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PHASE I STUDIES

Metabolism and disposition of the anticancer quinolone derivative vosaroxin, a novel inhibitor of topoisomerase II

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Summary *Background* Vosaroxin is a first-in-class anticancer quinolone derivative that is being investigated for patients with relapsed or refractory acute myeloid leukemia (AML). The primary objective of this study was to quantitatively determine the pharmacokinetics of vosaroxin and its metabolites in patients with advanced solid tumors. *Methods* This mass balance study investigated the pharmacokinetics (distribution, metabolism, and excretion) of vosaroxin in cancer patients after a single dose of 60 mg/m^2 ^{14}C–vosaroxin, administered as short intravenous injection. Blood, urine and feces were collected over 168 h after injection or until recovered radioactivity over 24 h was less than 1% of the administered dose (whichever was earlier). Total radioactivity (TRA), vosaroxin and metabolites were studied in all matrices. *Results* Unchanged vosaroxin was the major species identified in plasma, urine, and feces. N-desmethylvosaroxin was the only circulating metabolite detected in plasma, accounting for <3% of the administered dose. However, in plasma, the combined vosaroxin + N-desmethylvosaroxin $AUC_{0-\infty}$ was 21% lower than the TRA $AUC_{0-\infty}$, suggesting the possible formation of protein bound metabolites after 48 h when the concentration-time profiles diverged. The mean recovery of TRA in excreta was 81.3% of the total administered dose; 53.1% was excreted through feces and 28.2% through urine. *Conclusions* Unchanged vosaroxin was the major compound found in the excreta, although 10 minor metabolites were detected. The biotransformation reactions were demethylation, hydrogenation, decarboxylation and phase II conjugation including glucuronidation.

Keywords Vosaroxin · Mass balance · Pharmacokinetics · Metabolite profiling · Anti-cancer · ADME

Introduction

Vosaroxin is a first-in-class anticancer quinolone derivative that intercalates DNA and inhibits topoisomerase II, leading to cell cycle inhibition, double strand DNA breaks and apoptosis in replicating cells [1–3]. Vosaroxin's core quinolone scaffold is related to the quinolone antibacterials and is structurally distinct from other topoisomerase II inhibitors used as anticancer therapies, such as anthracyclines, anthracenediones, or epipidophyllotoxins. Due to its stable quinolone structure, vosaroxin is relatively minimally metabolized and not associated with significant production of free radicals, reactive oxygen species (ROS) or toxic metabolites [1–3]. Vosaroxin is currently in development for the treatment of acute myeloid leukemia (AML) [4–7]. Currently, there is no standard treatment for relapsed and refractory patients with AML. In a phase III randomized

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trial, vosaroxin in combination with an intermediate dose of cytarabine was compared with placebo plus cytarabine [5]. Vosaroxin plus cytarabine prolonged overall survival (OS) by 1.4 months (7.5 months versus 6.1 months with placebo plus cytarabine; hazard ratio [HR] 0.87, 95% confidence interval [CI] 0.73–1.02; unstratified log-rank $p = 0.061$) and the complete response rate with vosaroxin plus cytarabine was nearly double that with placebo plus cytarabine (30% [95% CI 25.3–35.1] versus 16% [95% CI 12.6–20.6], $p < 0.0001$). This clinical benefit observed in the overall VALOR study population is driven by the benefit in patients ≥60 years of age. Although the primary analysis of OS did not reveal statistically significant difference between treatment groups, prespecified subgroup analyses demonstrated a significant OS benefit in patients ≥60 years of age (OS 7.1 months vs 5.0 months with placebo/cytarabine; HR 0.75, 95% CI 0.62–0.92; $p = 0.003$) and in those with early relapse (OS 6.7 months vs 5.2 months; HR 0.77, 95% CI 0.59–1.00; $p = 0.039$).

The plasma pharmacokinetics (PK) of vosaroxin have been studied previously in clinical studies; after a short (< 10 min) intravenous (IV) injection, the elimination of vosaroxin from plasma appeared biphasic, with a rapid initial decline followed by a prolonged terminal phase [4, 5, 8]. Little is known, however, about the metabolism of vosaroxin in humans. The metabolism of vosaroxin was investigated in rats and in vitro using human, monkey, and rat liver microsomes [1]. In rats, 37.9% of the administered radioactive dose was recovered in bile, 32.5% in feces, and 19.6% in urine, which indicates excretion was partially through bile and partially through direct secretion into the intestine [1]. Metabolite profiles in rat plasma, urine, and bile, and after microsomal incubation, suggest that the metabolic pathways of vosaroxin include glucuronidation, oxidation, N-dealkylation, and O-dealkylation [1]. N-desmethylvosaroxin was the only metabolite identified with cytotoxic activity [1]. This metabolite has also been identified in the urine of patients [4]. The urinary excretion after an IV dose of 18 mg/m^2 or 27 mg/m^2 vosaroxin up to 24 h after administration was 2.35% for vosaroxin and 0.99% for N-desmethylvosaroxin.

The primary objective of this study was to quantitatively determine the PK (distribution, metabolism, and excretion) of ^{14}C–vosaroxin and its metabolites in patients with advanced solid tumors. The safety and tolerability of vosaroxin in these patients was a secondary objective.

Material and methods

Study design and treatment

This was a phase I, open-label, single-center study. The study was conducted in accordance with International Conference on Harmonisation guidelines for Good Clinical Practice, the code of Federal Regulations, and the European Union Directive. The protocol was approved by The Netherlands Cancer Institute Independent Ethics Committee. All patients provided written, informed consent at the time of screening.

The study consisted of 2 assessment periods. Period A (PK sampling period) took place on days 1–8 of cycle 1; during this period a single dose of radiolabeled ^{14}C–vosaroxin (60 mg/m^2, up to a maximum body surface area [BSA] of 1.67 m^2, containing approximately 100 μCi total dose of radioactivity) was administered as a short IV injection (over ≤10 min) on day 1, followed by 7 days of PK sampling on an inpatient basis. Patients who completed assessment in period A had the option to continue treatment in period B. Period B took place from day 9 of cycle 1 through day 28 of cycle 4; during this period, non-radiolabeled vosaroxin (60 mg/m^2, up to a maximum BSA of 2.0 m^2) was administered as a short IV injection on day 1 of cycles 2 through 4 on an outpatient basis, with weekly safety assessments.

Study drug and reference compounds

Vosaroxin, N-desmethylvosaroxin (Fig. 1), O-desmethylvosaroxin and N,O-bisdesmethylvosaroxin were provided by Sunesis Pharmaceuticals, Inc. (South San Francisco, CA, USA) and certified by Albany Molecular Research, Inc. (Albany, NY, USA). Labeled ^{14}C–vosaroxin (chemical purity 99.7%, radiochemical purity 98.6%) was prepared by Quotient Bioresearch (Cardiff, UK). The vosaroxin injection for period A was supplied by Quotient Clinical (Nottingham, UK) and contained a mixture of ^{14}C–vosaroxin and nonlabeled vosaroxin at 10 mg/mL (10 μCi/mL, 11 mL volume per vial). Each mL also contained 45 mg of D-sorbitol to maintain isotonicity between 270 and 310 mOsmol/kg. In addition, methanesulfonic acid, 2.6 mg/mL, was added to assist in the solubilization of vosaroxin at 10 mg/mL.

Patients

Patients ≥18 years of age with histologically or cytologically confirmed advanced solid tumors considered unresponsive to

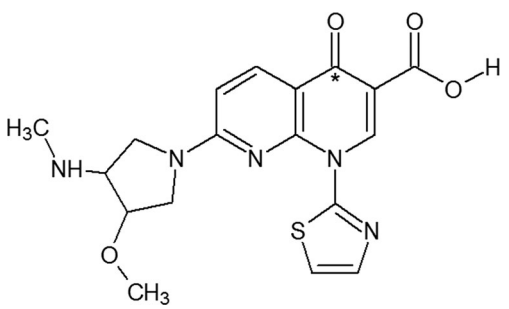

Fig. 1 Chemical structure of ^{14}C–vosaroxin. The asterisk in the ^{14}C–vosaroxin structure indicates the position of the ^{14}C–label

accepted available therapies were eligible for this study. Patients must have been able to understand and provide written informed consent. Other eligibility criteria included World Health Organization (WHO) performance status ≤2; adequate renal function (serum creatinine ≤1.5 x the upper limit of normal (ULN) and calculated creatinine clearance (CL_{CR}) ≥ 40 mL/min); and adequate hepatic function (bilirubin ≤1.5 x upper limit of normal [ULN], aspartate aminotransferase (AST) ≤ 2.5 x ULN, and alanine aminotransferase (ALT) ≤ 2.5 x ULN).

Exclusion criteria were prior treatment with vosaroxin within 60 days of enrollment; prior treatment with hematopoietic growth factors within 14 days of enrollment; pregnancy or lactation; presence of symptomatic brain metastases or active central nervous system (CNS) disease; New York Heart Association (NYHA) Class 3 or 4 heart disease, active ischemia, any uncontrolled, unstable cardiac condition; myocardial infarction within the previous 12 weeks; active, uncontrolled systemic infection; known positive test for hepatitis B surface antigen, hepatitis C antibodies, or human immunodeficiency virus (HIV); inflammatory bowel disease, occlusion of the gastrointestinal (GI) tract, significant constipation, or any significant obstruction of the GI tract; any significant obstruction of the urinary tract; history of biliary obstruction or cholecystectomy; known hypersensitivity to vosaroxin or any other components of the study treatment; any other serious medical or psychiatric condition that should preclude participation in the opinion of the investigator.

Sample collection and processing

Blood samples (6 mL) were collected via venipuncture or indwelling catheter prior to the start of ^{14}C–vosaroxin injection, at the end of injection (EOI), and 15 min, 30 min, 1 h, 1.5 h, 2 h, 4 h, 6 h, 8 h, 10 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, and 168 h after EOI. Samples were collected in dipotassium ethylene-diamine-tetraacetic acid (K_2EDTA) tubes, placed on ice, and centrifuged (2000 g, 4 °C, 10 min) within 30 min of collection. Plasma was isolated and aliquoted for total radioactivity (TRA) measurements and bioanalysis; one portion was stabilized with phosphoric acid for the screening of acyl glucuronides. In addition, 1 mL blood samples were collected 30 min, 8 h, 72 h, and 168 h after EOI for TRA analysis to determine the blood to plasma ratio of vosaroxin derived compounds.

When possible, a urine sample and feces sample was collected prior to the administration of ^{14}C–vosaroxin and used as a predose control. After ^{14}C–vosaroxin administration, urine samples were collected over 0–2 h, 2–4 h, 4–6 h, 6–12 h, and 12–24 h; thereafter, urine was collected over 24-h intervals. Feces samples were collected as voided. Urine and feces were collected through 168 h post- administration; if the TRA measured on the final scheduled collection day (144–168 h) was ≥1% of the administered radioactive dose, urine or feces collection was continued until the excreted TRA per day was less than 1% of the administered dose.

Plasma, urine, whole blood and feces homogenate aliquots were stored within the range of −70 °C and −80 °C.

Safety assessments

Adverse events (AEs) were graded according to National Cancer Institute Common Terminology Criteria for AEs (NCI CTCAE) version 4.03.

Total radioactivity analysis

The TRA in plasma, whole blood, urine and feces was determined by liquid scintillation counting (LSC). Plasma (0.2 mL) and urine (1 mL) samples were directly mixed with 10 mL liquid scintillation cocktail (Ultima Gold™, Perkin Elmer Inc., Waltham, MA, USA). To the whole blood samples (0.2 mL) 1 mL Solvable (Perkin Elmer Inc.), 0.1 mL 0.1 M EDTA, and 0.5 mL 30% hydrogen peroxide was added to dissolve and to decolorize the samples. Feces homogenates (0.2 mL) were first dissolved and decolorized using 1 mL Solvable (Perkin Elmer Inc.), 1 mL isopropanol, and 0.4 mL 30% hydrogen peroxide. The decolorization reaction was started by warming the samples in a shaking water bath of approximately 43 °C after which the samples were placed at a dark cool place for at least 1 h before adding liquid scintillation cocktail. Samples were counted on a Tri-Carb® 2800TR Liquid Scintillation Counter (Perkin Elmer Inc.). Quench correction was applied with a calibration curve of quenched radioactive reference standards. Samples were counted to a sigma 2 counting error of 1% or for a maximum of 60 min.

Concentrations of vosaroxin, N-desmethylvosaroxin, and O-desmethylvosaroxin in plasma and urine

Plasma and urine samples were analyzed using validated liquid chromatography tandem mass spectrometry (LC-MS/MS) to quantify unchanged vosaroxin and the metabolites N-desmethylvosaroxin and O-desmethylvosaroxin [9].

Plasma samples were pretreated to precipitate proteins; urine samples were diluted with human plasma to stabilize the analytes. Prepared plasma and urine extracts were injected on a Symmetry Shield RP8 column, and gradient elution was applied using 0.1% formic acid in water and acetonitrile-methanol (50:50, *v*/v); O-desmethylvosaroxin in urine was separated using a Synergi Hydro RP column using the same eluent. Analyses were performed with a triple quadruple mass spectrometer in positive-ion mode. A deuterated isotope of vosaroxin was used as internal standard. The validated plasma and urine assays quantify vosaroxin and N-

desmethylvosaroxin in the concentration range of 2–500 ng/mL, and O-desmethylvosaroxin in the concentration range of 4–500 ng/mL. Quality control samples were prepared and analyzed together with the study samples, and acceptance criteria for bioanalytical data during routine drug analysis, as described in the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines, were applied [10, 11].

Pharmacokinetic analysis

PK parameters for vosaroxin, N-desmethylvosaroxin, O-desmethylvosaroxin and TRA in plasma were estimated by noncompartmental methods using Phoenix® WinNonlin software (Version 6.4, Pharsight Corporation, Mountain View, CA, USA, 2003).

Metabolite profiling

The metabolite profiling approach consisted of two parts: 1) metabolite screening and identification and 2) metabolite quantification.

Pooling of samples

Pooled plasma, urine and feces samples were analyzed during the metabolite screening and identification using LC-LSC-MS^n. From each plasma collection time point up to 24 h (n = 6 per time point) 100 μL plasma was pooled to obtain a 600 μL sample. For plasma samples collected after 24 h (72 h and 96 h), 200 μL of each sample was pooled to obtain a 1200 μL sample. In addition, plasma samples stabilized with phosphoric acid were screened for acyl glucuronides. Urine and feces samples were pooled in 24-h intervals (intra-patient pools and subsequently inter-patient pools). Pooling accuracy was determined by calculating the difference between the theoretical radioactivity of the pooled sample to the actual radioactivity as measured using LSC model Tri-carb 2800 TR or Tri-carb 4810 TR (Perkin Elmer Life and Analytical Sciences, Inc., Waltham, MA), equipped with a quenching correction system.

Sample preparation

Plasma samples from EOI to 24 h were pretreated using protein precipitation by adding 900 μL acetonitrile/methanol (50:50, *v*/v) to 300 μL plasma followed by mixing (10 s), shaking (10 min at 1250 rpm) and centrifugation (10 min at 23,100 g). The clear supernatant was evaporated to dryness using nitrogen at 40 °C and reconstituted with 100 μL acetonitrile/water/formic acid (100:900:1, *v*/v/v). For the recovery calculations 20 μL of the final extract was used for LSC analysis and the remaining extract was transferred to an auto sampler vial and stored at −70 °C until further analysis. The plasma samples used for screening that were collected after 24 h were pretreated using protein precipitation by adding 3 mL acetonitrile/methanol (50:50, *v*/v) to 1 mL plasma followed by the same procedures as described above. The radioactivity recovery after sample pretreatment was determined. Urine samples were vortex mixed and directly injected onto the high-performance liquid chromatography (HPLC) system.

Feces samples were pretreated by adding 600 μL of 50 mM formic acid in acetonitrile/water (50:50, *v*/v) to 300 μL feces homogenate followed by mixing (10 s), ultrasonication (10 min), mixing (10 s) shaking (1 h at 1250 rpm) and centrifugation (10 min at 23,100 g). The clear final extract was transferred to an auto sampler vial and stored at −70 °C until further analysis and 20 μL of the final extract was used for LSC analysis for recovery calculations. The radioactivity recovery after sample pretreatment was determined.

LC-MS systems for metabolite profiling

Two chromatographic systems were used: one to obtain radiochromatographic data and normal resolution MS data (LC-LSC-MS^n) and the other to obtain high-resolution MS data (LC-MS^n). The chromatographic and MS settings on both systems were the same.

The LC-LSC-MS method used a Shimadzu LC-20 AD pump and SIL-HTc autosampler (4 °C) (Shimadzu, Kyoto, Japan). A Synergi Hydro RP 80 Å column (150 × 4.6 mm ID, 4 μm particles; Phenomenex, Torrance, CA, USA) was preceded by a 0.2 μm inlet filter (Upchurch Scientific, Oak Harbor, WA, USA) and warmed to 35 °C. Mobile phase A was 0.1% formic acid in water and mobile phase B was acetonitrile. The gradient started at 10% B for 2 min, increasing to 25% B over 43 min, then increasing to 90% over 2 min, after which the mobile phase was switched several times from 10% B to 90% B as a washing step, and finally the system was stabilized at 10% B for 3 min. The total run time was 60 min. The flow rate was 1 mL/min; a post-column splitter (Accurate Splitter, LC Packings, Sunnyvale, CA, USA) was used to direct one-quarter of the flow to the mass spectrometer and three-quarters of the flow to a fraction collector (LKB-FRAC-100, Amersham Biosciences AB, Uppsala, Sweden), which collected 1-min fractions in liquid scintillation vials. After addition of 4 mL scintillation cocktail, the TRA of each fraction was determined on the Tri-carb 2800TR liquid scintillation counter, using a counting time of 20 min. The resulting values were used to construct the radiochromatograms. If the peak in the radiochromatogram was above 14 dpm, the metabolite was quantified.

A linear ion trap mass spectrometer (LTQ XL, Thermo Electron, Waltham, MA) was used for the LC-LSC-MS analysis, applying positive ionization and using a scan range of

100–1100 amu. The sheath gas, aux gas, and sweep gas were 30, 15, and 5 arbitrary units. The spray voltage was 5.0 kV and the normalized collision energy was 35 V. Data-dependent acquisition using a predefined parent list was used to collect the MS data. Total ion current (TIC) chromatograms were obtained and MS/MS spectra were generated from each *m/z* signal above a threshold of 750 cps when a match was found with a (predicted) parent list. Spectra of unpredicted metabolites were obtained by comparing TIC chromatograms of predose samples with those of postdose samples. Most abundant ions at the retention time of the potential metabolite were selected and added to the parent list. Samples were re-injected and the MS/MS spectra of the unpredicted metabolites were obtained.

Based on the normal resolution MS results, one urine sample was selected that contained all potential metabolites and was analyzed with a high resolution mass spectrometer for accurate mass determination. The high resolution LC-MS setup consisted of an Ultimate 3000 Standard LC System (Dionex, Sunnyvale, CA, USA) with the autosampler set to 10 °C. A Synergi Hydro RP 80 Å column (150 × 0.5 mm ID, 4 μm particles; Phenomenex) was used with the same mobile phases and gradient described for the LC-LSC-MC analysis. This system was coupled to an LTQ Orbitrap XL system (Thermo Electron) using the same settings as described previously for the LTQ XL. Relative retention times were used to identify the metabolites and high-resolution MS^1 and MS^2 spectra of ions were obtained using a full data scan and a parent scan.

Metabolite identification and quantification

$LC\text{-}MS^n$ was used as primary tool to elucidate the molecular structures of the metabolites. The metabolites were identified by comparing the HPLC retention times and fragmentation patterns (MS^1, MS^2, and MS^3 spectra) to the results from the available reference standards. If reference standards were not available, the mass of the molecular ions (high-resolution if available) and fragmentation patterns were used to identify the metabolite. Because vosaroxin and N-desmethylvosaroxin in feces were not separated chromatographically, $LC\text{-}MS^n$ data from the ion trap MS were used to determine the ratio between these compounds.

Results

Patients and safety

Seven patients with solid tumors were enrolled in the study. One patient died of intracranial hemorrhage prior to treatment; therefore, six patients received treatment. Patient characteristics are summarized in Table 1.

Table 1 Baseline patient and disease characteristics

Characteristic	*N* = 6
Median (range) age, years	60.0 (42–71)
Sex, n (%)	
Male	4 (67)
Female	2 (33)
Race, n (%)	
Caucasian	6 (100)
Median (range) weight, kg	73.60 (52.0–86.0)
Median (range) height, cm	176.5 (162–187)
Median (range) body surface area, m^2 [range])	1.92 (1.5–2.1)
Primary cancer type, n (%)	
Colon	1 (17)
Gastric	1 (17)
Lung, non-small cell	1 (17)
Lung, small cell	1 (17)
Ovarian	1 (17)
Rectal	1 (17)
Median (range) time since diagnosis, mo	30.0 (18.5–76.1)
Disease stage at study entry, n (%)	
IV	5 (83%)
IVA	1 (17%)
ECOG performance status, n (%)	
0	5 (83%)
1	1 (17%)

ECOG Eastern Cooperative Oncology Group, *n* number

All 6 treated patients completed period A. One patient died of disease progression prior to period B; the remaining 5 patients received treatment with non-radiolabeled vosaroxin. Of these 5 patients, 1 patient completed 8 cycles of treatment and was ongoing at the time of database lock, and 4 patients discontinued treatment prior to completing all 4 planned treatment cycles (1 due to a serious treatment-emergent adverse event (TEAE) of grade 3 bone pain related to disease progression; 2 due to disease progression per Response Evaluation Criteria In Solid Tumors (RECIST) v 1.1; and 1 due to clinical disease progression). Among all treated patients, the median number of doses received was 2.5 (range 1 to 8), and the median duration of exposure was 43 days (range 1 to 203 days).

The most common TEAEs (occurring in 2 or more patients) were: neutrophil count decreased (n = 6), nausea (n = 4), abdominal pain (n = 2), constipation (n = 2), fatigue (n = 2), pyrexia (n = 2), and vomiting (n = 2). All other TEAEs were reported in 1 patient and indicative of the underlying disease and/or treatment. Two patients experienced one or more grade 3 TEAEs: grade 3 gastroesophageal reflux disease, neutrophil count decreased, white blood count (WBC) count decreased, bone pain and musculoskeletal pain were

each reported in 1 patient. Four patients experienced one or more grade 4 TEAEs: grade 4 neutrophil count decreased was reported in 4 patients and grade 4 WBC count decreased was reported in 1 patient. No patient had a grade 5 TEAE. Two patients had a serious TEAE (grade 3 bone pain and grade 2 gastroesophageal reflux disease); neither considered related to study treatment. Vosaroxin dose was reduced in 4 patients due to reduced WBC count (n = 2) or neutrophil count (n = 2).

There were no clinically meaningful shifts in hematology values, serum chemistry, urinalysis, vital signs and Eastern Cooperative Oncology Group (ECOG) performance status. As expected for this patient population, the majority of patients had low values related to WBC (and differential) counts both at baseline and throughout the study.

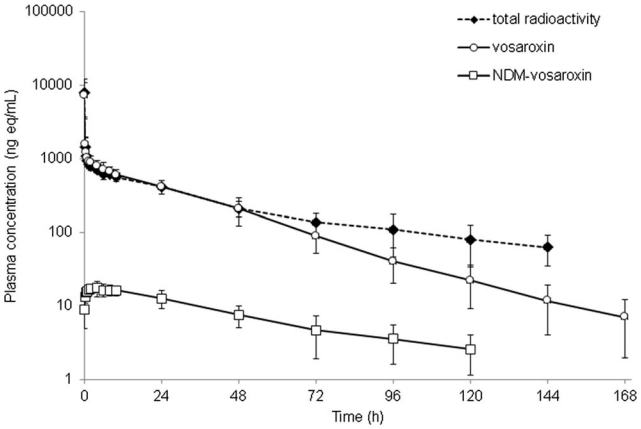

Fig. 2 Mean (± SD) log-linear plasma concentration-time curves of total radioactivity, vosaroxin, and N-desmethylvosaroxin (NDM-vosaroxin) in plasma

Quantitative bioanalysis and total radioactivity

Pharmacokinetics

Table 2 summarizes the mean PK parameters for TRA, vosaroxin, and N-desmethylvosaroxin after the administration of 60 mg/m^2 ^{14}C–vosaroxin. Corresponding plasma concentration-time curves are presented in Fig. 2. Plasma levels of O-desmethylvosaroxin were below the limit of quantification across all timepoints in all patients, so PK parameters were not derived for this metabolite.

Plasma concentrations of vosaroxin declined in a biphasic manner that was characterized by a short initial distribution phase followed by a prolonged elimination phase with measurable concentrations up to the last scheduled time point of 168 h. Median C_{max} was 7200 ng/mL and median t_{max} was 2 min, approximately coinciding with the end of the short infusion. The mean $AUC_{0-\infty}$ was 29,300 ng·hr./mL with a mean $t_{1/2}$ of 25.5 h. Plasma concentrations of N-desmethylvosaroxin were much lower than vosaroxin plasma concentrations, with a mean C_{max} of 18.3 ng/mL and $AUC_{0-\infty}$ was 963 ng·hr./mL. The shape of the concentration time curve for TRA in plasma was similar to those for vosaroxin and for the sum of vosaroxin and N-desmethylvosaroxin. TRA levels in plasma were similar to the sum of vosaroxin and N-desmethylvosaroxin levels up to 48 h, after which the elimination of vosaroxin and N-desmethylvosaroxin was somewhat faster than that of TRA, suggesting additional metabolism. The blood to plasma ratio of TRA was approximately 0.86 with no time dependency, indicating modest distribution to red blood cells.

Excretion balance

An overview of the average mass balance up to 168 h after administration is shown in Fig. 3. The mean total recovery in

Table 2 Mean (± SD) plasma pharmacokinetic parameters for total radioactivity, vosaroxin and its metabolite N-desmethylvosaroxin following a single intravenous injection of 60 mg/m^2 (100 μCi) ^{14}C–vosaroxin

Parameter	Vosaroxin	N-desmethyl-vosaroxin	Total radioactivity[b]
C_{max} (ng/mL)	7200 ± 3750	18.3 ± 2.75	7000 ± 3700
t_{max} (h)[a]	0.0417 [0.017–0.050]	3.03 [0.533–6.02]	0.0417 [0.017–0.050]
$AUC_{0-\infty}$ (ng·hr./mL)	29300 ± 7000	963 ± 240	38700 ± 8570
$t_{1/2}$ (hr)	25.5 ± 5.02	35.8 ± 9.29	35.1 ± 9.39
$V_{ss,normalized}$ (L/m^2)	56.0 ± 8.61	NA	62.5 ± 11.4
$CL_{normalized}$ (mL/h/m^2)	1830 ± 363	NA	1380 ± 267
V_z (L)	126 ± 32.1	NA	129 ± 32.7
CL (mL/h)	3440 ± 751	NA	2610 ± 620
CL_R (mL/h)	193 ± 94.9	NA	782 ± 201

NA Not applicable, C_{max}, maximum plasma concentration, t_{max} time at which the maximum plasma concentration was reached $AUC_{0\text{-inf.}}$ area under the plasma concentration-time curve from zero up to infinity $t_{1/2}$ terminal exponential half-life, $V_{ss,normalized}$ the estimated normalized volume of distribution $CL_{normalized}$ the normalized plasma clearance, V_z the apparent volume of distribution, CL plasma clearance CL_R renal clearance

[a] Median [range]

[b] Concentration unit for total radioactivity is ng eq/mL

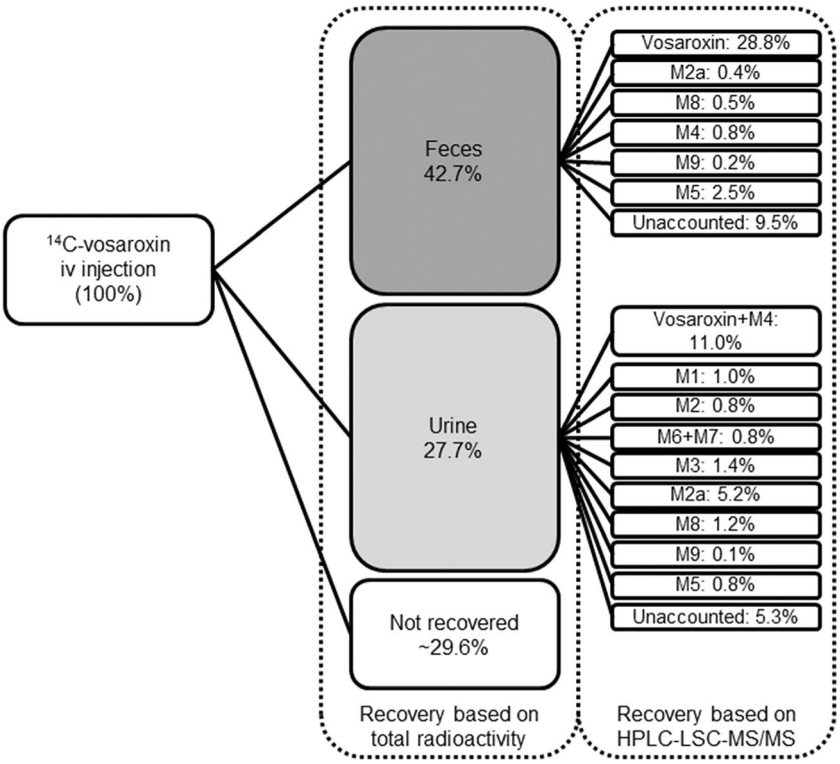

Fig. 3 Overview of the average mass balance of ^{14}C–vosaroxin in 6 cancer patients following a 60 mg/m^2 (100 μCi) intravenous injection of ^{14}C–vosaroxin. Data are from intrapatient pooled samples up to 168 h after administration

excreta after 168 h after administration represented 70.4% of the administered radioactivity. However, small amounts of radioactivity were still present in the last scheduled collection of excreta of some patients. As such urine and/or feces collections continued beyond 168 h. The mean total recovery in excreta after the total collection period (range from 168 h to 528 h postdose for individual patients) was 81.3% (SD ± 8.60) of the administered radioactivity.

Mean cumulative urinary recovery of TRA and unchanged vosaroxin plus vosaroxin metabolites (N-desmethylvosaroxin and O-desmethylvosaroxin) shown in Fig. 4. The mean cumulative urinary recovery of TRA over the sampling period up to 192 h was 28.2% (SD ± 2.65) of the administered radioactivity. Over this time interval, the urinary recovery of vosaroxin, N-desmethylvosaroxin and O-desmethylvosaroxin were 5.4%, 4.0%, and 0.2% of the administered dose, respectively. The sum of these three analytes account for 9.7% (compared with 28.2% cumulative recovery of TRA), indicating additional metabolism. Urinary excretion reached a plateau by 96 h to 120 h after administration.

The mean cumulative recovery of TRA in urine and feces up to 192 h is shown in Fig. 5. Substantial fecal excretion of TRA

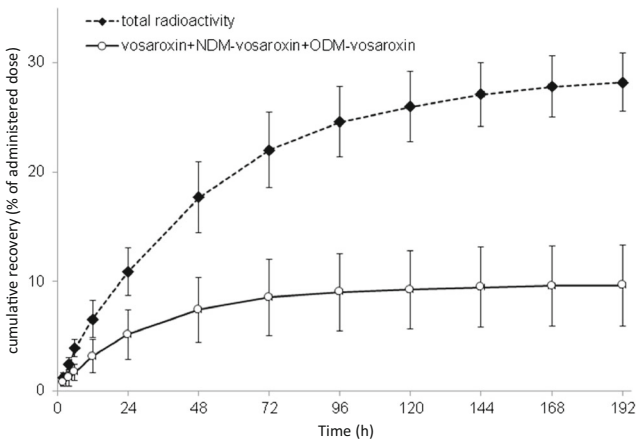

Fig. 4 Mean (±SD) cumulative urinary excretion of total radioactivity and sum of unchanged vosaroxin, N-desmethylvosaroxin (NDM-vosaroxin), and O-desmethylvosaroxin (ODM-vosaroxin) up to 168 h after a 60 mg/m^2 (100 μCi) intravenous injection of ^{14}C–vosaroxin

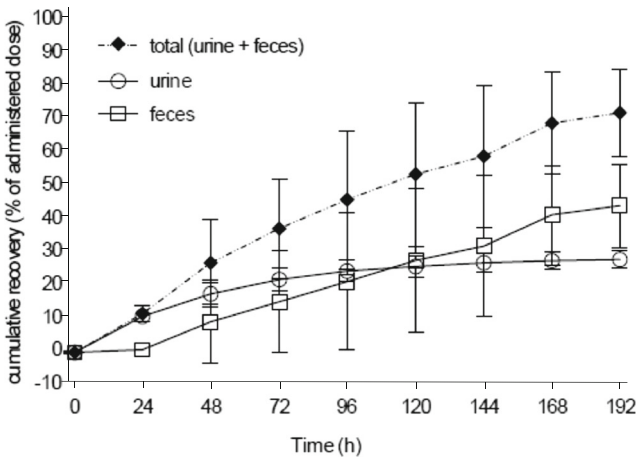

Fig. 5 Mean (± SD) cumulative recovery of ^{14}C–vosaroxin-derived total radioactivity in urine, feces, and in total for the period of 192 h following intravenous injection (60 mg/m^2) of ^{14}C–vosaroxin

was observed in these patients but occurred more slowly than via urine. After 168 h the total recovery of TRA in feces was 41.5% of the administered radioactivity. The mean cumulative recovery of TRA in feces over the entire sampling period up to 528 h was 53.1% (SD ± 7.00) of the administered dose.

Metabolite profiling

Plasma

Metabolite screening and identification in plasma was done with inter-patient pooled samples. The pooling accuracy was between −2.1% and 8.3%. The mean recovery of radioactivity in the inter-patient pooled plasma samples after pretreatment was 79.3% (76.3% in samples stabilized with phosphoric acid). Mean recovery decreased with sampling time, from 98.5% in the EOI sample to 54.2% in the 96 h sample, suggesting formation of protein-bound metabolites not extracted by sample pretreatment.

Similar radiochromatographic profiles were obtained for all plasma samples. Only vosaroxin (8) and N-desmethylvosaroxin (9) were present in plasma, with vosaroxin the predominant species present and N-desmethylvosaroxin contributing a small fraction of total exposure. In the plasma samples stabilized with phosphoric acid in order to detect and identify acyl glucuronides, no glucuronide species were found.

Urine

Intra- and inter-patient pooling was done for urine samples. Pooling accuracy for the inter-patient pooled samples (used for metabolite screening and identification) was between −6.3% and −0.5%. Pooling accuracy for the intra-patient pooled samples (used for the metabolite quantification) was between 8.6% and 1.5%. Initially, larger deviations in pooling accuracy were observed due to adsorption; however, the results reported here were achieved by thawing the samples at 2–8 °C and by using only one freeze/thaw cycle.

Figure 6 displays radiochromatograms showing the metabolite profile in urine over time from a representative patient. The peaks were designated based on the LC-MS^n data. Unchanged vosaroxin (9) and N-desmethylvosaroxin (8) were the predominant peaks found in urine and the metabolite profile did not change considerably over time. Table 3 summarizes the amount excreted in urine for each metabolite. Unchanged ^{14}C–vosaroxin (9) and N-desmethylvosaroxin (8) were not separated on the chromatographic system used for metabolic profiling; quantification of these two compounds using LC-MS/MS showed recovery of 5.4% of the administered dose for vosaroxin and 4.0% for N-desmethylvosaroxin. All other metabolites that were detected accounted for a small fraction of radioactivity. A mean of 5.3% of the administered radioactivity found in urine was not accounted for in the radiochromatograms.

Feces

Intra- and inter-patient pooling was done for feces samples. The mean sample pretreatment recovery of the radioactivity for all feces samples (including inter-patient pooled feces samples and intra-patient pooled samples) was 79.4%. The pooling accuracy of the inter-patient pooled samples (used for metabolite screening and identification) was between −1.4% and 10.5%. The pooling accuracy of the intra-patient pooled samples (used for the metabolite quantification) was between −4.8% and 6.7%.

Figure 6 displays radiochromatograms showing the metabolite profile of vosaroxin in feces over time from a representative patient. The peaks were designated based on the LC-MS^n data. Just as in urine, unchanged vosaroxin (9) was predominant in feces and the metabolite profile did not change over time. Unchanged ^{14}C–vosaroxin (9) accounted for a mean of 28.8% of the total administered dose. All other compounds accounted for small fractions of radioactivity (Table 3). A mean of 9.5% of radioactivity found in feces was not accounted for in the radiochromatograms.

Identification of metabolites

Metabolites were named in line with previously published data [1]. In addition, the metabolites were assigned a number according to the retention time of the metabolite as measured in this study. For example, M1 (1): M1 corresponds to the name provided in the earlier report [1] and (1) corresponds to the number assigned by retention time in this study. Supplementary Table 1 summarizes the accurate mass data and structural information for vosaroxin and its metabolites in human plasma, urine and feces samples. Supplementary Fig. 1 shows the proposed metabolite structure and fragmentation and the MS^2 and MS^3 spectra of the proposed metabolites. The proposed metabolic pathway in human is illustrated in Fig. 7. Structural characterization for each metabolite is described below.

Vosaroxin (9) The identity of vosaroxin (9) was confirmed by comparing to the MS, MS/MS spectrum, and retention time of the vosaroxin reference standard. The protonated molecular ion at *m/z* 402 gave product ions at *m/z* 384 and 358 (loss of hydroxyl and the total carboxylic acid group, respectively).

M1 (1) The MS spectrum of M1 (1) showed an addition of 176 Da relative to *m/z* 402, the mass of vosaroxin (9). The formation of a fragment ion at *m/z* 402 and the addition of 176 Da indicates the formation of a

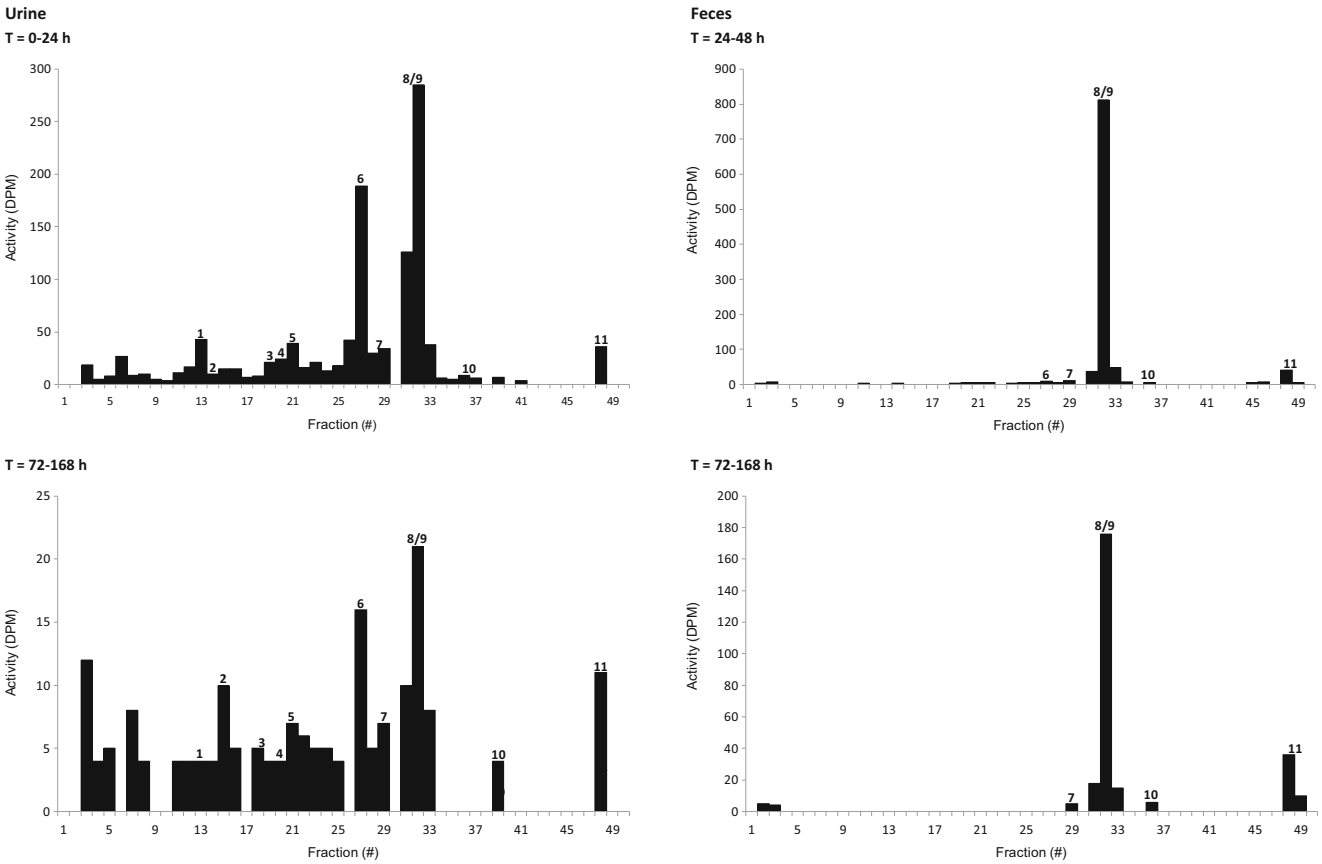

Fig. 6 Representative radiochromatograms of human urine and feces samples collected over different time intervals (urine: 0–24 h and 72–168 h; feces: 24–48 h and 72–168 h) after administration of a single intravenous 60 mg/m^2 dose of ^{14}C–vosaroxin. The numbers in the radiochromatograms correspond with the following ID numbers: 1 = M1, 2 = M2, 3 = M6, 4 = M7, 5 = M3, 6 = M2a, 7 = M8, 8 = M4, 9 = vosaroxin, 10 = M9, 11 = M5. Note that the scale of the y-axis is different in each radiochromatogram

glucuronide conjugate. At the retention time of M1 (1), MS and MS/MS spectra identical to the spectra of vosaroxin were observed, which supports the identification of M1 (1) as a glucuronide adduct. The determination of the accurate mass of this metabolite also supported this proposed identity.

M2 (2) The MS spectrum of M2 (2) showed an addition of 2 Da relative to the mass of vosaroxin (9). Two predominant product ions, *m/z* 360 and 386, also showed an addition of 2 Da relative to the product ions of vosaroxin. Two other product ions with *m/z* 269 and 332 were similar to the product ions of vosaroxin. This indicated addition of two hydrogen atoms on the pyrrolidine ring. The proposed structure is therefore dihydrovosaroxin, which was supported by the accurate mass data.

M6 (3) The MS spectrum of M6 (3) showed a loss of 28 Da relative to the mass of vosaroxin (9). Three predominant product ions, *m/z* 356, 330, and 304, also showed a loss of 28 Da relative to the product ions of vosaroxin. One product ion with *m/z* 269 was similar to the product ion of vosaroxin. This indicates the loss of two methyl groups. The MS, MS/MS spectrum and retention time of M6 (3) in the urine samples corresponded to the reference standard of M6 (3) which is N,O-bis-desmethylvosaroxin.

M7 (4) The MS spectrum of M7 (4) showed a loss of 14 Da relative to the mass of vosaroxin (9). Two other metabolites were identified with a loss of 14 Da compared with vosaroxin (9): M3 (5) and M4 (8) corresponding to ODM-vosaroxin and NDM-vosaroxin, respectively. The MS, MS/MS spectrum of M7 (9) in the urine samples corresponded to the reference of M3 (5), although the retention time did not correspond. Thus it is proposed that M7 (4) is possibly a conjugate of M3 (5); however, this could not be confirmed.

M3 (5) The MS spectrum of M3 (5) showed a loss of 14 Da relative to the mass of vosaroxin (9). This metabolite has previously been identified as ODM-vosaroxin [1]. The MS, MS/MS spectrum, and retention time of M3 (5) in the urine samples corresponded to the reference of M3 (5).

Table 3 Summary of vosaroxin metabolite profiling

Compound			Plasma exposure[1]		Amount excreted[1]			
Compound (ID number)	RT (min)	Proposed identity	*LSC* metabolite screening	*LC-MS/MS* Relative $AUC_{0\text{-}168h}$ (% of $AUC_{0\text{-}168h}$ of total ^{14}C) [2]	Urine (0-168 h)		Feces (0-168 h)[3]	Total (0-168 h)
					LSC metabolite screening	*LC-MS/MS* quanti-tation		
M1 (1)	12	Vosaroxin-glucuronide	ND	NQ	1.0%	NQ	ND	**NA**
M2 (2)	14	Dihydro-vosaroxin	ND	NQ	0.8%	NQ	ND	**0.8%**
M6 (3)	19	N,O-bis-desmethyl-vosaroxin	ND	NQ	0.8%[4]	NQ	ND	**0.8%**[4]
M7 (4)	19	O-desmethyl-vosaroxin related metabolite (phase II conjugate)	ND	<LLOQ		NQ	ND	
M3 (5)	20	O-desmethyl-vosaroxin	ND	NQ	1.4%	0.2%	ND	**1.4%**
M2a (6)	27	Dihydro-decarboxylic acid vosaroxin	ND	NQ	5.2%	NQ	0.4%	**5.6%**
M8 (7)	28–29	Unknown	ND	NQ	1.2%	NQ	0.5%	**1.7%**
M4 (8)	31	N-desmethyl-vosaroxin	NQ	2.5%	11.0%[5]	4.0%	0.8%	**4.8%**
Vosaroxin (9)	32	Parent drug	NQ	84.6%		5.4%	28.8%	**32.6%**
M9 (10)	35–37	Unknown	ND	NQ	0.1%	NQ	0.2%	**0.3%**
M5 (11)	47–48	Unknown	ND	NQ	0.8%	NQ	2.5%	**3.3%**
Radioactivity in matrix			NA		**27.7%**	NA	**42.7%**	**70.4%**
Supernatant after sample preparation			NA		NA[5]	NA	34.6%	**NA**
Total accounted for via radiochromatograms			NA		22.4%	NA	33.1%	**NA**
Unaccounted for (sample pretreatment loss + not detected in radiochromatogram)			NA		5.3%	NA	9.5%	**NA**

ID identification, *ND* not detected (<LOD (4 DPM)), *NA* not applicable, *NQ* detected but not quantified

[1] Fractions < LOD (4 DPM) were regarded as containing 0 DPM

[2] Calculated from plasma concentrations at EOI, 15 min, 30 min, 1 h, 2 h, 6 h, 8 h, 10 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h and 168 h after EOI (or up to the last point if no data was available)

[3] The amount excreted for feces was determined for 5 patients instead of 6 patients

[4] This is the total amount excreted of two metabolites (combined radioactive fractions)

[5] Urine samples were analyzed without sample pretreatment

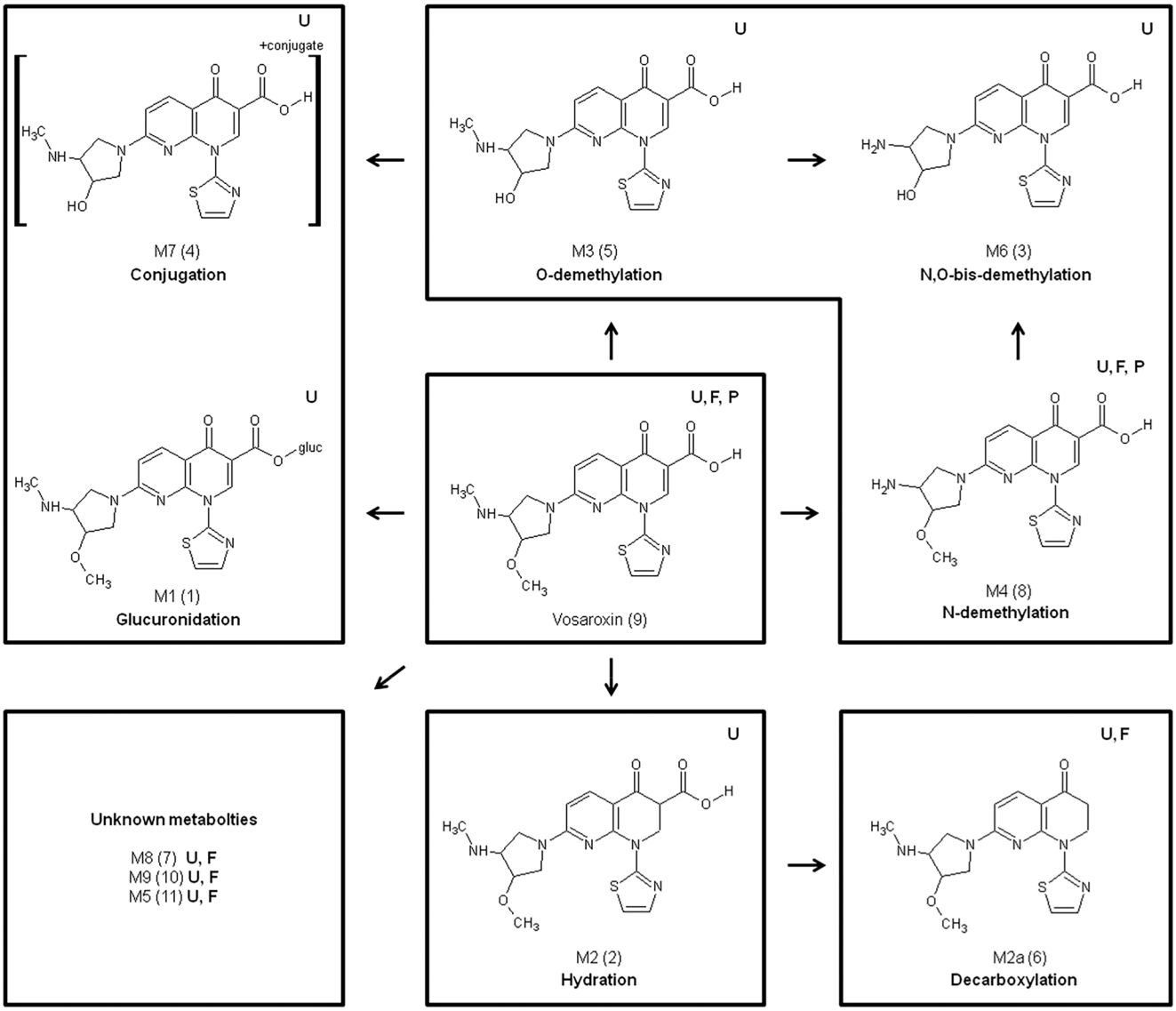

Fig. 7 Proposed metabolite pathway of vosaroxin including demethylation, hydrogenation, decarboxylation and phase II conjugation. The asterisk in the ^{14}C–vosaroxin structure indicates the position of the ^{14}C–label. F: feces; P: plasma; U: urine

M2a (6) The MS spectrum of M2a (6) showed a loss of 32 Da relative to *m/z* 402, the mass of vosaroxin (9) and a loss of 34 Da relative to *m/z* 404, the mass of M2 (2). This indicated the loss of a carboxylic acid compared with M2 (2). M2a was therefore proposed to be dihydrodecarboxylic acid vosaroxin which was supported by the accurate mass data.

M4 (8) The MS spectrum of M4 (8) shows a loss of 14 Da relative to the mass of vosaroxin (9). This metabolite has previously been identified as N-desmethylvosaroxin [1]. The MS, MS/MS spectrum, and retention time of M4 (8) in the urine samples corresponded to the reference of M3 (5).

Unknown metabolites Metabolites M8 (7), M9 (10), and M5 (11) did not yield detectable molecular ions when subjected to mass spectrometry analysis. Therefore, no structures are proposed for these potential metabolites.

Discussion

This study investigated the PK, including the identification of major circulating and excretory metabolites, of vosaroxin in adults with advanced solid tumors. In these patients, vosaroxin 60 mg/m^2 was generally well tolerated, with a median of 2.5 doses administered. The TEAEs most frequently reported were GI disorders, decreased neutrophil count, nausea, abdominal pain, constipation, fatigue, pyrexia, and vomiting, which is consistent with the previously reported safety profile of vosaroxin [4–8].

Plasma pharmacokinetics

The PK of ^{14}C–vosaroxin was investigated after a single infusion of ^{14}C–vosaroxin. Plasma concentration time profiles showed a biphasic elimination of vosaroxin, consisting of a rapid decline followed by a prolonged monophasic elimination, which was consistent with results from previous studies in cancer patients [4, 8]. The mean apparent volume of distribution (126 L) exceeded the estimated extracellular fluid volume of 18.2 L [12]), and the mean plasma CL (3440 mL/h or 57.3 mL/min) was approximately 4% of the estimated hepatic plasma flow rate of 1450 mL/min [12], suggesting that vosaroxin readily distributes beyond the extracellular space and is a low clearance drug.

Metabolite profile in humans

N-desmethylvosaroxin was the only metabolite detected in plasma. The relative $AUC_{0\text{-}168h}$ of N-desmethylvosaroxin was 2.5% of the total ^{14}C $AUC_{0\text{-}168h}$, which indicates that N-desmethylvosaroxin is not a major metabolite according to the FDA and ICH guidelines [10, 13]. In preclinical studies, N-desmethylvosaroxin exhibited cytotoxicity levels similar to vosaroxin [1]; however, as a minor metabolite it is expected that N-desmethylvosaroxin would provide only a slight contribution to the efficacy and toxicity of vosaroxin treatment. Although no additional metabolites were detected in plasma, the 21% difference between the $AUC_{0\text{-}inf}$ for vosaroxin and N-desmethylvosaroxin versus the TRA in plasma suggests the presence of additional metabolites. It is possible that protein bound metabolites were present that were not extracted during sample pretreatment and were therefore not detected.

Unchanged vosaroxin was the largest single component in both urine and in feces. In urine, N-desmethylvosaroxin and M2a (6) were relatively abundant vosaroxin-related compounds, with 8 other detected metabolites excreted in very low quantities. In feces, 5 metabolites were detected, but only minor metabolism was observed and unchanged vosaroxin was by far the largest single component. This minor metabolism can be explained by direct intestinal secretion which was previously observed in rats [1]. The high fraction of vosaroxin recovered in fecal material, taken together with the low percentage of unidentified radioactivity, suggests that biliary transport and metabolism is not a major component of vosaroxin clearance and that vosaroxin is not a highly metabolized drug.

The vosaroxin metabolites that were identified in this study were generally consistent with those previously identified in vitro and in rats [1]. Phase I metabolic conversions included O-demethylation, N-demethylation, and a combination, hydrogenation of the 4-pyridone and subsequent decarboxylation. Phase II metabolism, including glucuronidation of vosaroxin and an O-desmethylvosaroxin conjugate, was detected.

Excretion in humans and clinical implications

An average of 81.3% of the administered radioactivity was recovered in urine and feces over 22 days; approximately 69% was recovered within 7 days after the end of injection. The apparent high volume of distribution may indicate some tissue binding, which might explain why a higher percentage recovery was not observed; however, this recovery is not unusual [14] and is in line with previous mass balance study results in rats [1]. A higher recovery may have been obtained if the collection time had been further extended, but the added value of additional excretion data was considered limited.

Approximately 53.1% of the radioactivity was excreted in feces and 28.2% in urine. The mean renal clearance was 193 mL/h (3.22 mL/min) for vosaroxin and 782 mL/h (13.0 mL/min) for TRA, which is approximately 1% or less of the estimated renal blood flow rate of 1240 mL/min [12]. These results indicate that the major route of vosaroxin elimination is via feces, although both hepatic and renal processes contribute to the elimination of vosaroxin.

Conclusions

In conclusion, vosaroxin is a low clearance drug that readily distributes beyond the extracellular space and shows a biphasic elimination. No major metabolites were detected in plasma, urine, or feces. N-desmethylvosaroxin was the only circulating metabolite detected and accounted for <3% of the total administered dose. Additional minor metabolites were identified in urine and metabolite profiling indicated that vosaroxin is metabolized through demethylation, hydrogenation, decarboxylation and phase II conjugation, including glucuronidation. Vosaroxin was excreted primarily as unchanged drug in feces, with feces being the major route of elimination (53.1%) versus renal (28.2%), indicating that vosaroxin is not a highly metabolized drug. Vosaroxin appeared to be well tolerated in patients with advanced solid tumors, with a safety profile consistent with previous reports.

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Compliance with ethical standards

Conflict of interest GCJ is employed by Sunesis Pharmaceuticals. JAF was previously employed by and is a consultant for Sunesis Pharmaceuticals. DRM is president of Projections Research ink, a consulting company to the pharmaceutical industry. CMN, LL, HR, ADRH, MM-R, JHMS, and JHB have no conflicts of interest to declare.

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Ethical approval All procedures in this study were conducted in accordance with the ethical standards of the International Conference on Harmonisation guidelines for Good Clinical Practice, the code of Federal Regulations, and the European Union Directive. The protocol was approved by The Netherlands Cancer Institute Independent Ethics Committee.

Informed consent Written, informed consent was obtained from all individual participants at the time of screening.

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