ABSTRACT:
We examined the pharmacokinetics and metabolism of the experimental nucleoside reverse transcriptase inhibitor compound stampidine in mice, dogs, and cats. Also reported is the identification of p-bromophenyl sulfate (p-Br-Ph-S) as a major in vivo metabolite of stampidine. Liver cytosol was shown to take part in the hydrolysis of stampidine to form alaninyl-STV-monophosphate (Ala-STV-MP), 2',3'-dideoxy-3'-deoxythymidine (STV), and p-bromophenol; p-bromophenol was further sulfonated by sulfotransferase to form p-Br-Ph-S. Notably, plasma concentrations of stampidine >4 logs higher than its IC50 value can be achieved in both dogs and cats after its p.o administration at a 100-mg/kg dose level. In dogs as well as cats, stampidine was metabolized to yield micromolar concentrations of the active metabolites Ala-STV-MP and STV, which is similar to the metabolism of stampidine in mice. These findings encourage the further development of this new antiviral agent for possible clinical use in human immunodeficiency virus-infected patients.

Stampidine is a novel aryl phosphate derivative of stavudine, which inhibits the replication of human immunodeficiency virus (HIV\(^+\))-1 in human peripheral blood mononuclear cells at nanomolar concentrations (Venkatachalam et al., 1998; Vig et al., 1998; Uckun and Vig, 2000). Stampidine is substantially more potent than stavudine against primary clinical HIV-1 isolates. Importantly, stampidine was active against phenotypically and/or genotypically nucleoside reverse transcriptase inhibitor (NRTI)-resistant HIV with low nanomolar to sub-nanomolar IC50 values (Uckun et al., 2002c). Similarly, stampidine inhibited the replication of laboratory HIV-1 strains and primary clinical HIV-1 isolates with non-nucleoside reverse transcriptase inhibitors binding site mutations (K103N, V106N, Y179I, Y181C, and Y188L) and/or a phenotypically non-nucleoside reverse transcriptase inhibitor-resistant profile with low nanomolar to sub-nanomolar IC50 values. Notably, stampidine exhibited dose-dependent and potent in vivo anti-HIV activity in Hu-PBL-SCID mice against a genotypically and phenotypically NRTI-resistant clinical HIV-I isolate at nontoxic dose levels (Uckun et al., 2002b).

We recently studied the in vivo metabolism of this new anti-HIV agent in rodent species (Chen et al., 2001; Uckun et al., 2002a,b). In mice and rats, stampidine was found to form two active metabolites, namely, alaninyl-STV-monophosphate (Ala-STV-MP) and STV (Chen et al., 2001; Uckun et al., 2002b,c). The goal of the present study was to extend our studies to large animal species. Herein, we report the pharmacokinetics and metabolism of stampidine in dogs and cats. Also reported is the identification of a novel in vivo metabolite of stampidine and its pharmacokinetics in mice, dogs, and cats.

Materials and Methods

Anti-HIV Drugs and Chemicals. HPLC-grade reagents and deionized, distilled water obtained from Milli-Q purification system (Millipore, Medford, MA) were used in this study. Acetonitrile was purchased from Burdick and Jackson (Allied Signal Inc., Muskegon, MI). Hydrochloric acid was purchased from Fisher Scientific (Fair Lawn, NJ). Ammonium phosphate, adenosine 3'-phosphate 5'-phosphosulfate (PAPS), porcine liver esterase (catalog no. E2884), thymine, and phosphoric acid were purchased from Sigma-Aldrich (St. Louis, MO). Microcrystalline Cellulose NF (Avicel PH 101) was obtained from FMC Bioproducts (Newark, DE). Magnesium stearate NF was obtained from Spectrum Chemicals (Gardena, CA), and hard gelatin capsules sizes 4 and 00 were obtained from Capsugel Corporation (Greenwood, SC). The synthetic procedures for the preparation of stampidine and STV-5[para-bromophenyl methoxyalaninyl phosphate] have been described in detail (Fig. 1) previously (Venkatachalam et al., 1998; Vig et al., 1998; Uckun and Vig, 2000).

Stampidine capsules were used in the pharmacokinetic studies in both dogs and cats. A homogenous mixture of stampidine, microcrystalline cellulose, and magnesium stearate was prepared using a mortar and pestle. This homogenous mixture was filled manually into the bottom halves of size 4 and size 00 hard gelatin capsules; the upper halves of the shells were placed and the two halves were locked. The stampidine contents in size 4 and size 00 capsules were 250 mg of stampidine per capsule, and the fill weights were 400 mg.

The pooled human liver microsomes (catalog no. H161) and cytosol preparations were purchased from Gentest (Woburn, MA). The liver microsomes

1 Abbreviations used are: HIV, human immunodeficiency virus; NRTI, nucleoside reverse transcriptase inhibitor; Ala-STV-MP, alaninyl-STV-monophosphate; STV, 2',3'-dideoxy-3'-deoxythymidine; HPLC, high-performance liquid chromatography; PAPS, adenosine 3'-phosphate 5'-phosphosulfate; P450, cytochrome P450; p-Br-Ph-S, p-bromophenyl sulfone; TLC, thin layer chromatography; AUC, are under the concentration-time curve; PLE, porcine liver esterase; MS, mass spectrometry; RT, retention time.

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an ultraviolet spectrophotometer; and 1 H NMR, 13 C NMR, and 31 P NMR
were recorded. All chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification. All reactions were carried out with reagents that are not detected in animals.

from ICR/CD-1 mice, Sprague-Dawley rats, Dunkin-Hartley guinea pigs, New Zealand White rabbits, beagle dogs, and cynomolgus monkeys were purchased from In Vitro Technologies (Baltimore, MD). The protein concentrations in both liver microsomes and cytosol preparations, and specific activities of each cytochrome P450 (P450) isoform in the liver microsomes were provided in the data sheets by the manufacturer.

Synthesis and Characterization of Newly Proposed Assigned Metabolites. All chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification. All reactions were carried out under nitrogen. Melting points are not corrected; UV spectra were determined with an ultraviolet spectrophotometer; and 1 H NMR, 13 C NMR, and 31 P NMR spectra were determined on the Oxford 300-MHz spectrometer (Varian, Palo Alto, CA) using an automated broadband probe. Chemical shifts are expressed in ppm downfield from an internal reference of tetramethylsilane. The following compounds were synthesized and characterized as potential metabolites of stampidine:

**p-Bromophenyl Phosphate.** p-Bromophenyl phosphate was synthesized as follows: 54.0 µl (3.0 mmol) of H2O was added to a solution of 200 mg (0.69 mmol) p-bromophenyl phosphorodichloridate in anhydrous toluene at 0°C. The solution was stirred at room temperature for 3 h then at 50°C for 1 h. The solvent was removed under reduced pressure; a white crystallization of 175 mg (100%) was obtained. 1 H NMR (CD3OD) δ 7.12-7.15 (d, 2H, J = 9.0 Hz, Ar-2,6), 7.47-7.50 (d, 2H, J = 9.0 Hz, Ar-3,5); 13 C NMR (CD3OD) δ 117.1, 122.1, 132.4, 150.8. 31 P NMR δ -4.87; UV λmax = 222 nm; MS(EI) 251/253 (100%).

**2',3'-Didehydro-3'-deoxythymidine 5'- (methylxylanolyl Phosphate) Triethylammonium Salt.** The synthesis of this compound was started with equimolar phosphorylation of methoxyalanine, followed by equimolar substitution with STV, and subsequent hydrolysis with triethylammonium bicarbonate buffer (Scheme 1). To a solution of L-alanine methyl ester hydrochloride (1.41 g, 10 mmol) and phosphorus oxychloride (0.94 ml, 10 mmol) in anhydrous dichloromethane (50 ml) at −78°C was added a solution of triethylamine (2.80 ml, 20 mmol) in anhydrous dichloromethane (50 ml) dropwise while stirring. The reaction mixture was allowed to warm up to room temperature, followed by stirring overnight. The reaction mixture was filtered and rinsed with anhydrous ether under suction. The filtrate was concentrated under reduced pressure to yield methoxyxylanolyl phosphorodichloridate (2.17 g, 99% yield) as a colorless oil. STV (0.22 g, 1 mmol), methoxyxylanolyl phosphorodichloridate (0.59 ml, 3 mmol), and triethylamine (0.56 ml, 4 mmol) were dissolved in anhydrous tetrahydrofuran (10 ml) and stirred overnight. To this solution, 10 ml of triethylammonium bicarbonate buffer was added at 0°C with stirring. The mixture was concentrated and purified with flash chromatography (CHCl3/MeOH = 1:1) and preparative TLC (CHCl3/MeOH/H2O = 10:5:1), yielding 4.0 mg of the target compound. 1 H NMR (CD3OD) δ 1.25 to 1.33 (m, 12H, Ala-CH3 and CH2CH3), 1.94 (s, 3H, 5-CH3), 3.31 (q, 6H, CH3CH2), 3.67 (s, 3H, OCH3), 3.70 (s, 2H, 5'-H), 3.78 to 3.87 (m, 1H, N-CH), 3.97 to 4.00 (m, 1H), 4.94 (s, 1H, 4'-H), 5.86 (d, 1H, J = 6.0 Hz, 1'-H), 6.42 (d, 1H, J = 8.7 Hz, 2'-H), 6.96 (m, 1H, 3'-H), 7.70 (s, 1H, 6-H); 13 C NMR (CD3OD) δ 11.6, 20.5, 47.1 to 48.8, 50.3, 51.3, 66.5, 85.8, 89.7, 110.7, 115.7, 122.3, 126.3, 131.9, 134.1, 138.0, 151.6, 152.3, 165.1. 31 P NMR δ 6.23; UV λmax = 266 nm; MS(EI) 196 (40%), 267 (92%), 388 (100%).

**5'-{4-(Bromophenolphospho)-2',3'-didehydro-3'-deoxythymidine Triethylammonium Salt.** To a solution of 250 mg (0.87 mmol) of p-bromophenyl phosphorodichloridate in anhydrous pyridine at 0°C, 3.0 ml of 195 mg (0.87 mmol) of N-(Hydroxy-4-bromophenoxyphosphinyl)-L-alanine methyl ester hydrochloride (1.41 g, 10 mmol) and phosphorus oxychloride (0.94 ml, 10 mmol) in anhydrous dichloromethane (50 ml) at −78°C was added a solution of triethylamine (2.17 g, 99%) as a colorless oil. STV (0.22 g, 1 mmol), methoxyxylanolyl phosphorodichloridate (0.59 ml, 3 mmol), and triethylamine (0.56 ml, 4 mmol) were dissolved in anhydrous tetrahydrofuran (10 ml) and stirred overnight. To this solution, 10 ml of triethylammonium bicarbonate buffer was added at 0°C with stirring. The mixture was concentrated and purified with flash chromatography (CHCl3/MeOH = 1:1) and preparative TLC (CHCl3/MeOH/H2O = 10:5:1), yielding 4.0 mg of the target compound. 1 H NMR (CD3OD) δ 1.25 to 1.33 (m, 12H, Ala-CH3 and CH2CH3), 1.94 (s, 3H, 5-CH3), 3.31 (q, 6H, CH3CH2), 3.67 (s, 3H, OCH3), 3.70 (s, 2H, 5'-H), 3.78 to 3.87 (m, 1H, N-CH), 3.97 to 4.00 (m, 1H), 4.94 (s, 1H, 4'-H), 5.86 (d, 1H, J = 6.0 Hz, 1'-H), 6.42 (d, 1H, J = 8.7 Hz, 2'-H), 6.96 (m, 1H, 3'-H), 7.70 (s, 1H, 6-H); 13 C NMR (CD3OD) δ 11.6, 20.5, 47.1 to 48.8, 50.3, 51.3, 62.5, 86.2, 89.7, 110.9, 125.8, 134.6, 137.5, 152.1, 161.6, 176.2; 31 P NMR δ 6.23; UV λmax = 266 nm; MS(EI) 196 (40%), 267 (92%), 388 (100%).
mg, 0.69 mmol) in anhydrous pyridine (2.0 ml) was added to a solution of p-bromophenyl phosphorodichloridate (200 mg, 0.69 mmol) in anhydrous pyridine at 0–C, over 20 min. After stirring at room temperature for 8 h and at 50–C for 2 h, the solvent was removed under reduced pressure. The residue was treated with acetonitrile (3.0 ml) and 1.0 M triethylammonium bicarbonate buffer solution (2.0 ml) and stirred for 2 h. The solvent was removed in vacuo and the residue was purified by flash chromatography on silica gel (CHCl3/MeOH = 1:1) to obtain a white solid (45 mg, 62%). An analytical sample was obtained through preparative TLC (CHCl3/MeOH/H2O = 10:5:1). 1H NMR (CD3OD) δ 1.28 (t, 9H, CH3CH2), 1.32 (s, 3H, Ala-CH3), 3.18 (q, 6H, CH2CH3), 3.61 (3H, OCH3), 3.88 (m, 1H, N-CH2), 7.11 (d, 2H, J = 4.8 Hz, Ar-2,6), 7.37 (d, 2H, J = 8.4 Hz, Ar-3,5); 13C NMR (CD3OD) δ 117.2, 123.2, 131.9, 152.1; UV λmax = 220 nm; MS/ELI 336/338 (100%).

4-Bromophenyl Sulfate. To a mixture of p-bromophenol (1.0 g, 5.77 mmol) and sulfuryl chloride/pyridine complex (0.93 g, 5.84 mmol), anhydrous pyridine (12 ml) was added. The resulting suspension was stirred for 4 h and then at 50°C for 4 h. After removal of all solvent under reduced pressure, H2O (50 ml) was added and extracted using chloroform three times. The solution was concentrated in vacuo and the residue was purified by flash chromatography on silica gel (CHCl3/MeOH) to obtain a white solid (935 mg, 64%). 1H NMR (CD3OD) δ 7.19–7.22 (d, 2H, J = 9.0 Hz, Ar-2,6), 7.43 to 7.46 (d, 2H, J = 9.0 Hz, Ar-3,5); 13C NMR (CD3OD), δ 117.2, 123.2, 131.9, 152.1; UV λmax = 254 nm; MS/ELI 251/253 (100%), 171/173 (20%).

Identification of a New in Vivo Stampidine Metabolite. Sample Pretreatment. In brief, each plasma sample (200 μl) was mixed 1:4 with acetone (800 μl) and vortexed for at least 30 s. After centrifugation, the supernatant was transferred into a clean tube and was dried under nitrogen. A 50% methanol in 200 mM HCl was used to reconstitute the extraction residue, and 40 μl was injected into the HPLC.

HPLC conditions. The HPLC system used for these studies was a Hewlett Packard (Palo Alto, CA) series 1100 instrument equipped with a quaternary pump, an autosampler, an automatic electronic degasser, an automatic thermostatic column compartment, a diode array detector, and a computer with Chemstation software for data analysis (Chen et al., 1999a,c.e). The analytical column used was a Zorbax SB-Phenyl (5 μm; Agilent Technologies, Kenner, LA) column attached to a guard column (Agilent Technologies). The column was equilibrated before data collection. The linear gradient mobile phase (flow rate = 1.0 ml/min) was used 100% A/0% B at 0 min, 88% A/12% B at 20 min, and 8% A/92% B at 30 min (A, 10 mM ammonium phosphate buffer, pH 3.7; B, acetonitrile). The detection wavelength was 268 nm and the peak width, response time, and slit were set at >0.03 min, 0.5 s and 4 nm, respectively.

Mass spectrometry: Mass spectrometry was carried out using atmospheric pressure ionization electrospray and a high-energy-dynode electron multiplier that are connected to the liquid chromatography system (Agilent Technologies) (Chen et al., 1999f; Chen and Uckun, 2000). Liquid chromatographic conditions were described as above except that flow rate was 0.5 ml/min. High-purity nitrogen was provided by Nitrogen Generator (Agilent Technologies). The conditions for mass spectrometry were set at a fragmentor voltage of 75 V, drying gas flow of 10 l/min, nebulizer pressure of 25 psig, and drying gas temperature of 350°C. Both positive and negative ion monitoring with scanning were performed at a capillary voltage of 3500 V.

Animals. Female BALB/c mice (6–8 weeks old) (Tacomic Farms, German-town, NY) and female CD-1 mice (6–8 week old) (Charles River Laboratories, Inc., Wilmington, MA) were housed in an USDA-accredited animal care facility under standard environmental conditions (12-h light/dark photoperiod, 22 ± 1°C, 60 ± 10% relative humidity). All rodents were housed in microisolator cages (Lab Products, Inc., Maywood, NJ) containing autoclaved bedding. Mice were allowed free access to autoclaved pelleted food and tap water throughout the study.

Male beagle dogs (Canis familiaris) (body weight 10–12 kg) were obtained from Harlan (Indianapolis, IN) at 8 months of age. The dogs were housed in the Minneapolis Veterans Administration animal research facility that is approved by the American Association for Accreditation of Laboratory Animal Care. Specific pathogen-free male or female domestic cats (body weight 2.9–6.2 kg) were obtained from Liberty Research, Inc. (Waverley, NY), Cedar River Laboratories (Mason City, IA), or Harlan. All husbandry and experimental contact made with the cats maintained specific pathogen-free conditions (12-h light/dark photoperiod, 18–29°C, 30–70% relative humidity). The cats were housed in galvanized gang cages with Plexiglas in a well ventilated room with no air recirculation. The animal rooms were cleaned daily and cat litters were changed daily. Cats were provided with dry and wet Purina cat chow as well as tap water ad libitum. Cats were acclimated to study room conditions for at least 5 days before initiation of the experiment.

All animal studies were approved by Parker Hughes Institute Animal Care and Use Committee and all animal care procedures conformed to the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, Washington DC, 1996). Dog studies were also approved by the Veterans Administration Hospital Subcommittee on Animal Studies and cat studies were also approved by the University of Florida Animal Care and Use Committee.

Pharmacokinetic Studies in Animals. A solution of stampidine dissolved in dimethyl sulfoxide was administered i.v. to mice via tail vein injection at a dose of 100 mg/kg. Two to five mice per strain per time point were used for pharmacokinetic studies. Blood samples (~500 μl) were obtained from the ocular venous plexus by retro-orbital venipuncture at 0, 2, 5, 10, 15, 30, 45, 60, 120, 240, and 360 min after i.v. injection. To determine the pharmacokinetics of stampidine after its oral administration to mice, 12-h fasted mice were given a bolus dose of 100-mg/kg stampidine via gavage using a stainless steel ball-tipped feeding needle. Blood sampling time points were 0, 2, 5, 10, 15, 30, 45, 60, 120, 240, and 360 min after the gavage.

For pharmacokinetic studies in both dogs and cats, animals were fasted overnight and treated with oral stampidine capsules at a dose of 100 mg/kg. Blood samples (~1 ml) were obtained from the femoral veins of dogs and cephalic veins of cats at 0, 10, 20 (only in cats), 30, 40 (only in cats), and 45 min (only in dogs) and 1, 2, 4, 6, 8, 12, and 24 h from treated animals after oral administration. Hepatized blood samples were immediately centrifuged at 7000g for 2 min to separate the plasma fraction from the whole blood. The plasma samples were then processed immediately using the extraction procedure described above.

Noncompartmental analysis and parameter calculations were carried out using the WinNonlin Professional version 3.0 (Pharsight, Mountain View, CA) pharmacokinetics software (Chen et al., 1999b,d,f; Uckun et al., 1999a,b). The data were weighted as 1/y. The elimination half-life was estimated by linear regression analysis of the terminal phase of the plasma concentration-time profile. The area under the concentration-time curve (AUClast) from the time of dosing to the last measurable concentration was calculated based on the linear trapezoidal method.

Incubation of Stampidine with Liver Microsomes and Cytosol Preparations. The incubation mixtures of stampidine with either human or nonhuman liver microsomes contained the following components (Uckun et al., 2002d) at the indicated final concentrations (200 μl): 1× PBS, 1 mg/ml microsomes, 10 mM glucose 6-phosphate, 2 units/ml glucose-6-phosphate dehydrogenase, and 5 mM MgCl2. The mixtures were preincubated at 37°C for 5 min. The metabolite incubation was initiated by the addition of stampidine. After incubation for 10 min, the reaction was stopped by addition of 800 μl of acetonitrile and the suspension was thoroughly mixed using a vortex device. The mixtures were extracted and separated through the HPLC conditions as described above.

To study the saponification reactions, we first tested whether saponification is enzymatically catalyzed. To this end, we monitored the formation of p-bromophenyl sulfate in a reaction medium containing human liver cytosols without PAPS as a saponification donor as well as in a reaction medium containing PAPS but no cytosol. The contribution of sulfotransferase to the metabolism of p-bromophenol and stampidine was examined at the indicated final concentrations (200 μl): 1× phosphate-buffered saline, 1 mg/ml cytosol, and 5 mM MgCl2. The mixtures were preincubated at 37°C for 5 min. The metabolic incubation was initiated by the addition of either p-bromophenol or stampidine at a final concentration of 100 μM. After incubation for 30 min, the reaction was stopped by the addition of 800 μl of acetonite and thoroughly mixed using a vortex device. The mixtures were extracted and separated through the HPLC conditions as described above except that the detection wavelength was set at 222 nm. Porcine liver esterase (PLE) has been found to be involved in the activation of STV aryl phosphoramide (Saboulard et al., 1999). Therefore, we compared the amounts of cats used in the experiment.
Results

Identification of a New Stampidine Metabolite in Plasma. We have previously reported the identification of ala-STV-MP and STV as two of the three major in vivo metabolites of stampidine (Chen et al., 2001). However, the identity of the third metabolite eluting at a retention time of 26.8 min has remained elusive (Chen et al., 2001). Online liquid chromatography-MS analysis of murine plasma samples revealed that this candidate metabolite has an m/z of the same intensity of molecular ion at 250.9/252.9 and fragment ion at 170.9/172.9. These data prompted the hypothesis that the candidate metabolite may be bromophenyl phosphate. However, the synthesized p-bromophenyl

![Mass spectrum for new metabolite.](image)

Computer simulation for isotope distribution for both p-bromophenyl sulfate (A1) and p-bromophenyl phosphate (B1); the mass spectrum for the metabolite isolated from mice treated with stampidine (A2) and for the synthetic authentic p-bromophenyl phosphate (B2).
phosphate had a retention time of 11.6 min, which is significantly shorter than the observed retention time of the candidate metabolite (26.8 min) (Fig. 3).

We next sought to determine whether the candidate metabolite was \( p\)-Br-Ph-S that has the same MS spectrum. A small amount of the metabolite was separated from the plasma of mice treated with stampidine and subjected to MS analysis using a high-resolution QStar mass spectrometer (Applied Biosystems, Foster City, CA). Taurocholic acid was used as the internal standard. We first simulated the possible isotope distribution for both \( p\)-bromophenyl phosphate and \( p\)-Br-Ph-S. The \( p\)-Br-Ph-S has simulated isotope patterns of 250.9014, 251.9014, 254.8914, 253.9014, 254.8914, 255.8914, and 256.8914 (Fig. 2A1), whereas isotope patterns for \( p\)-bromophenyl-phosphate yielded a possible \( m/z \) of 250.9109, 251.9109, 252.9009, 253.9109, and 254.9109 (Fig. 2B1). The actual isotope distribution for the isolated metabolite from stampidine-treated mice shown in Fig. 2A2 matched very well with that of \( p\)-Br-Ph-S, whereas the actual isotope distribution for the synthesized authentic bromophenyl-phosphate

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**Figure 3.** HPLC chromatograms for possible hydrolysis metabolite.

A, proposed hydrolysis metabolites. B, HPLC chromatograms for separation of the proposed metabolites. C, HPLC chromatogram from a BALB/c mice at 15 min post-i.v. injection (100 mg/kg stampidine).
matched very well with the simulated isotope distribution profile of bromophenyl-phosphate (Fig. 2B). In addition, the synthesized authentic p-Br-Ph-S had the same retention time of 26.8 min as the in vivo metabolite and the authentic p-Br-Ph-S can be coeluted with the in vivo metabolite when spiked into the plasma from stampidine-treated animals. Therefore, the candidate metabolite corresponding to the distinct HPLC peak at a retention time of 26.8 min was unambiguously identified as p-Br-Ph-S.

To study in greater detail the in vivo metabolism pathway of stampidine, we next synthesized several proposed hydrolysis products of stampidine, including \( N \)-(hydroxy-4-bromophenoxypylnyl)-l-aniline-methyl ester; 4-bromophenol-dihydrogen phosphate; 2,3'-didehydro-3'-deoxythymidine-5'-(4-bromophenyl)-phosphate; 2,3'-didehydro-3'-deoxy-thymidine-5'-methoxy-l-analinnyl phosphoramide; STV, MP, and STV containing the identified metabolites STV, Ala-STV-MP, and p-Br-Ph-S (Fig. 3A). These compounds can be clearly separated using established HPLC conditions (Fig. 3B). We compared the retention times of the synthesized compounds to the peaks detected in the HPLC chromatograms of plasma extracts from mice treated with 100 mg/kg stampidine (Fig. 3C). There were only three matches: Ala-STV-MP (RT of 15.6 min), STV (RT of 18.6 min), and p-bromophenyl sulfate (RT of 26.8 min). No additional candidate in vivo metabolite peaks, matching the retention times of the other in vivo metabolite when spiked into the plasma from stampidine-treated animals. Therefore, neither of these two metabolites is likely to contribute to the anti-HIV activity of stampidine.

We next set out to evaluate the pharmacokinetics of p-Br-Ph-S in mice after i.v. and p.o. administration of 100 mg/kg stampidine (Fig. 4). The estimated pharmacokinetic parameter values are presented in Table 1. After i.v. and p.o. administration of 100 mg/kg stampidine, p-Br-Ph-S had a maximum plasma concentration of 328.6 ± 20.1 and 372.2 ± 53.6 \( \mu \text{M} \), and AUC of 351.5 ± 84.0 and 878.4 ± 106.8 \( \mu \text{M} \cdot \text{h} \), respectively. The elimination half-life values were 143.1 ± 12.7 and 139.4 ± 22.9 min, respectively. After i.v. and p.o. administration of 100 mg/kg stampidine, 20.0 ± 4.9 and 19.4 ± 5.9 min, respectively, were required to reach the maximum plasma p-Br-Ph-S concentration.

**Table 1**

<table>
<thead>
<tr>
<th>Route of Administration</th>
<th>( C_{\text{max}} ) (( \mu \text{M} ))</th>
<th>AUC (( \mu \text{M} \cdot \text{h} ))</th>
<th>( t_{1/2} ) (min)</th>
<th>( T_{\text{max}} ) (min)</th>
</tr>
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<tbody>
<tr>
<td>i.v.</td>
<td>328.6 ± 20.1</td>
<td>351.5 ± 84.0</td>
<td>143.1 ± 12.7</td>
<td>20.0 ± 4.9</td>
</tr>
<tr>
<td>p.o.</td>
<td>372.2 ± 53.6</td>
<td>878.4 ± 106.8</td>
<td>139.4 ± 22.9</td>
<td>19.4 ± 5.9</td>
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\( N = 2 \) BALB/c and 5 CD-1 mice per time point.

In dogs as well as cats, stampidine was metabolized to yield the active metabolites ala-STV-MP and STV as well as p-Br-Ph-S, which is similar to the results obtained in mice (Chen et al., 2001; Uckun et al., 2002a) (Table 2; Fig. 5). However, Ala-STV-MP was detected in the blood samples of only one of the four dogs.

**Involvement of Liver Enzymes in the Metabolism of Stampidine.** The identification of p-Br-Ph-S as the third major in vivo metabolite of stampidine prompted the hypothesis that stampidine metabolism results in generation of p-bromophenol that is then converted to p-Br-Ph-S via sulfonation. To study the role of sulfonylation reactions in the metabolism of stampidine, we first tested whether sulfonylation of p-bromophenol can be enzymatically catalyzed. No p-Br-Ph-S was formed in a reaction medium containing human liver cytosol without PAPS as a sulfonation donor, or in a reaction medium containing PAPS but no cytosol. In contrast, in a complete reaction medium containing 100 \( \mu \text{M} \) p-bromophenol, the formation rate of p-Br-Ph-S (mean ± S.E.M.) was 281.2 ± 14.3 pmol/min/mg cytosolic protein.

Using the same enzymatic reaction conditions, we found that similar amounts of Ala-STV-MP, STV, and p-bromophenol can be detected when stampidine is incubated with human liver cytosol either in presence or absence of PAPS (Fig. 6B versus C). However, a significantly higher amount of p-Br-Ph-S was formed in the presence versus the absence of PAPS (the formation rate was 512.3 ± 27.2 versus 4.7 ± 4.7 pmol/min/mg protein) \( (p < 0.001) \) (Table 3; Fig. 6). As expected, no significant metabolite was observed when cytosol was omitted from the reaction medium (Fig. 6A).

PLE has been found to be involved in the activation of STV aryl phosphoramide (Saboulard et al., 1999). Our results showed that in the presence or absence of PAPS, PLE-containing medium alone produced similar amount of Ala-STV-MP, STV, p-bromophenol, and trace amount of p-Br-Ph-S. By comparing the metabolism profiles of PLE versus cytosol, we observed that stampidine was less stable in the PLE-containing medium than in the cytosol. Although the formation of Ala-STV-MP and p-bromophenol was significantly higher in the presence of PLE versus cytosol \( (p < 0.05) \), PLE itself does not significantly contribute to the formation of p-Br-Ph-S (Table 3). The
addition of liver cytosol to the PLE reaction medium rendered stampidine less stable and increased the formation of STV, but not of Ala-STV-MP and p-bromophenol. As expected, the presence of PAPS in both the PLE medium and cytosol reaction medium results in significant formation of p-Br-Ph-S [formation rate 176.1 ± 9.9 versus 7.5 ± 7.5 pmol/min/mg cytosol protein (p < 0.001)] (Table 3). It was interesting to find that the formation rate of p-Br-Ph-S in the presence of PLE and cytosol was significantly lower than that in the presence of cytosol alone [176.1 ± 9.9 versus 512.3 ± 27.2 pmol/min/mg cytosol protein (p < 0.001)] (Table 3).

We next examined whether the cytochrome P450 system is involved in the metabolism of stampidine. Stampidine was stable when incubated with human or nonhuman liver microsomes, and no potential metabolite peaks were observed in the HPLC chromatograms in the presence of the NADPH-regenerating system (data not shown). Under identical conditions, human liver microsomes (catalog no. H161) can metabolize a control compound, 4-(4’-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline (Uckun et al., 2002d), to form 7-O-demethylated metabolite at the rate of 17.6 ± 1.4 pmol/min/mg protein. These strongly argue against a significant contribution of the cytochrome P450 system to the metabolism of stampidine.

**Discussion**

Our results indicate that stampidine can be quickly biotransformed to two active phase I metabolites, Ala-STV-MP and STV, and a phase II metabolite, p-Br-Ph-S (Fig. 1). Ala-STV-MP was considered as an intra- and/or extracellular depot form of STV and/or STV-MP (Balzarini et al., 1996a,b; Naesens et al., 1998), whereas STV is a clinically used anti-HIV drug (Lea and Faulds, 1996). The formation of the major metabolite Ala-STV-MP is consistent between the in vitro (Venkatachalam et al., 1998) and in vivo systems (Vig et al., 1998; Chen et al., 2001); however, the formation of STV was found in plasma but not in cells.

In this report, we identified a significant amount of p-Br-Ph-S in animals treated with stampidine and trace amounts of p-bromophenol that could not be quantitated. p-Bromophenol has a very low toxicity, because the oral LD₅₀ value for p-bromophenol was over 500 mg/kg (Sigma-Aldrich MSDS for 4-bromophenol). As a metabolite for bromobenzene, p-bromophenol has been shown not to contribute to the toxicity of bromobenzene (Monks et al., 1982; Dankovic and Billings, 1985). Further metabolism of p-bromophenol to form a glutathione conjugate was also found to be nontoxic in vivo (Monks et al., 1984). The sulfonation pathway generally results in detoxification and facilitates the elimination of the metabolites (Negishi et al., 2001). There-

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**TABLE 2**

Estimated pharmacokinetic parameter values for stampidine and its metabolites in dogs and cats

<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Measured</th>
<th>Tₘₐₓ</th>
<th>Cₘₐₓ</th>
<th>t₁/₂</th>
<th>AUCₘₐₓ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dogs (N = 4)</td>
<td>Stampidine</td>
<td>112.8 ± 46.2</td>
<td>15.4 ± 6.1</td>
<td>108.6 ± 28.8</td>
<td>23.1 ± 5.4</td>
</tr>
<tr>
<td></td>
<td>Ala-STV-MP</td>
<td>45.0</td>
<td>4.7</td>
<td>51.0</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>STV</td>
<td>270.0 ± 30.0</td>
<td>39.3 ± 5.4</td>
<td>201.6 ± 34.8</td>
<td>262.0 ± 25.3</td>
</tr>
<tr>
<td></td>
<td>p-Br-Ph-S</td>
<td>161.3 ± 48.0</td>
<td>284.3 ± 59.5</td>
<td>246.4 ± 32.1</td>
<td>1732.1 ± 366.4</td>
</tr>
<tr>
<td>Cats (N = 3)</td>
<td>Stampidine</td>
<td>106.2 ± 57.0</td>
<td>7.5 ± 1.6</td>
<td>50.5 ± 15.8</td>
<td>7.1 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>Ala-STV-MP</td>
<td>115.8 ± 54.6</td>
<td>6.3 ± 1.3</td>
<td>82.2 ± 9.0</td>
<td>15.6 ± 3.9</td>
</tr>
<tr>
<td></td>
<td>STV</td>
<td>312.0 ± 26.4</td>
<td>72.8 ± 9.2</td>
<td>282.0 ± 21.0</td>
<td>766.5 ± 98.3</td>
</tr>
<tr>
<td></td>
<td>p-Br-Ph-S</td>
<td>120.0 ± 0.0</td>
<td>656.1 ± 98.5</td>
<td>252.2 ± 41.1</td>
<td>3892.8 ± 794.4</td>
</tr>
</tbody>
</table>

a Ala-STV-MP was detected in the blood samples of only one of the four dogs.
b Two of the cats were treated with 100 mg/kg stampidine twice in two independent experiments. The remaining cat was treated with 100 mg/kg stampidine only once. Thus, the results were obtained in five pharmacokinetic experiments involving three cats.
c Elimination half-life was estimated by linear regression analysis of the terminal phase of the plasma concentration-time profile.

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**FIG. 5.** Metabolite pharmacokinetics in dogs and cats.

Plasma Ala-STV-MP (A), STV (B), and p-bromophenyl sulfate (C) concentrations as a function of time after oral administration of 100 mg/kg stampidine to the both dogs (N = 4) and cats (N = 3).
The metabolism of stampidine may involve demethylation first followed by the release of \( p \)-bromophenol, a theory consistent with recent reports of other STV aryl phosphoramidate derivatives using an in vitro system by Sabould’s group (Sabould et al., 1999; Siddiqui et al., 1999). Demethylated stampidine (Fig. 1) was, however, very difficult to synthesize; several attempts were made to cleave the methyl ester of stampidine, but in all cases either no reaction occurred or unwanted side reactions were observed. An analog of stampidine with a \( tert \)-butyl ester on the alanine residue was synthesized and subjected to a variety of mildly acidic conditions expected to provide the carboxylic acid. Every attempt to remove the \( tert \)-butyl group resulted in loss of bromophenol. Stampidine did not readily lose the bromophenyl group under these conditions. It is possible that de-bromophenyl stampidine was formed but was subsequently metabolized rapidly so that it cannot be observed experimentally. This was further supported by the hydrolysis of stampidine using the pig liver esterase because we observe Ala-\( \text{STV-MP} \), STV and \( p \)-bromophenol, but no other significant peaks.

It has been established that the metabolism of aryl phosphoramides or nucleotides may be mediated by carboxyesterases, phosphodiesterase, ribonucleoside phosphoramidase (Egron et al., 1998; McGuigan et al., 1998; Naesens et al., 1998). Our results showed that the cytochrome P450 system is not significantly involved in the metabolism of stampidine. The formation of \( p \)-Br-Ph-S must be catalyzed by sulfotransferase, which transfers a sulfuryl group from \( 3’ \)-phosphoadenosine \( 5’ \)-phosphosulfate to hydroxy or amino groups (Duffel et al., 2001; Negishi et al., 2001). The positive results after incubation of \( p \)-bromophenol with human liver cytosol in the presence of PAPS further support this hypothesis. Our results also showed that PLE is not involved in the sulfonation of \( p \)-bromophenol to form \( p \)-Br-Ph-S. In the presence of PAPS, formation of \( p \)-Br-Ph-S was significantly slower with the combination of both PLE and liver cytosol versus cytosol alone. This may be caused by the higher concentrations of Ala-\( \text{STV-MP} \) and \( p \)-bromophenol but less stampidine in the reaction system. However, the possibility that hydrolyzed products may inhibit the sulfonation reaction requires further study.

Sulfotransferases have been shown to be present in almost all animal species, including mice, rats, and dogs and also in human beings, but it should be noted that sulfotransferases are found to be genetically polymorphic in humans (Coughtrie et al., 1999; Nagata and Yamazoe, 2000; Evans and Ingelman-Sundberg, 2001). In addition, the level of available \( 3’ \)-phosphoadenosine \( 5’ \)-phosphosulfate in plasma, which has been found to affect acetaminophen sulfonation (Falany, 1997), may also influence the stampidine sulfonation. Therefore, there may be some variation in the further metabolism of \( p \)-bromophenol released from stampidine. There is no significant difference in metabolite pharmacokinetics (\( C_{\text{max}} \), \( T_{\text{max}} \), and \( t_{1/2} \)) of

\[ \text{Table 3} \]

\begin{table}
\begin{center}

Involvement of liver enzymes in metabolism of stampidine

All data (the concentrations at the end of the 10-min incubation) are expressed as the mean ± S.E.M. values from at least three separated experiments. The data in parentheses are the rate for the formation of \( p \)-bromophenol sulfate (picomoles per minute per milligram of liver cytosol).

<table>
<thead>
<tr>
<th>Reaction Conditions</th>
<th>Stampidine</th>
<th>Ala-( \text{STV-MP} )</th>
<th>STV</th>
<th>( p )-Br-Ph</th>
<th>( p )-Br-Ph-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>102.0 ± 1.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cytosol-PAPS</td>
<td>85.6 ± 2.0</td>
<td>11.7 ± 0.1</td>
<td>2.5 ± 0.2</td>
<td>3.1 ± 1.1</td>
<td>0.1 ± 0.1 (4.7 ± 4.7)</td>
</tr>
<tr>
<td>Cytosol + PAPS</td>
<td>65.2 ± 5.0</td>
<td>11.3 ± 0.5</td>
<td>5.1 ± 0.4</td>
<td>0.8 ± 0.3</td>
<td>15.4 ± 0.8 (512.3 ± 27.2)</td>
</tr>
<tr>
<td>PLE-PAPS</td>
<td>25.1 ± 0.5</td>
<td>49.6 ± 0.6</td>
<td>1.8 ± 0.1</td>
<td>15.8 ± 0.2</td>
<td>0.6 ± 0.4</td>
</tr>
<tr>
<td>PLE + PAPS</td>
<td>20.9 ± 2.7</td>
<td>47.2 ± 1.1</td>
<td>1.9 ± 0.2</td>
<td>12.7 ± 2.2</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Cytosol + PLE-PAPS</td>
<td>15.1 ± 0.9</td>
<td>51.2 ± 2.3</td>
<td>5.5 ± 1.0</td>
<td>8.5 ± 0.5</td>
<td>0.2 ± 0.2 (7.5 ± 7.5)</td>
</tr>
<tr>
<td>Cytosol + PLE + PAPS</td>
<td>4.1 ± 2.5</td>
<td>44.3 ± 1.8</td>
<td>5.4 ± 1.3</td>
<td>20.0 ± 5.1</td>
<td>5.3 ± 0.3 (176.1 ± 9.9)</td>
</tr>
</tbody>
</table>

\( p \)-Br-Ph, \( p \)-bromophenol.
Metabolism of New Anti-HIV Agent Stampidine

p-Br-Ph-S in mice after intravenously and orally administered stampidine, except for significantly higher systemic exposure (AUC) of p-Br-Ph-S after oral administration of stampidine compared with intravenous injection. Cats treated p.o. with 100 mg/kg stampidine have the highest plasma concentrations of p-Br-Ph-S and AUC, whereas there are similar elimination half-lives of p-Br-Ph-S in mice, dogs, and cats. The relative contributions of intestinal wall metabolism versus liver first-pass metabolism to the observed metabolism of stampidine after oral administration will be the subject of future studies.

Notably, stampidine inhibits the replication of primary clinical HIV-1 isolates with subnanomolar to nanomolar IC₅₀ values (Uckun et al., 2002c). Our results provided direct evidence that plasma concentrations >4 logs higher than its IC₅₀ value can be achieved in both dogs and cats after its p.o administration at a 100-mg/kg dose level. In dogs as well as cats, stampidine was metabolized to yield the active metabolites ala-STV-MP and STV, which is similar to the pharmacokinetic profiles of stampidine in mice. The pharmacokinetics and metabolism profiles, and potent anti-HIV activity of stampidine warrant the further development of this new antiviral agent for possible clinical use in HIV patients.

Acknowledgments. We thank Zhaoai Zhu, Jason Thoen, Hao Chen, Thao Tran, Greg Mitcheltree, Krista Wyvell, Christina Tague, and Heidi Bergstrom for skillful technical assistance.

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