Several minor peaks corresponding to <3% or 5% of the dose in urine and feces, respectively, were attributed to other minor metabolites or to chromatographic background noise.

Metabolite Identification

In total, 12 metabolites were quantified by RAD and their structures characterized based on HRMS spectra. LC retention times of the metabolites identified by HRMS were in agreement with the corresponding radio-chromatographic peaks identified by RAD. HRMS data were used to propose a molecular composition, and product ion spectra were used to propose metabolite structures. The structures and presence of balcinrenone and six metabolites (M2 and M5–9) in samples were confirmed by comparing LC retention times and MS spectra with the synthesized standards. Tentative structures of the remaining six metabolites (M1, M3, M4, M10–12) were proposed based on their fragment ions and a comparison of the product ion spectra of synthesized standards of balcinrenone and metabolite analogs. Representative product ion spectra and diagnostic fragments of balcinrenone and its metabolites are shown in Supplemental Fig. 1 and Supplemental Table 3. Proposed biotransformation pathways of balcinrenone are shown in Fig. 5.

In summary, the major metabolic pathways after oral administration in humans were via oxidation of balcinrenone, which resulted in the formation of the majority of metabolites. Several hydroxylated metabolites (M1, M2, and M12), and hydroxylations followed by N-dealkylation (M9), ring opening (M6, M11 and M13), further oxidation to carboxylic acid (M5 and M10), or hydrolysis (M8) were identified in human plasma, urine, and feces. Hydrolyzed product, M7, and *N*-acetylated secondary metabolite M14 were also detected. Under positive ionization MS conditions, metabolites M10 and M12 underwent significant MS in-source fragmentation; therefore, only the protonated molecules with the depletion of water were detected. However, under negative MS conditions, the intact deprotonated molecules of M10 and M12 were detected, and their molecular compositions were confirmed. Metabolites M11 and M12 were coeluted radio-chromatographic peaks; however, they were separated by MS detection.

Human Plasma Protein Binding

Following method optimization, the plasma protein binding of balcinrenone was determined after an 18-hour equilibrium dialysis. The percentage unbound concentrations were 17.3%, 17.6%, 18.6%, and 20.5% at concentrations of 0.1, 1, 10, and 100 μM balcinrenone, respectively.

P450 Phenotyping in Vitro

Using human recombinant P450s, CYP3A4 was shown to be the P450 contributing the most to P450-mediated metabolism of balcinrenone. The fractions of total metabolism occurring via CYP3A4, CYP2D6, and

TABLE 2

Quantitative estimates of balcinrenone and metabolites in excreta and plasma (AUC_{0-24h} pool) following a single oral administration to healthy volunteers at a target dose of 100 mg (8 MBq)

Compound	Mass Shift (Da)	Biotransformation	$Urine_{0-24\ h}$ (% dose)	$Feces_{0-120\ h}$ (% dose)	$Plasma_{0-24\ h}$ (% $AUC_{0-24\ h}$)
Balcinrenone	–	Parent	22	6.2	54.8
M10	48	Hydroxylated M5	NQ	1.0	ND
M8[a]	−40	M5 or M6 hydrolysis	2.2	2.3	NQ
M5[a]	32	Oxidation to form ring opened carboxylic acid	1.3	3.9	NQ
M11[b]	4	N-demethylation and oxidation to form ring opened alcohol	0.2	0.8	ND
M12[b]	16	Hydroxylation	–	–	–
M6[a]	18	Hydroxylation to form ring opened alcohol	0.5	8.5	NQ
M13	18	Hydration	0.3	1.6	NQ
M1	16	Hydroxylation	1.8	0.3	5.1
M14	2	N-acetylated M8	ND	3.5	ND
M2[a]	16	Hydroxylation	2.2	4.2	2.4
M9[a]	−14	N-demethylation	3.8	1.0	4.4
M7[a]	−13	Amide hydrolysis	1.5	0.8	3.7
Sum[c]	–	–	35.8	34.3	70.4
Total[d]	–	–	42.7	45.3	–

AUC, area under the curve; ND, not detected by RAD or MS; NQ, not quantified (detected by MS but RAD peak area below the limit of quantification [7 cpm in plasma and 10 cpm in excreta] in radio-chromatograms); RAD, radioactivity detection.
[a]Structure confirmed by comparing LC retention time and MS spectrum with the synthesized standard.
[b]Coeluting metabolites (liquid chromatography).
[c]Sum of integrated radioactive peaks expressed as percent of administered dose in excreta and percent of AUC_{0-24h} in plasma.
[d]Total radioactivity in samples expressed as percent of administered dose in excreta as derived from study D6402C00002.

CYP3A5 were determined to be 91%, 3%, and 6%, respectively. In all other P450 isoforms, balcinrenone was metabolically stable, and CL_{int} could not be determined. Following incubation of balcinrenone in human hepatocytes, in the presence and absence of ketoconazole, CL_{int} values were determined to be 0.28 and 0.021 $\mu l \cdot min^{-1} \cdot (10^6\ cells)^{-1}$, respectively, and the fraction of total hepatic metabolism occurring via CYP3A4/5 was determined to be 93%.

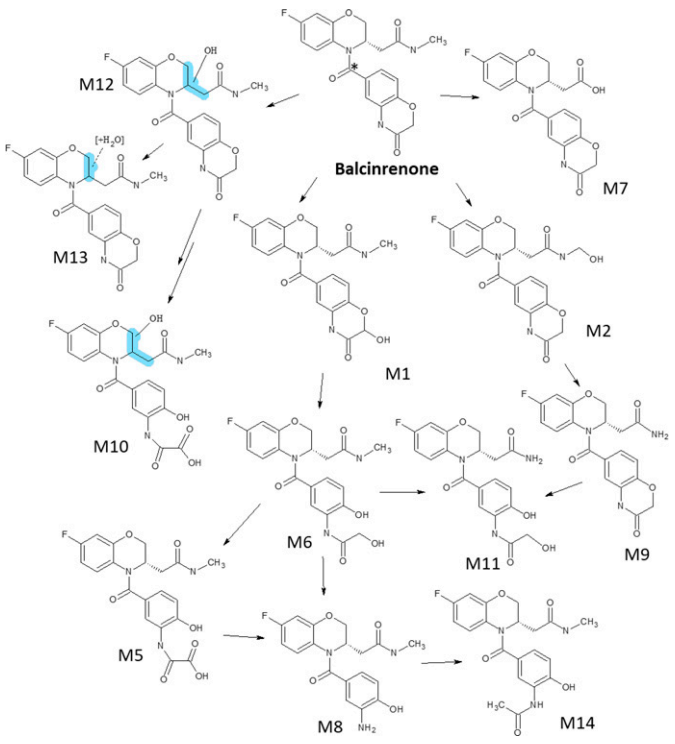

Fig. 5. Proposed metabolic pathways of [^{14}C]balcinrenone after a single oral dose. *Designates the site of ^{14}C labeling. Structures of M2 and M5–9 were confirmed by comparison with synthetic standards.

In Vitro Drug Transporter Studies

The potential of balcinrenone to be a substrate of human efflux transporter P-gp was evaluated in MDCK-MDR1 cell monolayers. At a balcinrenone concentration of 1 μM, efflux ratios in the absence and presence of P-gp inhibitor cyclosporin A were determined to be 52 and 1.1, respectively, suggesting that balcinrenone is a substrate of P-gp.

The potential of balcinrenone to be a substrate of human efflux transporter BCRP was evaluated in Caco-2 cell monolayers. At a balcinrenone concentration of 1 μM, efflux ratios in the absence and presence of the BCRP inhibitor fumitremorgin C were determined to be 41 and 18, respectively. The accompanying B–A P_{app} values in the absence and presence of the inhibitor were comparable, suggesting that balcinrenone is unlikely to be a substrate of BCRP.

The potential of balcinrenone to be a substrate of human renal SLC transporters OAT1, OAT2, OAT3, OCT2, MATE1, and MATE2-K was evaluated in HEK293 cells transfected with these transporters. Data generated on uptake ratios, at a 5 μM concentration, in the absence and presence of the inhibitors for the evaluated SLC transporters, suggested that balcinrenone is a potential substrate of OAT3, an unlikely substrate of OAT2, and not substrate of OAT1, OCT2, or MATE1. While data generated at a 5-μM concentration suggest that balcinrenone is a substrate of MATE2-K, taking the results of all concentrations evaluated into consideration, it was concluded that balcinrenone is unlikely to be a substrate of MATE2-K. Efflux and uptake ratios for balcinrenone in the evaluated cell lines expressing efflux and SLC transporters, respectively, are reported in Table 3 and Supplemental Tables 4–11.

Tolerability

Single doses of 100 mg balcinrenone and 100 μg i.v. [^{14}C]balcinrenone in period 1 and 100 mg oral [^{14}C]balcinrenone in period 2 were well tolerated by the healthy subjects enrolled in this study. All adverse

TABLE 3

Efflux and uptake ratios for balcinrenone in cell lines expressing human efflux and SLC transporters

Efflux ratios determined at 1 μM and uptake ratios at 5 μM concentration of balcinrenone.

Transporter	Ratio		Result/Conclusion
	Without inhibitor	With inhibitor	
P-gp	51.9	1.1	Is a substrate
BCRP	40.9	18.1	Unlikely a substrate[a]
OCT2	1.1	2.6	Not a substrate
OAT1	1.1	1.1	Not a substrate
OAT2	2.7	3.1	Unlikely a substrate
OAT3	2.1	1.5	Potential substrate
MATE1	1.1	1.1	Not a substrate
MATE2-K	2.1	0.9	Unlikely a substrate
	1.5[b], 1.6[c], 1.0[d]	NT[b], 1.4[c], NT[d]	

NT, not tested.
[a]No accompanying change in P_{app} B to A when incubated in the presence of inhibitor.
[b]Uptake ratio at a 0.5 μM concentration of balcinrenone.
[c]Uptake ratio at a 20 μM concentration of balcinrenone.
[d]Uptake ratio at a 50 μM concentration of balcinrenone.

events were mild, and none were considered to be related to [^{14}C]balcinrenone. Clinical laboratory evaluations, vital signs, electrocardiograms, and physical examinations revealed no clinically relevant findings.

Discussion

This study had a 2-period design. In period 1, an intravenous microtracer of [^{14}C]balcinrenone was administered shortly after a single oral dose of unlabeled balcinrenone formulated in a capsule to estimate the absolute bioavailability and basic PK parameters of balcinrenone. In period 2, [^{14}C]balcinrenone was administered as an oral suspension to characterize its ADME properties, including mass balance. This study design allowed capsule and suspension formulations of balcinrenone to be compared in the same subjects, with reduced data variability despite the small number of subjects enrolled.

After single oral administration of [^{14}C]balcinrenone, 94.1% of the administered radioactivity was recovered in excreta over a 168-hour sampling period, in line with guidelines (www.ema.europa.eu/en/documents/newsletter/news-bulletin-small-medium-sized-enterprises-issue-23_en.pdf). Similar amounts of total radioactivity were recovered between the urine and feces (45% and 49% of the dose, respectively). In urine, approximately half of the drug-related material could be assigned to intact parent compound (22% of the radioactive dose). In feces, only a minor portion of the radioactivity was assigned to balcinrenone (6% of the dose), suggesting no effect or a minor effect of transporters on the absorption of balcinrenone. Renal clearance of balcinrenone was estimated to be 5.4 l/h, which is more than f_u multiplied by glomerular filtration rate (i.e., 0.185 × 7.5 = 1.4 l/h), indicating excretion not only by glomerular filtration but also by involvement of active tubular secretion. The potential for balcinrenone to be a substrate of human efflux and SLC transporters was evaluated in vitro, with the results suggesting that P-gp and/or OAT3 contribute toward active renal elimination of balcinrenone. In two previous studies of single doses of balcinrenone and repeated twice-daily dosing in healthy subjects, renal clearance was consistent across the entire dose range evaluated (single dose, 5–1200 mg; repeated dosing, 50-300 mg bid) (Erlandsson et al., 2018; Whittaker et al., 2020). It can therefore be concluded that transporters involved in the renal clearance of balcinrenone are not saturated in this dose range. The low amounts of parent drug detected in feces could be explained by unabsorbed drug, and the fraction absorbed (f_{abs}) was estimated to be 94% following oral suspension administration. It is acknowledged that f_{abs} 94% might be somewhat underestimated because elimination via the bile cannot be ruled out. A quantitative mass balance diagram after oral administration of balcinrenone is given in Fig. 6.

The whole blood-to-plasma total radioactivity concentration ratios tended to indicate nonpreferential distribution of total radioactivity to the cellular components of whole blood. No or negligible saturation in the plasma protein binding (unbound percentage 17.3%–20.5%), in the concentration range evaluated (0.1–100 μM), suggests binding to plasma albumin rather than α-1-acid glycoprotein. Results also suggest that plasma protein binding of balcinrenone is constant at anticipated therapeutic exposures, high nM or low μM, provided albumin levels are normal.

To mitigate a biased disposition profile with a microdose, at exposure levels much lower than therapeutic levels, an intravenous microtracer was administered at anticipated C_{max} after an oral administration of 100 mg

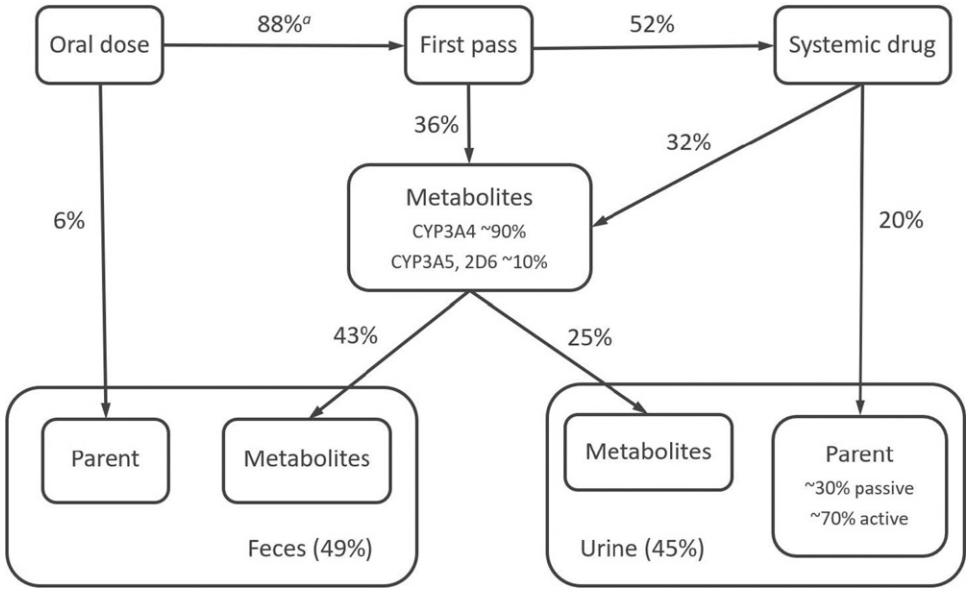

Fig. 6. Balcinrenone: quantitative mass balance diagram. Accounting for 94% recovery determined in mass balance part of the study.

unlabeled balcinrenone. According to ICH guideline M3(R2), the maximum microdose allowed is 100 μg, and the selected dose must be ≤1/100th of the no observed adverse effect level (determined in nonclinical safety studies) and <1/100th of the pharmacologically active dose (www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-m3r2-non-clinical-safety-studies-conduct-human-clinical-trials-marketing-authorisation_en.pdf). These criteria were both met for the selected microtracer of [^{14}C]balcinrenone in this study. A strength with using an intravenous microtracer rather than a high dose is that the study can be qualified by the existing oral toxicology studies, and no additional preclinical toxicology study with intravenous administration is required [ICH guideline M3(R2)]. To achieve the bioanalytical sensitivity needed, the low levels of radioactivity in plasma were determined using the ultrasensitive combustion AMS technique (van Duijn et al., 2014). The low radioactive dose (equivalent to 0.0207 millisievert) falls into the International Commission on Radiologic Protection category I, which is associated with a negligible risk to the dosed subjects (ICRP, 1991). Based on intravenous microtracer and coadministered therapeutic oral dose, the basic PK parameters of balcinrenone were estimated for the first time in humans. The short half-life of 4.2 hours, as determined after an intravenous dose, is likely to reflect the elimination half-life of balcinrenone. The longer terminal half-life of 12.3 hours, as determined following oral capsule administration, is likely affected by absorption rate limited elimination. Clearance and steady-state volume of distribution were estimated to 14.2 l/h and 37.8 l, respectively. Assuming the nonrenal clearance (8.8 l/h) is hepatic (CL_h), the fraction escaping first-pass hepatic metabolism (F_h) was estimated to be 90% using a liver blood flow value of 84 l/h ($F_h = 1 - CL_h/84$ l/h). A first-pass hepatic metabolism of only 10% is consistent with a marginal difference in $AUC_{0\text{-inf,total radioactivity}}/AUC_{0\text{-inf,balcinrenone}}$ ratio after oral and intravenous administration, estimated as 2.3 and 2.0, respectively. The absolute F was determined to be 52%, and with knowledge of F and F_h, $F_{abs} \cdot F_{gut}$ was estimated to be 58% ($F = F_{abs} \cdot F_{gut} \cdot F_h$). Utilizing the estimated F_{abs} 94%, the fraction escaping gut metabolism (F_{gut}) was estimated to be 62%.

Metabolite profiling and identification showed that the metabolism of [^{14}C]balcinrenone is mediated primarily by oxidative mechanisms (approximately 95%), with minor fractions metabolized via amide hydrolysis. This finding, considered alongside in vitro P450 phenotyping data, indicates that in vivo metabolism is primarily mediated by CYP3A4. Even though the fraction of balcinrenone eliminated via metabolism was estimated to be only 62% (renal 38%), the first-pass extraction in the intestine is high (f_{gut} about 62%), which is likely due to CYP3A4 metabolism. Thus, overall, it could not be ruled out that exposure to balcinrenone would be influenced by inhibition of CYP3A4. Therefore, a clinical drug-drug interaction study with the strong CYP3A4 inhibitor itraconazole was performed, demonstrating that balcinrenone is a moderately sensitive substrate of CYP3A4 (NCT03843060, data on file).

Following single oral administration of [^{14}C]balcinrenone suspension, the half-life of both balcinrenone and total radioactivity plasma profiles were similar, suggesting formation rate limited kinetics of metabolites. Four metabolites (M1, M2, M7, and M9) of balcinrenone were quantified and identified in the plasma, with M1 the most abundant and accounting for 5.1% of drug-related exposure in pooled $AUC_{(0\text{-}24h)}$ plasma. In total, balcinrenone and quantified major metabolites accounted for 70.4% of the radioactivity in the pooled $AUC_{0\text{-}24h}$ plasma. Numerous minor metabolites (each accounting for <2% of total radioactivity) were also found in plasma; however, no detailed structure characterization was conducted. Due to the exposure of all circulating metabolites accounting for <10% of the total drug-related-exposure, with no indication of longer half-life than the parent compound, no further safety assessment of these circulating metabolites was warranted (www.ema.europa.eu/en/documents/other/international-conference-harmonisation-technical-requirements-registration-pharmaceuticals-human-use_en.pdf; www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-m3r2-non-clinical-safety-studies-conduct-human-clinical-trials-marketing-authorisation_en.pdf; www.fda.gov/media/72279/download). According to the US Food and Drug Administration in vitro drug-drug interaction guidelines, there is no need to evaluate if a metabolite is a substrate of enzymes or transporters if its contribution to the overall pharmacological effect is <50% (https://www.fda.gov/regulatory-information/search-fda-guidance-documents/in-vitro-drug-interaction-studies-cytochrome-p450-enzyme-and-transporter-mediated-drug-interactions). Among the four circulating metabolites, three are less active than balcinrenone as determined in an MR reporter gene antagonist assay (data on file): M9 is 1.8-fold less active than balcinrenone, M2 is 7.3-fold less active than balcinrenone, and M7 is inactive. M1 is not available as a synthetic standard but is likely to be low active or inactive based on the structure/activity relationship established within this chemical series (Granberg et al., 2019). This, together with low exposure of the metabolites compared with balcinrenone, clearly indicated that their contribution to the overall pharmacological activity is negligible.

In summary, this human ADME study has provided invaluable insights on the disposition of balcinrenone. The absolute F, F_{abs}, fraction escaping first-pass hepatic metabolism (F_h), and fraction escaping gut metabolism (F_{gut}) were determined for the first time in humans. These data provided further insights into the potential risk for altered exposure of balcinrenone by inhibition of CYP3A4, both in the liver and intestine and in organ-impaired patients. There were no human circulating metabolites that warrant any further safety assessment nor exposures of active metabolites that could contribute to a meaningful effect on pharmacodynamic response identified. The low plasma exposure to metabolites suggests negligible risk of drug-drug interactions by any of the identified metabolites.

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Data Availability

Data underlying the findings described in this manuscript may be obtained in accordance with AstraZeneca's data sharing policy described at https://astrazenecagrouptrials.pharmacm.com/ST/Submission/Disclosure. Data for studies directly listed on Vivli can be requested through Vivli at www.vivli.org. Data for studies not listed on Vivli could be requested through Vivli at https://vivli.org/members/enquiries-about-studies-not-listed-on-the-vivli-platform/. AstraZeneca Vivli member page is also available outlining further details: https://vivli.org/ourmember/astrazeneca/.

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Drug Metabolism and Disposition (manuscript ID: DMD-AR-2022-001240)

Supplemental Materials

Mass Balance and ADME Properties of Balcinrenone Following Oral Administration in Combination with Intravenous Microtracer in Healthy Subjects

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Supplemental Methods

Sample Preparation for Total Radioactivity Determined by Liquid Scintillation Counting (LSC)

Whole blood: Duplicate weighed aliquots of whole blood (ca. 0.3 g) were solubilized using tissue solubilization fluid (ca. 2 ml, AquiGest, Meridian Biotechnologies Ltd.). Following solubilization, addition of EDTA di sodium salt (aq) (ca. 100 µl), hydrogen peroxide bleaching (ca. 300 µl in 100 µl additions, 30% solution), and incubation, samples were mixed with scintillation fluid (Gold Star, Meridian Biotechnologies Ltd.) and were taken for direct determination of radioactivity by LSC.

Plasma: Duplicate samples of plasma (0.3 ml, where possible) were taken and mixed with 0.6 ml of phosphate buffered saline (0.01 M). Samples were mixed with scintillation fluid (Gold Star, Meridian Biotechnologies Ltd.) and were taken for direct determination of radioactivity by LSC.

Urine: The weight of each urine sample received was recorded before combining samples per subject per time interval. Duplicate weighed aliquots (ca. 1–5 g) were taken from each bulk sample, mixed with scintillation fluid (Gold Star, Meridian Biotechnologies Ltd.), and taken for direct determination of radioactivity by LSC.

Feces: The weight of each fecal sample received was recorded before combining samples per subject per time interval. Each bulk sample was homogenized with a volume of water sufficient to produce a paste, using a commercial blender. The total

homogenate weight was recorded. Duplicate weighed aliquots (ca. 0.3–0.6 g) of each fecal homogenate were taken and combusted using an Automatic Sample Oxidizer (Model 307 Tri-Carb®, Perkin Elmer) prior to measurement of radioactivity by LSC.

High-Resolution Mass Spectrometry (HRMS) Instrument Settings for Metabolite Identification

The mass spectrometry (MS) source settings were as follows: capillary voltage, 0.5 kV; sample cone voltage, 40 V. The source and desolvation temperatures were set to 150°C and 550°C, respectively. The same settings were used in negative mode. For the low energy MS^E acquisition, the trap energy was set to 4 V and transfer energy to 0 V, while for the high energy MS^E acquisition, the trap energy was ramped from 15 to 45 V and the transfer energy was set to 20 V. Tandem mass spectrometry (MS/MS) acquisition was performed with the trap energy at 2 eV and the transfer energy ramped from 15 to 45 eV, with the quadrupole mass window set to 1 Da for precursor ion selection. All data were acquired in centroid mode with a mass range of 100–1000 *m/z*. Leucine-enkephalin was used as an internal calibrant for the accurate mass measurements and the MS resolution was 20,000.

In Vitro Human Plasma Protein Binding

The equilibrium dialysis device (RED Device, Pierce, Thermo Fischer Scientific, USA) was used for dialysis against phosphate buffer pH 7.4 for 18 hours at 37°C. Samples were analyzed by high-performance liquid chromatography with tandem mass

spectrometry (HPLC-MS/MS) (LC-30A, Shimadzu, Japan and API 4000, Sciex, MA, USA). Plasma stability and time to equilibrium were initially investigated using rat plasma and were then confirmed in human plasma using the final assay condition. All experiments were carried out in triplicates at concentrations of 0.1, 1, 10, and 100 μM balcinrenone. Frozen human plasma, generated using K2-EDTA anticoagulant from healthy participants, was used in the method optimization and final analyses.

In Vitro CYP Phenotyping

Balcinrenone was incubated for 25 minutes with 10 human recombinant cytochrome P450s: CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 3A4, and 3A5. After 0, 5, 10, 15, and 25 minutes, aliquots were taken from the reaction mixture and quenched by acetonitrile, followed by analysis of balcinrenone using LC-MS/MS (Acquity UPLC-Xevo TQD mass spectrometer, Waters, UK). Intrinsic clearance (CL_{int}) values were calculated from the slope of exposure versus time and used in combination with intersystem extrapolation factors, and abundance in liver, to calculate the fraction of total P450 mediated CL_{int} for each CYP isoform. In addition, balcinrenone was incubated in human hepatocytes for 180 minutes in the presence and absence of the potent and selective CYP3A4/5 inhibitor, ketoconazole. The reactions were stopped after 30, 60, 120, and 180 minutes, followed by analysis of balcinrenone using LC-MS/MS. CL_{int} values were determined both in the presence and absence of ketoconazole, from which fraction of total CL_{int} via CYP3A4/5 was calculated.

In Vitro Drug Transporter Studies

MDCK-multidrug resistance 1 gene (MDR1) and Caco-2 cells were seeded onto Millipore Multiscreen Transwell plates and cultured in Dulbecco's Modified Eagle Medium at 37°C. Media was changed on Day 3 and every 2 or 3 days for MDCK-MDR1 and Caco-2 cells, respectively, and the transporter substrate identification study was performed on day 4 (MDCK-MDR1) and on day 18–22 post cell seeding (Caco-2). Prior to assay, the mature cell monolayers were rinsed twice with pre-warmed (37°C) transport buffer (Hank's Balanced Salt Solution containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES] and 4.45 mM glucose, pH 7.4) on both basolateral and apical surfaces, and then pre-incubated with transport buffer in both apical and basolateral compartments for 30 minutes at 37°C. Permeability was assessed (triplicate wells per condition) in both the apical to basolateral (A–B) and basolateral to apical (B–A) directions. After pre-incubation, transport buffer was removed, and the appropriate solutions were added to the donor and receiver compartments. Donor solutions in transport buffer (final dimethyl sulfoxide concentration of ≤1 % v/v) contained the test compound at four concentration levels (1, 10, 50, and 150 µM), and the fluorescent cell monolayer integrity marker, lucifer yellow (100 µM). As reference inhibitors, cyclosporin A (10 µM) and fumitremorgin C (10 µM) were used for P-glycoprotein 1 (P-gp) and breast cancer resistance protein (BCRP), respectively, and co-incubated with 1 and 10 µM of balcinrenone. Receiver compartment solutions in transport buffer contained dimethyl sulfoxide (at ≤1 % v/v) and where applicable, the reference inhibitor.

Incubations were carried out at 37°C for 90 minutes. After the incubation period, exposure of balcinrenone was quantified by LC-MS/MS (Acquity Binary Solvent Manager coupled with a Xevo-TQ-S micro triple quadrupole mass spectrometer; Waters, UK) in apical and basolateral compartment samples, and in donor dosing solutions (C_0), to estimate apparent permeability (P_{app}) and assess recovery of balcinrenone in this assay setup. P_{app} values for A–B and B–A were calculated according to Equation 1, and efflux ratios were determined by dividing P_{app} (B–A) by P_{app} (A–B).

Equation 1: $P_{app} = (dQ/dt) / (A \bullet C_0)$

P_{app}: Apparent permeability ($cm \bullet sec^{-1} \bullet 10^{-6}$)

dQ/dt: Rate of drug transport ($pmol \bullet sec^{-1}$)

A: Surface area of the membrane (cm^2)

C_0: Initial donor concentration (nM)

For renal solute carrier (SLC) transporters, organic anion transporters (OAT1, OAT2, OAT3), organic cation transporter 2 (OCT2), and multidrug and toxin extrusion transporters (MATE1 and MATE2-K), all cell lines were seeded and cultured in Dulbecco's Modified Eagle Medium (containing sodium butyrate for MATE cells and corresponding control cells) in 24-well poly-D-lysine coated plates. Media was changed 3–4 hours post-seeding and the cells were cultured for 24 hours at 37°C. Prior to the transporter identification study, cells were washed twice with pre-warmed

uptake buffer (Hank's Balanced Salt Solution containing 10 mM HEPES, pH 7.4), then left to pre-incubate in uptake buffer for 10 minutes (MATE cells and corresponding control cells were pre-incubated in uptake buffer solution containing 40 mM ammonium chloride for 20 minutes). After pre-incubation, the uptake buffer was removed, and the appropriate test solutions were added to the 24-well plate. Incubations containing balcinrenone (0.5, 5, 20, and 50 µM test concentration, and 5 and 20 µM also in the presence of reference inhibitor) were performed in transporter transfected cells and control (empty vector) cells for 0.5, 2, and 20 minutes at 37°C. Solvent concentration of dimethyl sulfoxide was below 1.2 % (v/v). All assay conditions were tested in triplicate wells. As reference inhibitors, probenecid (100 µM) was used for OAT1 and OAT3, cimetidine (100 µM) for MATE1 and MATE2-K, verapamil (200 µM) for OCT2, and indomethacin (100 µM) for OAT2. The reactions were terminated by removing the incubation solutions and immediately washing the cells twice with ice cold uptake buffer and putting the plates on ice. The cells were then lysed for at least 30 minutes with water, protein extracted by the addition of two volumes of methanol followed by centrifugation at 4000 rpm for 5 minutes at 4°C, and lysate supernatants were quantified for balcinrenone (pmol/ml) against a matrix matched standard curve by LC-MS/MS (Agilent HP 1290 LC system, Agilent Technologies, UK, coupled with 5500 Qtrap mass spectrometer, Sciex, USA). The determined concentrations of balcinrenone in cell lysates (pmol/ml) were normalized to the protein (mg/ml) content of each well to calculate cell concentrations (pmol/mg). The cell concentrations of balcinrenone in transporter-expressing cells were divided

by that determined in control cells to produce an uptake ratio at each combination of incubation time and substrate concentration.

Supplemental Tables

Supplemental Table 1. Demographic characteristics

Characteristic	Statistics	Total (*N* = 8)
Height (cm)	Mean	178.8
	Standard deviation	6.6
	Median	176.5
	Minimum	170
	Maximum	187
Weight (kg)	Mean	81.7
	Standard deviation	9.6
	Median	80.4
	Minimum	68.4
	Maximum	97.0
BMI (kg/m^2)	Mean	25.5
	Standard deviation	2.2
	Median	25.1
	Minimum	23.4
	Maximum	29.3
Sex	Male	8

Age (years)	Mean	41.6
	Standard deviation	9.2
	Median	42.5
	Minimum	30
	Maximum	56
Race	White	8

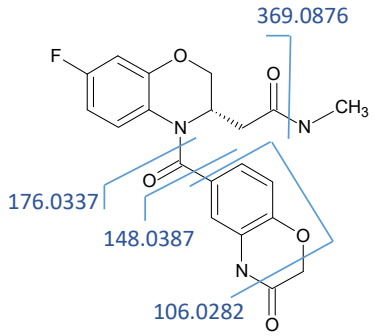
BMI, body mass index.

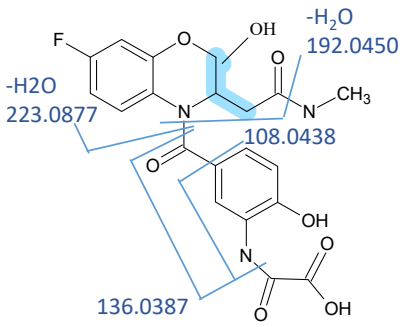
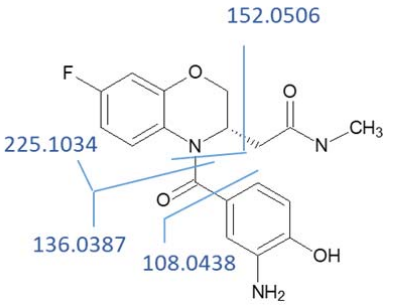
Supplemental Table 2. Mean cumulative of fraction of total radioactivity excreted in urine, feces, and total (urine + feces) expressed as a percentage of the radioactive dose administered following a single oral dose of 100 mg [^{14}C]balcinrenone in the fasted state (Period 2 only)

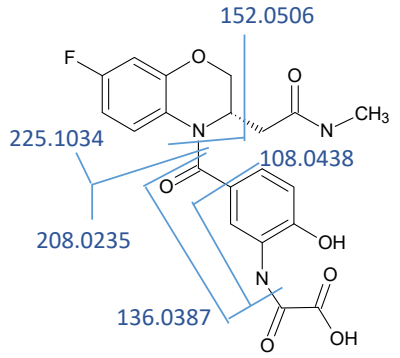
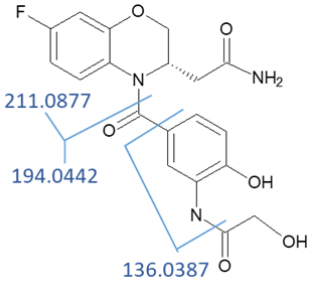
Collection Interval (h)	Cumulative Fraction of Dose Excreted		
	Urine (%) [*n* = 7]	Feces (%) [*n* = 7]	Urine + Feces (%) [*n* = 7]
0–6	25.615 (8.168)	–	–
0–12	38.005 (5.334)	–	–
0–24	42.728 (5.338)	4.645 (12.122)	47.373 (13.421)
0–48	44.326 (5.098)	16.390 (15.620)	60.716 (15.827)
0–72	44.854 (5.085)	32.134 (14.997)	76.988 (13.663)
0–96	45.067 (5.158)	44.195 (6.422)	89.262 (4.959)
0–120	45.134 (5.190)	46.858 (3.329)	91.992 (3.757)
0–144	45.168 (5.200)	48.713 (2.106)	93.880 (4.622)
0–168	45.188 (5.207)	48.916 (2.115)	94.104 (4.836)
Total amount excreted (nmol equivalent)	117,000 (13500)	127,000 (5480)	244,000 (12500)

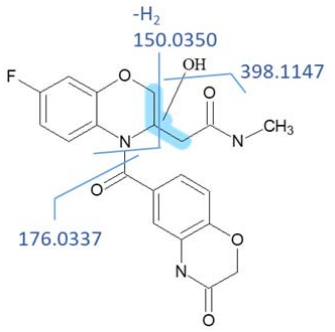
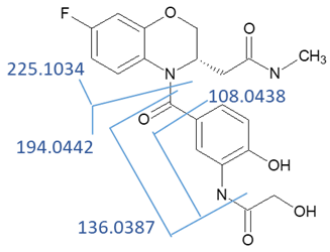
All values are arithmetic mean (standard deviation).

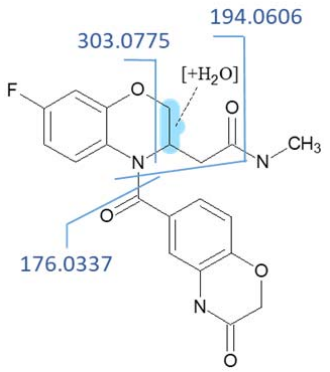
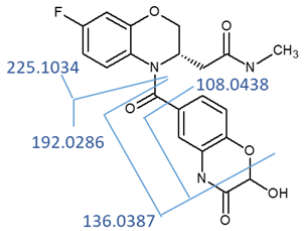
Supplemental Table 3. Observed precursor ions, proposed elemental compositions, and transformations of balcinrenone and metabolites

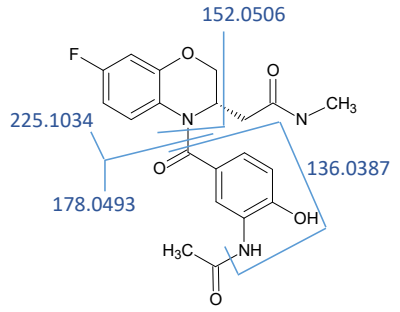
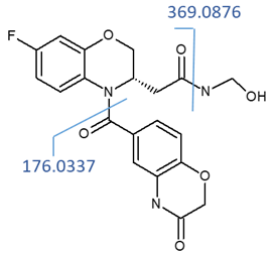
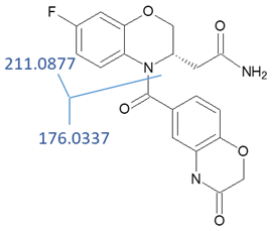
Compound	LC Retention Time (min)	ESI mode	Theoretical *m/z*	Observed *m/z*	Error (mDa)	Proposed Molecular Composition	Structure and MS/MS Key Fragment
Balcinrenone	38.5	ESI+	400.1303	400.1299	–0.4	$C_{20}H_{18}FN_3O_5$	

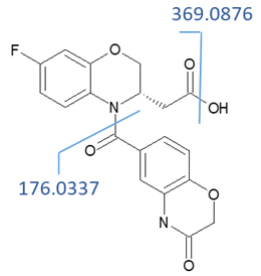
M10	3.4	ESI+[a] ESI–	430.1045 446.1005	430.1059 446.1006	1.4 0.1	$C_{20}H_{18}FN_3O_8$ – H_2O $C_{20}H_{18}FN_3O_8$	
M8 (AZ13689810)	7.8	ESI+	360.1354	360.1352	–0.2	$C_{18}H_{18}FN_3O_4$	

M5 (AZ13837259)	10.8	ESI+	432.1202	432.1194	–0.8	$C_{20}H_{18}FN_3O_7$	
M11	12.4 (12.26)[b]	ESI+	404.1252	404.1252	0.0	$C_{19}H_{16}FN_3O_6$	

M12	12.4 (12.31)[b]	ESI+[a] ESI–	398.1147 414.1107	398.1146 414.1097	–0.1 –1.0	$C_{20}H_{18}FN_3O_6$ – H_2O $C_{20}H_{18}FN_3O_6$	
M6 (AZ13825409)	16.4	ESI+	418.1409	418.1406	–0.3	$C_{20}H_{20}FN_3O_6$	

M13	18.8	ESI+	418.1409	418.1409	0.0	$C_{20}H_{20}FN_3O_6$	
M1	21.1	ESI+	416.1252	416.1256	0.4	$C_{20}H_{18}FN_3O_6$	

M14	22.7	ESI+	402.1459	402.1468	0.9	$C_{20}H_{20}FN_3O_5$	
M2 (AZ13773702)	27.7	ESI+	416.1252	416.1257	0.5	$C_{20}H_{18}FN_3O_6$	
M9 (AZ13656504)	31.4	ESI+	386.1147	386.1143	–0.4	$C_{19}H_{16}FN_3O_5$	

M7 (AZ13588176)	40.3	ESI+	387.0987	387.0983	–0.4	$C_{19}H_{15}FN_2O_6$	

ESI, electrospray interface; LC-MS/MS, liquid chromatography with tandem mass spectrometry.

[a]Protonated molecule underwent significant MS in-source depletion of water. The observed *m/z* under ESI+ condition corresponded to the dehydrated compound molecule (loss of H_2O).

[b]Co-eluting RAD peak; however, partially separated in LC-MS/MS chromatogram with the LC retention time in parentheses.

Supplemental Table 4. Bidirectional apparent permeability of balcinrenone across MDCK-MDR1 cell monolayers in the absence and presence of the P-gp reference inhibitor cyclosporin A

Compound (concentration)	Direction	P_{app} (cm/s x 10^{-6})					Mean % recovery	Efflux ratio
		Rep 1	Rep 2	Rep 3	Mean	SD		
Balcinrenone (1 µM)	A-B	0.232	0.230	9.712	0.391	0.278	91.6	52
	B-A	20.9	19.1	21.0	20.3	1.07	93.4	
Balcinrenone (10 µM)	A-B	0.314	0.482	0.350	0.382	0.0885	99.1	50
	B-A	20.4	17.9	19.3	19.2	1.25	98.4	
Balcinrenone (50 µM)	A-B	0.188	0.465	0.279	0.311	0.141	76.5	68
	B-A	22.4	20.2	20.8	21.1	1.14	74.4	
Balcinrenone (150 µM)	A-B	ND	ND	0.395	0.395	NC	68.6	44
	B-A	16.7	17.1	17.7	17.2	0.503	74.1	
Balcinrenone	A-B	1.97	2.14	1.53	1.88	0.315	84.9	1.1

(1 μM) + reference inhibitor	B-A	2.39	1.86	2.11	2.12	0.265	84.0	
Balcinrenone (10 μM) + reference inhibitor	A-B	4.07	1.75	1.38	2.40	1.46	80.0	0.8
	B-A	1.97	1.78	2.10	1.95	0.161	77.2	
	B-A	66.2	61.6	55.1	61.0	5.58	75.6	

NC, not calculated; ND, not determined due to sampling error.

Supplemental Table 5. Bidirectional apparent permeability of balcinrenone across Caco-2 cell monolayers in the absence and presence of the BCRP reference inhibitor fumitremorgin C

Compound (concentration)	Direction	P_{app} (cm/s x 10^{-6})					Mean % recovery	Efflux ratio
		Rep 1	Rep 2	Rep 3	Mean	SD		
Balcinrenone (1 µM)	A-B	0.857	0.893	0.981	0.910	0.0638	84.9	41
	B-A	38.1	36.7	36.7	37.2	0.808	92.0	
Balcinrenone (10 µM)	A-B	1.02	0.850	0.867	0.912	0.0936	76.5	36
	B-A	32.8	32.6	34.1	33.2	0.814	86.4	
Balcinrenone (50 µM)	A-B	0.966	0.848	0.865	0.893	0.0638	74.3	40
	B-A	34.1	36.8	36.7	35.9	1.53	78.8	
Balcinrenone (150 µM)	A-B	0.847	0.723	0.822	0.797	0.0656	88.1	42
	B-A	32.0	34.1	34.5	33.5	1.34	86.8	
Balcinrenone	A-B	1.83	1.66	1.86	1.78	0.108	89.9	18

(1 μM) + reference inhibitor	B-A	31.6	32.3	33.1	32.3	0.751	89.6	
Balcinrenone (10 μM) + reference inhibitor	A-B	1.82	1.77	1.74	1.78	0.0404	79.0	15
	B-A	28.2	27.7	26.0	27.3	1.15	83.1	

Supplemental Table 6. Uptake rate of balcinrenone into OAT1 and empty vector (control) transfected HEK293 cells

Balcinrenone concentration (μM)	Incubation time (min)	Cell line	Uptake rate (pmol/mg)		Uptake ratio
			Mean	SD	
0.5	0.5	OAT1	4.32	0.591	1.2
		Control	3.75	1.02	
	2	OAT1	13.2	1.63	1.0
		Control	12.9	2.08	
	20	OAT1	16.0	1.22	0.9
		Control	17.1	3.65	
5	0.5	OAT1	53.8	2.38	1.1
		Control	47.5	6.12	
	2	OAT1	119	13.2	1.0
		Control	116	10.3	

	20	OAT1	146	7.21	1.1
		Control	128	2.31	
20	0.5	OAT1	224	18.1	1.2
		Control	192	20.8	
	2	OAT1	453	30.3	1.0
		Control	462	8.96	
	20	OAT1	554	20.4	1.1
		Control	515	4.93	
50	0.5	OAT1	457	8.89	1.0
		Control	475	58.6	
	2	OAT1	977	106	0.9
		Control	1080	25.2	
	20	OAT1	1280	61.1	1.1

		Control	1170	15.3	
5 + Reference inhibitor	0.5	OAT1	48.3	3.83	1.1
		Control	42.9	4.79	
	2	OAT1	125	5.51	0.8
		Control	159	28.8	
	20	OAT1	175	4.04	0.7
		Control	240	29.0	
20 + Reference inhibitor	0.5	OAT1	189	9.50	1.1
		Control	175	9.71	
	2	OAT1	482	41.2	0.8
		Control	593	206	
	20	OAT1	619	14.2	0.9
		Control	713	84.2	

Supplemental Table 7. Uptake rate of balcinrenone into OAT3 and empty vector (control) transfected HEK293 cells

Balcinrenone concentration (μM)	Incubation time (min)	Cell line	Uptake rate (pmol/mg)		Uptake ratio
			Mean	SD	
0.5	0.5	OAT3	10.1	0.377	2.1
		Control	4.92	1.43	
	2	OAT3	20.4	2.58	2.5
		Control	8.08	1.75	
	20	OAT3	22.6	5.05	1.7
		Control	13.2	0.0577	
5	0.5	OAT3	105	15.5	2.1
		Control	49.3	7.84	
	2	OAT3	201	17.5	1.8
		Control	110	16.9	

	20	OAT3	223	7.64	1.2
		Control	179	25.2	
20	0.5	OAT3	356	35.6	2.0
		Control	175	7.21	
	2	OAT3	698	128	1.6
		Control	428	47.8	
	20	OAT3	735	78.5	1.5
		Control	502	92.1	
50	0.5	OAT3	806	40.0	2.1
		Control	385	21.5	
	2	OAT3	1860	320	1.9
		Control	968	63.9	
	20	OAT3	650	165	1.0

		Control	1670	448	
5 + Reference inhibitor	0.5	OAT3	67.5	8.29	1.5
		Control	44.2	2.72	
	2	OAT3	95.7	6.92	1.0
		Control	94.5	6.04	
	20	OAT3	173	13.9	1.4
		Control	127	21.2	
20 + Reference inhibitor	0.5	OAT3	287	46.1	1.7
		Control	172	26.5	
	2	OAT3	439	22.5	1.3
		Control	348	31.8	
	20	OAT3	672	91.5	1.2
		Control	558	80.3	

Supplemental Table 8. Uptake rate of balcinrenone into OCT2 and empty vector (control) transfected HEK293 cells

Balcinrenone concentration (μM)	Incubation time (min)	Cell line	Uptake rate (pmol/mg)		Uptake ratio
			Mean	SD	
0.5	0.5	OCT2	16.6	7.49	2.7
		Control	6.24	0.428	
	2	OCT2	20.3	1.90	0.9
		Control	23.1	3.04	
	20	OCT2	28.2	1.93	0.8
		Control	36.3	5.52	
5	0.5	OCT2	115	18.2	1.1
		Control	104	33.8	
	2	OCT2	219	5.20	1.1
		Control	194	13.4	

	20	OCT2	285	13.1	1.1
		Control	270	37.6	
20	0.5	OCT2	577	139	1.6
		Control	367	151	
	2	OCT2	851	41.0	1.1
		Control	794	45.7	
	20	OCT2	1040	37.9	1.0
		Control	1090	145	
50	0.5	OCT2	1190	128	1.7
		Control	709	96.6	
	2	OCT2	1730	90.7	1.1
		Control	1600	90.7	
	20	OCT2	2220	37.9	1.0

		Control	2240	106	
5 + Reference inhibitor	0.5	OCT2	151	17.0	2.6
		Control	59.1	10.5	
	2	OCT2	173	13.6	0.9
		Control	199	28.6	
	20	OCT2	220	10.2	0.8
		Control	274	49.2	
20 + Reference inhibitor	0.5	OCT2	354	44.2	0.9
		Control	378	75.6	
	2	OCT2	740	71.2	1.1
		Control	659	46.9	
	20	OCT2	1020	33.6	1.1
		Control	948	47.6	

Supplemental Table 9. Uptake rate of balcinrenone into MATE1 and empty vector (control) transfected HEK293 cells

Balcinrenone concentration (μM)	Incubation time (min)	Cell line	Uptake rate (pmol/mg)		Uptake ratio
			Mean	SD	
0.5	0.5	MATE1	17.4	5.96	0.9
		Control	20.2	4.65	
	2	MATE1	27.1	4.00	1.1
		Control	23.8	2.57	
	20	MATE1	23.3	0.458	0.7
		Control	34.9	13.3	
5	0.5	MATE1	153	12.4	1.1
		Control	140	11.6	
	2	MATE1	336	44.2	1.1
		Control	318	68.3	

	20	MATE1	287	5.86	0.8
		Control	359 (n=2)[a]	ND	
20	0.5	MATE1	660	121	1.4
		Control	485	30.0	
	2	MATE1	1200	250	1.0
		Control	1250	503	
	20	MATE1	1100	45.1	0.6
		Control	1840 (n=2)[a]	ND	
50	0.5	MATE1	1480	104	1.1
		Control	1380 (n=2)[a]	ND	
	2	MATE1	2590	348	0.9
		Control	2970	968	
	20	MATE1	2690	70.2	0.7

		Control	3620 (n=2)[a]	N.D.	
5 + Reference inhibitor	0.5	MATE1	118	15.5	1.1
		Control	112	44.5	
	2	MATE1	248	33.0	1.5
		Control	166	17.6	
	20	MATE1	289	7.94	0.9
		Control	316	8.33	
20 + Reference inhibitor	0.5	MATE1	530	34.1	1.7
		Control	317	139	
	2	MATE1	1060	166	0.9
		Control	1150	280	
	20	MATE1	1330	123	0.8
		Control	1590 (n=2)[a]	ND	

ND, not determined.

[a]Replicate excluded due to protein outlier.

Supplemental Table 10. Uptake rate of balcinrenone into MATE2-K and empty vector (control) transfected HEK293 cells

Balcinrenone concentration (μM)	Incubation time (min)	Cell line	Uptake rate (pmol/mg)		Uptake ratio
			Mean	SD	
0.5	0.5	MATE2-K	19.1	0.723	1.5
		Control	13.0	2.43	
	2	MATE2-K	39.4	3.97	1.3
		Control	30.8	8.64	
	20	MATE2-K	27.0	4.27	0.9
		Control	31.7	5.17	
5	0.5	MATE2-K	296	34.7	2.1
		Control	143	39.8	
	2	MATE2-K	438	78.5	1.2
		Control	359	114	

	20	MATE2-K	414	101	0.9
		Control	483	120	
20	0.5	MATE2-K	1020	37.3	1.6
		Control	647 (n=2)[a]	ND	
	2	MATE2-K	1490	211	1.2
		Control	1210	191	
	20	MATE2-K	1480	17.3	0.7
		Control	2060	764	
50	0.5	MATE2-K	2000	46.2	1.0
		Control	2040	302	
	2	MATE2-K	3110	340	1.3
		Control	2420 (n=2)[a]	ND	
	20	MATE2-K	3610	221	0.9

		Control	3820	385	
5 + Reference inhibitor	0.5	MATE2-K	136	49.5	0.9
		Control	156	30.9	
	2	MATE2-K	227	20.2	1.0
		Control	233 (n=2)[a]	ND	
	20	MATE2-K	349	23.6	0.8
		Control	423	39.0	
20 + Reference inhibitor	0.5	MATE2-K	692	86.3	1.4
		Control	505	69.3	
	2	MATE2-K	1100	106	1.1
		Control	1030	169	
	20	MATE2-K	1840	26.5	1.3
		Control	1390	445	

ND, not determined.

[a]Replicate excluded due to protein outlier.

Supplemental Table 11. Uptake rate of balcinrenone into OAT2 and empty vector (control) transfected HEK293 cells

Balcinrenone concentration (μM)	Incubation time (min)	Cell line	Uptake rate (pmol/mg)		Uptake ratio
			Mean	SD	
0.5	0.5	OAT2	9.52 (n=2)[a]	ND	2.5
		Control	3.79	0.965	
	2	OAT2	35.4 (n=2)[a]	ND	3.2
		Control	11.1	2.50	
	20	OAT2	27.2	4.57	1.6
		Control	16.8	1.76	
5	0.5	OAT2	132 (n=2)[a]	ND	2.7
		Control	49.5	11.7	
	2	OAT2	158	3.61	1.0
		Control	151	43.6	

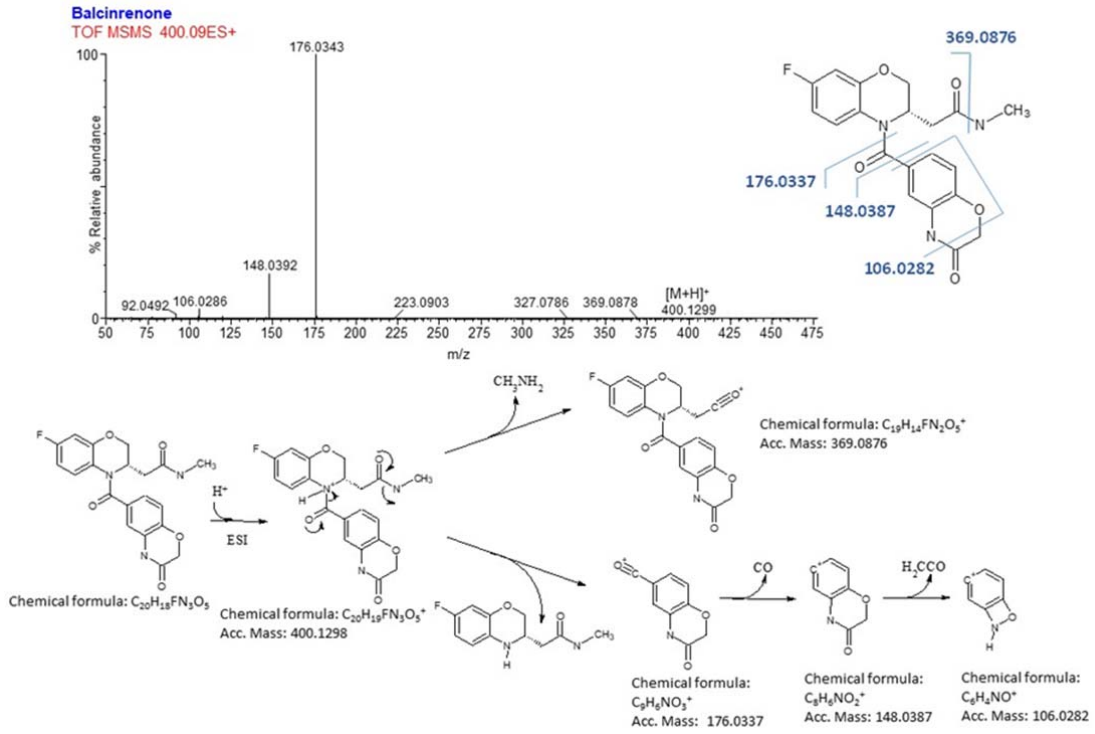
	20	OAT2	243	19.8	1.9
		Control	125	5.51	
20	0.5	OAT2	553 (n=1)[a]	ND	2.8
		Control	195	16.8	
	2	OAT2	971	209	1.9
		Control	522	93.6	
	20	OAT2	1040	138	2.0
		Control	513	64.2	
50	0.5	OAT2	1760	367	3.6
		Control	483	71.8	
	2	OAT2	3990	178	2.3
		Control	1760	454	
	20	OAT2	2650	286	1.9

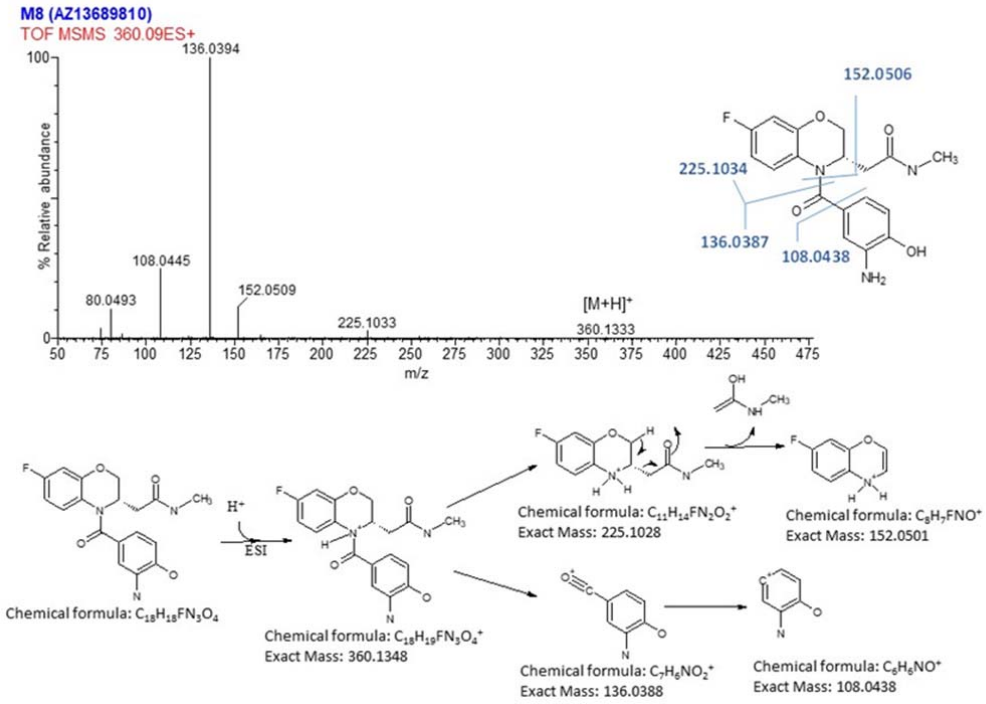
		Control	1400	155	
5 + Reference inhibitor	0.5	OAT2	183 (n=2)[a]	ND	3.1
		Control	58.4	2.52	
	2	OAT2	206	68.6	1.7
		Control	124	22.8	
	20	OAT2	188	14.6	1.3
		Control	148	7.77	
20 + Reference inhibitor	0.5	OAT2	534	111	2.3
		Control	230	40.4	
	2	OAT2	553	7.57	0.9
		Control	610	157	
	20	OAT2	641	42.3	1.2
		Control	531	8.14	

ND, not determined.

[a]Replicate excluded due to protein outlier.

Supplemental Fig. 1. High-resolution mass spectrometry product ion spectra of protonated synthetic standards of balcinrenone and M8 and their proposed fragmentation pathways





ESI, electrospray interface.