Metabolism of (+)-1,4-Dihydro-7-(trans-3-methoxy-4-methylamino-1-pyrrolidinyl)-4-oxo-1-(2-thiazolyl)-1,8-naphthyridine-3-carboxylic Acid (Voreloxin; Formerly SNS-595), a Novel Replication-Dependent DNA-Damaging Agent

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ABSTRACT:

Voreloxin is a replication-dependent DNA-damaging agent that intercalates DNA and inhibits topoisomerase II, resulting in double-stranded DNA breaks, irreversible G2 arrest, and rapid apoptosis. The topoisomerase II-associated DNA intercalation and DNA damage produced by voreloxin are highly selective and show selectivity for proliferating cells. These targeted DNA-protein interactions may contribute to the broad clinical responses observed with voreloxin to date. Preclinical pharmacokinetic studies in rats and monkeys showed that voreloxin has dose-proportional exposure, low to moderate clearance, and a long half-life (Hoch and Silverman, 2007). In vitro studies using rat, monkey, and human liver microsomes indicated that voreloxin undergoes minimal oxidative and conjugative metabolism. In vivo data in rats and monkeys showed that metabolic pathways for voreloxin include glucuronide conjugation, oxidation, N-dealkylation, and O-dealkylation.

Voreloxin is a replication-dependent DNA-damaging agent that intercalates DNA and inhibits topoisomerase II, resulting in double-stranded DNA breaks, irreversible G2 arrest, and rapid apoptosis. The topoisomerase II-associated DNA intercalation and DNA damage produced by voreloxin are highly selective and show selectivity for proliferating cells. These targeted DNA-protein interactions may contribute to the broad clinical responses observed with voreloxin to date. Preclinical pharmacokinetic studies in rats and monkeys showed that voreloxin has dose-proportional exposure, low to moderate clearance, a moderate half-life, and low interindividual variability. In humans, pharmacokinetic estimates show dose linear increase in exposure, low clearance, and a long half-life (Hoch and Silverman, 2007). In vitro studies using rat, monkey, and human liver microsomes indicated that voreloxin undergoes minimal oxidative and conjugative metabolism (Hoch et al., 2005). In addition, in vitro profiling suggests that rat metabolism is a good model of voreloxin human metabolism. In mass balance studies, greater than 90% of drug was eliminated within 48 h of intravenous administration of 5 mg/kg [14C]voreloxin to rats, with a majority (~78%) of the radioactivity recovered in the first 24 h. Nearly 80% of recovered radioactivity was eliminated in the feces, and additional studies using bile duct-cannulated (BDC) rats indicated biliary and direct intestinal secretion are routes of elimination (Hoch et al., 2005).

The current investigation focuses on the identification of in vivo metabolites collected after intravenous administration of voreloxin to male Sprague-Dawley rats. We observed five unique metabolites plus one degradation product of a phase I-mediated metabolite and two chemically rearranged isomers of an acyl glucuronide.

Materials and Methods

Chemicals and Reagents. Except as specified below, all the chemicals were purchased from Sigma-Aldrich (St. Louis, MO). High-performance liquid
chromatography (HPLC) solvents were analytical grade and purchased from EMD Biosciences (San Diego, CA). Flo-Scint III was used as the scintillation mixture in on-line radioactivity detection, and OptiPhase “SuperMix” (PerkinElmer Life and Analytical Sciences, Waltham, MA) was used for liquid scintillation counting. Voreloxin was synthesized at Dainippon Sumitomo Pharmaceutical Co., Ltd. (Osaka, Japan) and was 99.9% pure. [14C]Voreloxin was synthesized with the radiolabel incorporated on the C-2 carbon of the naphthyridine ring with a specific activity of 137 μCi/mg and a radiochemical purity of 97.1%. Authentic metabolite standards of O-desmethyl-voreloxin, N-desmethyl-voreloxin, and dihydrodecarboxylic acid-voreloxin were synthesized at Sunesis Pharmaceuticals, Inc. (South San Francisco, CA) according to published procedures (Okada et al., 1993; Tomita et al., 2002) and had purities of greater than 90%. Pooled microsomes and expressed cytochrome P450 (P450) and UDP glucuronosyltransferase (UGT) enzymes were purchased from BD Biosciences (San Jose, CA).

Metabolite Profile in Vitro. To determine the P450-dependent metabolite profile generated in the presence of liver microsomes, 1 or 10 μM voreloxin was incubated in the absence and presence of NADPH cofactor with pooled sex liver microsomes from rats, monkeys, and humans. Incubations were performed in 100 mM sodium phosphate buffer, pH 7.4, containing 3.3 mM MgCl₂, 1 mg/ml liver microsomal protein, and 1 mM NADPH for 60 min. Metabolism of voreloxin by phase II UGT enzymes was investigated using liver microsome fractions supplemented with UDP-glucuronic acid (UDPGA) according to Fisher et al. (2000). Reactions were for 60 min with 100 μM voreloxin and initiated with 5 mM UDPGA. The por-forming agent alamethicin was added to liver microsomes at a concentration of 100 μg/ml protein, and the mixture was incubated for 30 min on ice before the addition of voreloxin and UDPGA. Saccharic acid (5 mM) was added to inhibit the action of glucuronidases on conjugates formed during the incubation. The oxidative metabolites were profiled using an API 4000 mass spectrometer (Applied Biosystems, Foster City, CA) coupled to a turbo electrospay ionization source. Before mass spectrometry (MS) analysis, chromatography was performed to achieve some degree of separation between the parent and the metabolites. Conjugative metabolites were profiled using an HPLC coupled to a UV detector set to 350 nm. The HPLC system consisted of an Agilent Technologies (Palo Alto, CA) 1100 binary pump and a Phenomenex (Torrance, CA) Synergi Hydro-RP column (150 × 2 mm, 4 μm, 80 Å particle size). Mass spectral profiles of metabolites were obtained using two experiments: MS full scans to identify metabolites and selected ion scans to compare the metabolic profile across the three species.

To determine specific P450 enzymes responsible for oxidative metabolism of voreloxin, baculovirus-expressed enzymes were used. Individual P450 enzyme incubations were set up to contain one of the following enzymes: CYP3A4, CYP2D6, CYP2C9, CYP1A2, or CYP2C19. The amount of enzyme added to the incubation mixture was scaled so that the individual P450 activity was equal to the activity of that enzyme in the human liver microsome incubation. All the other reaction conditions were identical to those described above. Baculovirus-expressed human UGT isoforms were screened to identify UGT enzymes capable of generating the glucuronide conjugates of voreloxin. The reaction conditions were identical to those described above with UGTs substituted for liver microsomes. UGT isoforms screened included UGT1A1, UGT1A4, UGT1A3, UGT2B7, UGT1A6, UGT1A9, UGT2B4, UGT1A8, and UGT1A10.

Metabolite Identification. Metabolites were characterized in plasma, urine, and bile samples collected from rats receiving voreloxin. Urine samples were analyzed without further preparation. Bile samples were diluted in water before injection at a 1:50 ratio. Plasma samples were extracted using the same acetonitrile protein precipitation method used in the pharmacokinetic analysis described. Lower limits of detection for voreloxin and metabolites M1, M2a, M3, and M4 were determined using standard curves prepared by spiking compound in plasma. For the proposed acyl glucuronide metabolite, M1, the concentration of the plasma spiked standards was also compared against nonextracted samples of the same concentration. Aliquots of urine and bile


Metabolism of voreloxin by phase II UGT enzymes was investigated using liver microsomes at a concentration of 100 μM voreloxin and initiated with 5 mM UDPGA. The pore-forming agent alamethicin with a specific activity of 137 μCi/mg was synthesized with the radiolabel incorporated on the C-2 carbon of the naphthyridine ring with a specific activity of 137 μCi/mg and a radiochemical purity of 97.1%. Authentic metabolite standards of O-desmethyl-voreloxin, N-desmethyl-voreloxin, and dihydrodecarboxylic acid-voreloxin were synthesized at Sunesis Pharmaceuticals, Inc. (South San Francisco, CA) according to published procedures (Okada et al., 1993; Tomita et al., 2002) and had purities of greater than 90%. Pooled microsomes and expressed cytochrome P450 (P450) and UDP glucuronosyltransferase (UGT) enzymes were purchased from BD Biosciences (San Jose, CA).

Pharmacokinetic. Total radioactivity in plasma from rats administered [14C]voreloxin was determined by addition of OptiPhase Supermix and subsequent measurement using a liquid scintillation counter (1450 Micobeta Trilux; PerkinElmer Wallac, Gaithersburg, MD). Voreloxin and metabolite concentrations in plasma samples were measured after protein precipitation with acetonitrile. Voreloxin was separated from metabolites on a reverse-phase HPLC column with an Agilent Technologies 1100 system. Chromatography was carried out on a 250 × 4.6-mm, 4-μm C18 Synergi Hydro column (Phenomenex) using a binary mobile phase consisting of a mixture of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). The flow rate was 0.75 ml/min with the following gradient: solvent A/solvent B 90:10 for 2 min, changed from 90:10 to 70:30 from 2 to 45 min, changed from 70:30 to 10:90 from 45 to 47 min, held at 10:90 from 47 to 49 min, changed from 10:90 to 90:10 from 49 to 50 min, held at 90:10 from 50 to 52 min, changed from 90:10 to 10:90 from 52 to 55 min, and changed from 10:90 to 90:10 from 55 to 57 min; the column was allowed to equilibrate at 90:10 before the next injection. The HPLC system was coupled to a Radiochrom 610TR Flow Scintillation Analyzer equipped with a 500-μl liquid cell (PerkinElmer Life and Analytical Sciences). A quantitative assessment of individual radiolabeled peaks in the plasma was made by multiplying the percentage of peak area of the compound of interest by the total quanified radioactivity. Pharmacokinetic parameters were analyzed with WinNonlin version 4.1 (Pharsight, Mountain View, CA) software using noncompartmental analysis. The following parameters were estimated: t₁/₂, Cₚ, area under the curve (AUC₀–∞), AUC₀–t, clearance, and Vss.

Routes and Rate of Excretion. Radioactivity in urine and bile was counted directly after addition of tissue-dissolving solution and scintillation liquid. Radioactivity in feces was determined after combustion. Radioactivity in each sample was measured for 5 min with a liquid scintillation counter.

Metabolite Identification. Metabolites were characterized in plasma, urine, and bile samples collected from rats receiving voreloxin. Urine samples were analyzed after further preparation. Bile samples were diluted in water before injection at a 1:50 ratio. Plasma samples were extracted using the same acetonitrile protein precipitation method used in the pharmacokinetic analysis described. Lower limits of detection for voreloxin and metabolites M1, M2a, M3, and M4 were determined using standard curves prepared by spiking compound in plasma. For the proposed acyl glucuronide metabolite, M1, the concentration of the plasma spiked standards was also compared against nonextracted samples of the same concentration. Aliquots of urine and bile
samples from each time interval collected were analyzed by reverse-phase HPLC/MS/MS using the HPLC gradient system and column described above.

The characterization of metabolites was performed with an API 4000 (Applied Biosystems/MD Sciex, Foster City, CA) triple quadrupole mass spectrometer using turbo electrospray ionization operated in positive ion mode with the following source parameters: source temperature 650°C, ion spray voltage 5.5 kV, and declustering potential set to 46 V.

**Isolation of Voreloxin Glucuronide.** Voreloxin glucuronide adduct was isolated from rat bile collected between 0 and 12 h postadministration using solid-phase extraction. A strata-X 6-ml column (Phenomenex) was preconditioned with 6 ml of methanol followed by 6 ml of water. One milliliter of bile was loaded and rinsed with 6 to 10 ml of water. Analytes were eluted from the column with methanol and collected in silanized glass tubes, evaporated to dryness under nitrogen, and reconstituted in 50:50 acetonitrile/0.17% methane sulfonic acid in water solution. The samples were reconstituted then separated using reverse chromatography as described above. Samples were freeze-dried overnight using a lyophilizer (VirTis, Gardiner, NY) kept frozen at −20°C until reconstituted and analyzed.

**Cleavage of Voreloxin Glucuronide.** Glucuronide conjugate isolated from rat bile was subjected to cleavage under acidic and basic conditions, and with β-glucuronidase. Freeze-dried conjugate was reconstituted in water to a concentration of 0.1 mg/ml. For cleavage under acidic and basic conditions, an aliquot of the isolated glucuronide (100 µl) was incubated with HCl or NaOH at a final concentration of 0.1 N for 30 min at 37°C. For β-glucuronidase cleavage, a 100-µl aliquot of the reconstituted glucuronide was added to 100 µl of 100 mM sodium phosphate buffer, pH 7.4, to achieve a final activity of 5000 units/ml β-glucuronidase from Escherichia coli (Sigma-Aldrich) and incubated at 37°C for 30 min. Control samples were treated similarly but without the addition of acid, base, or β-glucuronidase. Acidic and basic reactions were stopped by addition equal molar base or acid to return the reaction mixture to neutral pH, and immediately analyzed. Reactions with β-glucuronidase were terminated by the addition of an equal volume of acetonitrile followed by analysis. Samples were chromatographically separated on a reverse-phase HPLC column with an Agilent Technologies 1100 system coupled to a UV detector set to 350 nm. Separation of the glucuronide conjugate and voreloxin was achieved on a 150 × 4.6-mm, 4-µm C18 Synergi Hydro column (Phenomenex) using mobile phase A of 0.1% formic acid in water and mobile phase B, acetonitrile. The flow rate was 0.8 ml/min with the following gradient: 0 to 1 min held at 5% B followed by a linear gradient to 50% B at 7 min and held for 1 min; 8 to 9 min ramping to 5% B and held for the completion of the run.

**Synthesis of Metabolite M2a.** Metabolite M2a was synthesized in a manner analogous to that of a similar compound (Tsuzuki et al., 2004a). Voreloxin was N-protected with di-tert-butyl dicarbonate in 1 M NaOH, treated with NaBH4 in MeOH, followed by 1 M HCl to give 7-[S,S]-3-amino-4-methoxy-pyrrolinyl-1-yl]-1-thiazol-2-yl-2,3-dihydro-1H-[1,8]naphthyridin-4-one hydrochloride (metabolite M2a).

**Synthesis of Metabolite M3 and M4.** The N- and O-des-methyl metabolites were synthesized in a manner analogous to that of an earlier reported synthesis of voreloxin (Tsuzuki et al., 2004b). (S,S)-3-tert-Butyryloxycarbonylamino-4-hydroxy-pyrrolidin-1-carboxylic acid tert-butyl ester was selectively O-methylated with NaH and MeI in tetrahydrofuran and then deprotected with HCl in dioxane to give the (S,S)-3-amino-4-methoxy-pyrrolidinyl dihydrochloride needed for synthesis of metabolite M3. The same (S,S)-3-tert-butyrylcarbonylamino-4-hydroxy-pyrrolidin-1-carboxylic acid tert-butyl ester was O-protected as a silyl ether with tert-butylmethylsilyl chloride and imidazole in dichloromethane, N-methylated with NaH and MeI in N,N-dimethylformamide, and deprotected by tetrabutylammonium fluoride in tetrahydrofuran followed by HCl in dioxane to give the (S,S)-3-methylamino-4-hydroxy-pyrrolidinyl dihydrochloride needed for synthesis of metabolite M4. The above intermediates were taken on to 1,4-dihydro-7-[S,S]-3-amino-4-methoxy-pyrrolinyl[1]-4-oxo-1-(2-thiazolyl)-1,8-naphthyridine-3-carboxylic acid (metabolite M3) and 1,4-dihydro-7-[S,S]-3-hydroxy-4-methylaminopyrrolinyl[1]-4-oxo-1-(2-thiazolyl)-1,8-naphthyridine-3-carboxylic acid (metabolite M4) as described previously (Tsuzuki et al., 2004a).

**Activity of Voreloxin and Metabolites.** Cell proliferation assays were performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium cytoxic assay as described previously (Mosmann, 1983; Hansen et al., 1989).

In brief, 96-well tissue culture-treated flat-bottom plates (Costar 3595; Corning Inc., Corning, NY) were plated with 4000 trypsinized HCT116 cells at 100 µl/well and incubated overnight. Stock concentrations (100×) of voreloxin and metabolite reference standards were prepared in dimethyl sulfoxide (DMSO) at 5 µM and serially diluted 2-fold in DMSO in a 96-well polypropylene v-bottom plate (Costar 3363; Corning Inc.). DMSO dilutions (5 µl) were then added to 45 µl of supplemented RPMI 1640 medium, and 10 µl/well of this mixture was added to plates containing HCT116 cells. Plates were incubated for 72 h at 37°C in an incubator (5% CO2). Cell viability was then recorded as the difference in absorbance at 595 nm between compound-and DMSO-treated cells using a Bio-Rad Benchmark Microplate Reader (Bio-Rad, Hercules, CA). Data were plotted and IC50 values calculated using least-squares regression using Prism 4 software (GraphPad Software Inc., San Diego, CA).

**Results**

**Metabolite Profiles after Microsomal Incubations.** Incubation of voreloxin with liver microsomes from human, monkey, and rat in the presence of NADPH resulted in the formation of three metabolites, M2a, M3, and M4, suggesting involvement of P450 enzymes in voreloxin metabolism. Previous studies monitoring voreloxin stability after 60-min incubation showed >97%, >74%, and >85% unchanged voreloxin remained after incubation with human, monkey, and rat liver microsomes, respectively (data not shown). Formation of these metabolites was similar across the species tested, with metabolite M4 being the most prevalent species. Metabolites M2a, M3, and M4 had MH+ molecular ions at m/z 360, 388, and 388, respectively. To assess which human P450 enzymes mediate the metabolism seen in human liver microsomes, voreloxin was incubated with Supersomes containing individually expressed cytochrome CYP3A4, CYP2D6, CYP2C9, CYP2C19, or CYP1A2 enzymes. The results show that CYP3A4, CYP2D6, and CYP1A2 play a role in the P450-mediated metabolism of voreloxin. P450s CYP2C9 and CYP2C19 do not contribute to the in vitro metabolism of voreloxin.

When incubated with liver microsomes in the presence of UDPGA, voreloxin formed one metabolite, M1. The M1 metabolite was formed in the presence of both human and rat liver microsomes. Incubation of voreloxin with baculovirus-expressed human UGT isoforms indicated several UGT isoforms, including UGT1A1, UGT1A4, UGT1A3, UGT2B7, UGT2B15, and UGT1A8, were capable of conjugating voreloxin. UGT isoforms that showed little or no metabolic activity against voreloxin included UGT2B17, UGT2B4, UGT1A6, UGT1A10, and UGT1A9.

**Pharmacokinetics of Total Radioactivity and Voreloxin.** The plasma concentration-time profiles of total radioactivity and voreloxin after intravenous administration to rats are shown in Fig. 2. Pharmacokinetic parameters were summarized in Table 1. Plasma concentration-time profiles for voreloxin and total radioactivity were similar, declined in a biphasic fashion, and resulted in terminal half-lives of 6.3 and 5.4 h, respectively. Pharmacokinetic parameters were similar for voreloxin and total radioactivity, with a maximum plasma concentration at time 0 (C0) of 4.2 µg/ml, AUC of 17 µg h/ml, clearance of 10 ml/min/kg, and a volume of distribution at steady state of 4 l/kg.

**Routes and Rate of Excretion.** Bile, urine, and fecal excretion after intravenous administration of [14C]voreloxin in rats is summarized in Table 2. Forty-eight hours after intravenous injection, 37.9, 32.5, and 19.6% of the administered radioactivity were excreted into the bile, feces, and urine, respectively. Biliary excretion followed a logarithmic pattern, with half the radioactivity excreted by 6 h post-dose. In bile and urine, the majority of radioactivity was recovered by 24 h postdose, whereas radioactivity in feces continued to increase.
between 24 and 48 h postdose. Together with the small amounts of radioactivity recovered in the carcass, total recoveries of radioactivity were >95% of the dose within the first 48 h.

**Metabolite Profiles in Plasma, Urine, and Bile.** HPLC radiochromatograms from plasma (4 h), urine (6–8 h), and bile (4–6 h) after a 10-mg/kg i.v. bolus injection of [14C]voreloxin are shown in Fig. 3. Based on the retention time, voreloxin (P) was the largest peak observed in plasma, urine, and bile. One additional metabolite peak (M4) was observed in plasma. In urine, six metabolite peaks were detected in addition to voreloxin (M1, M2, M3, M2a, M4, and M5). In addition to five of the metabolite peaks detected in urine, two additional peaks (M1a and M1b) were observed in bile. Metabolite peak M2 was not observed in the depicted bile sample but was observed at other collection intervals. The contribution of voreloxin and its metabolites to total radioactivity over the 12-h collection interval is summarized in Table 3. Parent drug was the predominant component, accounting for 96, 35, and 30% of the radioactivity in plasma, urine, and bile, respectively. The remaining radioactivity in plasma (3%) was caused by M4. In urine, metabolite peaks M4, M5, M2a, and M1 accounted for 16, 15, 12, and 10% of the radioactivity, respectively. Metabolite peaks M2 and M3 were minor, and each accounted for 3% of the total radioactivity. In bile, metabolite peaks M5, M1, M2a, and M1b accounted for 17, 14, 12, and 10% of the total radioactivity, respectively. Total radioactivity of metabolite peaks M1a, M4, M2, and M3 accounted for less than 10% each.

**Identification of Voreloxin Metabolites.** Voreloxin metabolites in rat bile were structurally characterized using triple quadrupole LC/MS/MS. Protonated molecules, MS/MS spectra, and proposed structures are summarized in Table 4. The LC/MS/MS spectrum for voreloxin is shown in Fig. 4. In addition to the MH+ molecular ion at m/z 402, predominant product ions with m/z 384, 332, 269, and 243 were observed. The molecular ion at m/z 384 was the result of a loss of water [18 atomic mass units (amu)] from voreloxin. Loss of 70 amu (carboxylic acid and C-2/C-3 atoms of the naphthyridinone ring) generated the product ion with m/z 332. Additional loss of the N- and O-methyl substituents on the pyrrolidine ring coupled with saturation on the pyrrolidine ring resulted in the product ion with m/z 269. The product ion at m/z 243 is consistent with a core fragment of the naphthyridine ring. The minor product ion at m/z 274 resulted from cleavage at the pyrrolidine-nitrogen.

Metabolite peak M1 had an MH+ molecular ion at m/z 578 and generated a fragment ion at m/z 402, resulting from the loss of 176 amu, diagnostic of a glucuronide conjugate. Additional product ions observed at m/z 384, 332, and 269 were identical to the product ions of voreloxin, supporting the identification of M1 as glucuronide adduct. Metabolite M1 isolated from bile collected from rats dosed with voreloxin underwent hydrolysis on treatment with β-glucuronidase. Disappearance of the metabolite peak corresponded to a 1:1 appearance of a voreloxin peak. Moreover, treatment of M1 with 0.1 N sodium hydroxide or 0.1 N hydrochloric acid resulted in complete disappearance of the metabolite peak within 30 min. These results supported the notion that M1 was an ester glucuronide. Metabolites M1a and M1b showed identical molecular and product ions to M1, also suggesting they were glucuronide metabolites.

Metabolite M2 showed an MH+ molecular ion at m/z 404, an addition of 2 amu compared with voreloxin. Predominant product ions observed were at m/z 360, 306, and 243. The molecular ion at m/z 360 was consistent with loss of the carboxylic acid moiety (44 amu). Additional loss of the N- and O-methyl substituents on the pyrrolidine ring led to the product ion at m/z 297. The product ions at m/z 217 and 247 were consistent with cleavage at the pyrrolidine-nitrogen. The structure of this metabolite could not be confirmed because a synthetic reference standard was not available.

A product ion spectrum related to that of metabolite M2 was observed for metabolite M2a. The molecular ion for this metabolite was at m/z 360, indicating a loss of the carboxylic acid compared with metabolite M2. Similar to metabolite M2, metabolite M2a generated product ions at m/z 318, 306, 297, 243, 217, 205, and 193. Identity of
metabolite M2a as dihydrodecarboxylic acid voroelixin was confirmed with an authentic reference standard, which shared identical retention time and MS/MS spectrum with M2a.

Metabolite M4 had an MH⁺ molecular ion at m/z 388, indicating a loss of 14 amu compared with voroelixin. The fragmentation pattern for M4 was similar to that of voroelixin and included loss of water (18 amu) to result in a product ion at m/z 370, loss of 70 amu to result in a product ion at m/z 318, and formation of the product ions at m/z 269 and 243 also observed for voroelixin. Chemically synthesized N-desmethyl-voroelixin had the same retention time as M4 and showed an identical fragmentation pattern on MS/MS analysis, confirming identity of M4 as N-desmethyl-voroelixin.

Metabolite M3 also had an MH⁺ molecular ion at m/z 388, indicating a loss of 14 amu compared with voroelixin. The fragmentation pattern for M3 was identical to that observed for M4. Chemically synthesized O-desmethyl-voroelixin had the same retention time as M3, confirming the identity of M3 as O-desmethyl-voroelixin. Metabolite M5 did not yield a detectable molecular ion when subjected to mass spectral analysis; therefore, no structure could be proposed for this nonpolar metabolite.

LC/MS/MS analysis of plasma and urine samples confirmed the

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**TABLE 2**

<table>
<thead>
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<th>Sample</th>
<th>Cumulative Excretion ± S.E.M. (%Dose) at Specific Time Points</th>
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<td></td>
<td>0–0.5 h</td>
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<td>Bile</td>
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<tr>
<td>Urine</td>
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</tr>
<tr>
<td>Feces</td>
<td>N.D.</td>
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<tr>
<td>Carcass</td>
<td>N.D.</td>
</tr>
<tr>
<td>Total</td>
<td>2.8 ± 0.4</td>
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</tbody>
</table>

N.D., not determined.

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**TABLE 3**

<table>
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<tr>
<th>Peak</th>
<th>Retention Time</th>
<th>Mean % of Dose</th>
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<td></td>
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<tr>
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<tr>
<td>M5</td>
<td>49.9</td>
<td>15</td>
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</table>

N.D., not detected.

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**Fig. 3.** Representative radiochromatograms of voroelixin (P) and its metabolites (M#) in plasma (A), urine (B), and bile (C) after a 10-mg/kg i.v. bolus injection of [14C]voroelixin to rats.
identity of the metabolite peaks shown in Fig. 3 as those described for bile. Lower limit of detection for metabolites for which standards were available were at least 5 ng/ml. Peak area response for the glucuronide metabolite, M1, spiked in plasma before extraction was identical to glucuronide prepared in pure solution at the same concentrations.

### Cytotoxicity of Voreloxin and Metabolites

Cytotoxicity of voreloxin, dihydrodecarboxylic acid voreloxin (M2a), O-desmethyl-voreloxin (M3), and N-desmethyl-voreloxin (M4) is summarized in Table 5. N-Desmethyl-voreloxin showed activity that was similar to that of voreloxin, whereas the O-desmethyl and the dihydrodecarboxylic acid metabolites were inactive at the highest concentrations tested.

### Discussion

Voreloxin (formerly SNS-595) is a novel naphthyridine analog currently under investigation for the treatment of platinum-resistant ovarian cancer and acute myeloid leukemia. The present study describes the pharmacokinetics and metabolism of this novel chemotherapeutic. Pharmacokinetic parameters of voreloxin in nonclinical studies showed a favorable profile: systemic clearance was low at 11% of the rat liver blood flow; volume of distribution at steady state was high, indicating distribution outside the vasculature; and terminal half-life was 5.4 h. Pharmacokinetic estimates based on voreloxin were similar to those determined based on total radioactivity, suggest-
ing low levels of circulating metabolites. Indeed, a more detailed analysis of plasma samples confirmed that voroxin contributed to 97% of the radioactivity in plasma.

In vitro metabolism of voroxin was studied in microsomes from rat and monkey, the two species used in nonclinical toxicology studies, and compared with that in microsomes from human. Voroxin is relatively stable to oxidative and conjugative metabolism. In microsomes supplemented with NADPH, greater than 85, 74, and 97% of parent drug remain after 1 h with either 1 or 10 μM voroxin in rat, monkey, and human microsomes, respectively. We observed O- and N-desmethyl-voroxin and dihydrodecarboxylic acid voroxin in rat, monkey, and human microsomal incubations. In all the species, the N-desmethyl metabolite, M4, was the predominant species observed based on AUC of the ionized species. Both rat and human microsomes were capable of generating a glucuronide conjugate of voroxin in microsomes supplemented with UDGA, but high concentrations (100 μM) of voroxin were required to observe this metabolite, suggesting that its binding affinity to the UGT enzyme(s) is weak.

Metabolite M5 was not observed in microsomal incubations supplemented with either NADPH or UDGA. Cross-species comparison of the oxidative and conjugative metabolite profiles after microsomal incubation indicates that microsomal metabolism in the rat is a good predictor of human microsomal metabolism.

We used baculovirus-expressed human P450 enzyme preparations to predict which enzyme(s) are responsible for generating the observed oxidative metabolites. Incubations containing human CYP3A4, CYP2D6, CYP2C9, CYP1A2, or CYP2C19 showed that CYP3A4 and CYP1A2 are capable of generating the N-desmethyl, O-desmethyl, and dihydrodecarboxylic acid metabolites. Cytochrome CYP2D6 generated the dihydrodecarboxylic acid metabolite, M2a, probably through oxidation of voroxin to the dihydro species, M2, which chemically degrades to M2a. Cytochrome CYP1A2 was also important because, along with CYP3A4, it was the only other isoform capable of generating all the oxidative metabolites.

Identification of the metabolites in rat bile allowed us to propose the metabolic scheme shown in Fig. 5. Metabolites identified by LC/MS/MS analysis and synthesized reference standards include N-desmethyl-voroxin, O-desmethyl-voroxin, and dihydrodecarboxylic acid voroxin. Whereas N- and O-desmethyl-voroxin are primary metabolites, the dihydrodecarboxylic acid metabolite, M2a, is probably a degradation product of dihydro-voroxin (metabolite M2).

Low-level presence of a species at m/z 404, a molecular weight

<table>
<thead>
<tr>
<th></th>
<th>Vorexin</th>
<th>M2a</th>
<th>M3</th>
<th>M4</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT 116</td>
<td>0.42</td>
<td>&gt;5</td>
<td>&gt;5</td>
<td>0.51</td>
</tr>
</tbody>
</table>

**TABLE 5**

Cytotoxicity of voroxin and metabolites M2a, M3, and M4.

**Fig. 5.** Proposed metabolic pathways for voroxin in rat.
consistent with the proposed structure for M2, and the commonly observed instability of β-ketoacids support this hypothesis.

For many xenobiotics, glucuronidation constitutes a major route of elimination and is the second most common clearance mechanism listed for the top 200 most-prescribed drugs on the market (Williams et al., 2004). Most common conjugations occur at hydroxyl, amino, and carboxylic acid centers, but conjugation at carbon centers has been observed (Miners et al., 2004). In the case of carboxylate conjugation, the biologically generated 1-O-β-glucuronide metabolite, unlike other types of glucuronides, is relatively labile in vitro and in vivo and can also undergo chemical rearrangement of the glucuronide to form isomers of the acyl glucuronide (Spahn-Langguth and Benet, 1992; Wang and Dickinson, 1998). In the acyl migration, the aglycone is transferred to the C-2, C-3, or C-4 position of the glucuronic acid ring (Spahn-Langguth et al., 1996; Bailey and Dickinson, 2003).

Here, identity of metabolite M1 as the acyl glucuronide of vorloxin could not be definitively confirmed via a synthesized reference standard. Chemical synthesis was attempted but failed to yield the desired glucuronide. The limited solubility of vorloxin in appropriate solvents prevented direct glucuronidation reactions. Addition of protecting groups on the secondary amine functionality increased the solubility of vorloxin sufficiently to allow the formation of the acyl glucuronide. However, attempts to remove the protecting group also resulted in cleavage of the desired glucuronide. Nevertheless, several lines of evidence support identification of metabolite M1 as the acyl glucuronide of vorloxin: 1) acyl glucuronidation is commonly described for carboxylic acid-containing drugs, including fluoroquinolone antibiotics (Dalvie et al., 1996; Ramji et al., 2001; Tachibana et al., 2005), which are structurally related to vorloxin; 2) a glucuronide isolated from rat bile was relatively labile and completely converted to vorloxin under acidic and basic conditions and after incubation with β-glucuronidase; 3) the presence of multiple peaks for M1 in bile and not in urine suggests possible intra-acyl rearrangement of the acyl glucuronide in a pH-dependent manner. Intrahepatic rearrangement of acyl glucuronides occurs more readily under the alkaline conditions of bile (Hyneck et al., 1988; Frank et al., 1989).

Radioactivity was observed in bile, urine, and feces, indicating that vorloxin is removed through hepatic clearance (38%), renal clearance (20%), and by direct intestinal secretion (33%). In urine and bile, unchanged vorloxin contributed to 35 and 30% of the radioactivity and constituted the largest single peak. Formation of metabolites M2/M2a, M3, and M4 is most likely mediated by the P450 system. This pathway of elimination contributed 34 and 21% of the radioactivity in urine and bile, respectively. Finally, elimination by UGT glucuronosyltransferases contributed to 10 and 32% of the radioactivity in urine and bile, respectively. Formation of metabolites unchanged vorloxin contributed to 35 and 30% of the radioactivity in bile (Hyneck et al., 1988; Frank et al., 1989).

In conclusion, in the present study vorloxin showed favorable pharmacokinetic properties in nonclinical species. Elimination occurred renal, hepatically, as well as by direct intestinal secretion. Four primary metabolites were identified, and the human enzymes capable of producing them were profiled. In rats, excretion of unchanged vorloxin, phase II and P450-mediated metabolism played roles in the excretion of vorloxin.

References


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