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Preclinical and First-In-Human Phase I Clinical Evaluation of Stampidine, a Potent Anti-HIV Pharmaceutical Drug Candidate

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Abstract

The rationally designed novel anti-HIV drug candidate Stampidine exhibited (a) remarkable subnanomolar to low nanomolar *in vitro* ARV potency against genotypically and phenotypically NRTI-resistant primary clinical HIV isolates, non-nucleoside reverse transcriptase inhibitor (NNRTI)-resistant HIV-1 isolates, clinical non-B subtype HIV-1 isolates (subtypes A, C, F, and G) originating from South America, Asia, and sub-Saharan Africa with resistance to stavudine, adefovir and tenofovir, as well as recombinant HIV clones containing common patterns of RT mutations responsible for NRTI resistance such as multiple TAMs plus M184V, multiple TAMs plus T69 insertion, and Q151 complex (b) favorable, safety profile in mice, rats, dogs, and cats, and (c) promising prophylactic *in vivo* anti-retroviral activity in Hu-PBL-SCID mice as well as therapeutic anti-retroviral activity in FIV-infected domestic cats. Notably, in a placebo-controlled Phase I study involving 30 therapy-naïve adult HIV-infected adult patients, formulated GMP-grade oral Stampidine capsules did not cause dose-limiting toxicity at single dose levels ranging from 5 to 25 mg/kg. Taken together, the presented favorable preclinical and early clinical safety/activity profile of Stampidine warrants its further development as a new anti-HIV drug candidate.

Introduction

Human immunodeficiency virus type 1 (HIV-1) infection remains a global health concern affecting millions of individuals worldwide [1,2]. Combination antiretroviral therapy has become the standard of care for patients with HIV infection [3,4]. Antiretroviral (ARV) treatment regimens employing combinations of drugs from at least two of the three classes of ARV therapy, namely nucleoside analogue RT inhibitors (NRTI), non-nucleoside analogue RT inhibitors (NNRTI), and protease inhibitors, exhibit a potent and sustained antiviral effect and confer consistent long-term viral suppression in patients with HIV infection [3,4]. However, each of these drugs can select for drug-resistant viruses and the emergence of antiviral drug resistance limits their clinical benefit [5-8]. Acquired resistance to ARV agents hampers the long-term success of contemporary highly active antiretroviral therapy (HAART) regimens [5-8]. Likewise, pre-exposure prophylaxis (PrEP), an evolving new approach to HIV prevention in which ARV agents are used prior to potential HIV exposure in an attempt to reduce the likelihood of HIV infection post exposure in the context of unprotected heterosexual intercourse [9-13], has had limited success due to ARV resistance [14-19]. Notably, TDF, one of the PrEP arms of VOICE study, an NIH funded HIV prevention trial of the Microbicide Trials Network involving more than 5,000 women in Africa and evaluating oral PrEP agents TDF and Truvada (TDF/FTC), a vaginal microbicide gel formulation of TDF, and combinations thereof, has been discontinued based on the interim review of the data by the NIAID Prevention Trials DSMB demonstrating that oral TDF did not reduce HIV infection in participants receiving it [18]. Likewise, FEM-PrEP, a large PrEP trial of Truvada could not demonstrate efficacy in preventing HIV acquisition in women and was recently closed for futility [19]. Therefore, there is an urgent need for potent anti-HIV agents that are active against ARV-resistant HIV strains.

Stampidine (5'-[4-bromophenyl methoxy]aniliny]phosphate)-2',3'-didehydro-3'-deoxythymidine) (CAS 217178-62-6), is a rationally designed novel aryl phosphate derivative of stavudine (STV)/d4T (CAS 3056-17-5) that is being developed as a promising new drug candidate against ARV-resistant HIV [20,21]. NRTI form the backbone of contemporary combination ARV therapy regimens. The 5'-triphosphates of the NRTI family, which are generated intracellularly by the action of nucleoside and nucleotide kinases, are capable of competing with the natural deoxynucleoside triphosphates for binding to the RT primer:template complex and represent the biologically active form of NRTI responsible for their anti-HIV activity [22]. The rate-limiting step for the generation of the bioactive NRTI triphosphates is the conversion of the NRTI to their monophosphate derivatives. Stampidine was developed in an attempt to overcome the dependence

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Received December 22, 2011; **Accepted** January 20, 2012; **Published** January 24, 2012

Citation: Cahn P, Rolon MJ, Gun AM, Ferrari I, Dibirdik I, et al. (2012) Preclinical and First-In-Human Phase I Clinical Evaluation of Stampidine, a Potent Anti-HIV Pharmaceutical Drug Candidate. J AIDS Clinic Res 3:138. doi:[10.4172/2155-6113.1000138](https://doi.org/10.4172/2155-6113.1000138)

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of the NRTI stavudine (STV) on intracellular nucleoside kinase activation [20,21]. Stampidine has been shown to inhibit the replication of HIV-1 strain HTLV-III_B, HIV-2, as well as the AZT (ZDV)-resistant HIV-1 strain RT-MDR in human peripheral blood mononuclear cells at nanomolar concentrations [20,21]. Stampidine is a much more potent anti-HIV agent than STV and was active against phenotypically and/or genotypically NRTI-resistant HIV with low nanomolar to subnanomolar IC₅₀ values [20,21]. The superior anti-HIV-1 activity of Stampidine was attributed to the rapid formation of its active metabolite Ala-STV-MP. Cellular metabolic studies revealed that the *p*-Br group in the phenyl moiety of Stampidine contributes to its ability to undergo rapid hydrolysis yielding the key metabolite Ala-STV-MP in a TK-independent fashion [20,21,23]. The potency of Stampidine against genotypically and phenotypically NRTI-resistant HIV-1 isolates is likely due to the rapid kinetics of the generation of its active triphosphate metabolite yielding much higher inhibitor concentrations at the catalytic site sufficient to overcome the binding restrictions imposed by the NRTI resistance-associated RT mutations. As a lipophilic aryl phosphate derivative of STV, Stampidine can enter target cells easier than STV, which could also contribute to higher inhibitor concentrations at the catalytic site of HIV RT. In addition, the presence of Ala side chain may promote the binding and/or incorporation of the triphosphate metabolite of these prodrugs. Stampidine as well as its combinations with standard anti-HIV drugs are potent inhibitors of HIV-1 replication in human PBMCs, and replacement of either ZDV, ZDV + 3TC or STV in 3-drug cocktails with Stampidine resulted in greater anti-HIV potency *in vitro* [24]. Stampidine was found to have a favorable pharmacokinetics profile in mice, rats, dogs, and cats with 25 mg/kg or 50 mg/kg tolerable dose levels yielding micromolar plasma concentrations, which are 1,000-fold higher than its *in vitro* IC₅₀ value against HIV [25,26]. Drug metabolism studies conducted in multiple animal species have provided experimental evidence that Stampidine is quickly biotransformed to two active metabolites, Ala-STV-MP and STV with favorable pharmacokinetics [27]. Here, we report the very favorable preclinical and early clinical safety and ARV activity profile of Stampidine. In agreement with its preclinical safety profile, the first-in-human Phase I clinical trial of Stampidine reported herein showed that it is clinically safe and does not cause any dose-limiting toxicity at single dose levels of 5, 10, 15, 20 and 25 mg/kg.

Materials and Methods

HIV-1 isolates

36 HIV-1 clones were used in the present analysis, including 30 primary clinical HIV-1 isolates with ARV resistance [20,21,28] and a panel of recombinant HIV-1 infectious clones containing common patterns of RT mutations responsible for resistance to each of the currently available NRTI and submitted it to the NIH AIDS Research and Reference Reagent Program. The following 6 prototypical multi-drug resistant HIV-1 clones containing multiple NRTI resistance mutations were used: NIH-7324-1, NIH-7295-1, NIH-52534-2, NIH-1617-1, NIH-4755-5, NIH-56252-1 [28].

Anti-HIV potency assays

The sensitivity of clinical HIV-1 isolates and recombinant HIV-1 clones to Stampidine and other antiretroviral agents was tested using our previously published HIV-1 replication assays [24] (see Supplemental Material for detailed description). IC₅₀ value was defined as the concentration at which HIV-1 replication in peripheral blood mononuclear cells (PBMC) was inhibited by 50%.

MTS staining for PBMC viability to measure cytotoxicity

At assay termination, assay plates were stained with the soluble tetrazolium-based dye MTS (CellTiter 96 Reagent, Promega) to determine cell viability and quantify compound toxicity (see Supplemental Material for detailed description of the procedure).

Toxicity studies in animals

The toxicity of orally administered Stampidine was examined at multiple dose levels and according to distinct treatment regimens in BALB/c mice, Wistar albino rats, Beagle dogs (*Canis familiaris*), and FIV-infected shorthair domestic cats, as described in detail in the Supplemental Material. All animal studies were performed with approval from an Institutional Animal Care and Use Committee (IACUC) in strict compliance with relevant laws, the Animal Welfare Act, and Public Health Services Policy.

Assessment of the *in vivo* anti-retroviral activity of stampidine in HIV-1-infected Hu-PBL-SCID mice and FIV-infected domestic cats

The *in vivo* anti-HIV-1 activity of orally administered Stampidine was examined in a BL-3 containment facility with IACUC approval in the surrogate Hu-PBL-SCID mouse model of AIDS, as previously described [29] and reported in detail in the Supplemental Material. Human peripheral blood lymphocyte (Hu-PBL)-SCID mice were generated by reconstituting SCID mice by i.p. injection of 10⁷ peripheral blood mononuclear cells from seronegative volunteer donors. Two weeks after inoculation of the cells, mice were anesthetized with isoflurane and then challenged by i.p. injection of 1x10⁵ experimentally determined median 50% tissue culture infectious doses (TCID₅₀) of cell-free BR/92/019, a genotypically and phenotypically NRTI-resistant HIV-1 isolate. Two weeks after infection, Hu-PBL-SCID mice were electively killed, and their peritoneal lavage cells as well as spleen cells were examined for evidence of infection by determination of the viral RNA load (log₁₀[RNA copies per milligram of spleen tissue or per milliliter of peritoneal lavage fluid]) using an Organon Teknika Nuclisens HIV-1 QT assay kit (bioMerieux, Durham, N.C.). Chronically FIV-infected cats were previously inoculated intravaginally with FIV_{Bang}-infected FeT-J cells (5 × 10³ to 5 × 10⁶ cells/0.4 ml) >6 months prior to initiation of the study, as previously described [30]. At the time of the present study, all chronically infected cats were off antiretroviral therapy for 4 weeks and had viral culture evidence of FIV infection in their PBMC. At the indicated time points (pre-study, 2 weeks, 4 weeks, 6 weeks, and 8 weeks), the FIV load in peripheral blood mononuclear cells (PBMCs) was measured by virus isolation. The cells from Stampidine treated and placebo-treated cats were co-cultured in 25-cm² tissue culture flasks (total volume: 5 mL) for 3 weeks with 5x10⁶ T-cell enriched PBMCs from SPF cats in a total volume of 5 mL at a ratio of 1:1, 1:10, 1:100, 1:1000, 1:10,000, 1:100,000, or 1:1,000,000. Culture supernatants were harvested and cells were resuspended in fresh culture media every 3 days. FIV replication was monitored by measuring the levels of RT activities in the culture supernatants. The RT values were considered positive if the value was ≥ 10,000 cpm/mL. FIV load in PBMC is presented as the highest dilution (log-X) of infected cells from the cat needed to detect FIV in cocultured PBMCs from SPF cats by virus isolation (VI-RT values), whereby undiluted infected PBMC concentration is set at 1x10⁶ cells/mL. Thus, the FIV load can range from 1, which is equivalent to FIV detection at undiluted cell concentration of 1x10⁶ /mL, to 6, which is equivalent to FIV detection at a 1:1,000,000 dilution. The FIV load (VI-RT) was arbitrarily assigned a value of "0" when no FIV was detected even at 1x10⁶ /mL.

GMP-grade stampidine drug product and placebo control

The clinical grade Stampidine Drug Product was prepared in accordance with current Good Manufacturing Practices (cGMP) in the clinical manufacturing facility at the Division of Pharmaceutical Service at the College of Pharmacy at the University of Iowa (Iowa City, Iowa, U.S.A., also known as Iowa University Pharmaceuticals (IUP)) as previously reported [31]. The raw materials used in the preparation of the Stampidine Drug Product were Stampidine, microcrystalline cellulose, magnesium stearate, and a white opaque gelatin capsule (size 00). The GMP-grade Stampidine drug substance was obtained from Cardinal Health (formerly Magellan Laboratories, Inc.) (Morrisville, North Carolina, U.S.A.) following cGMP guidelines. The magnesium stearate, NF material was obtained from Spectrum Laboratory Products, Inc. (New Brunswick, New Jersey, U.S.A.) and the white opaque gelatin capsule (size 00) material was obtained from Capsugel (Greenwood, South Carolina, U.S.A.). The Stampidine-free placebo capsules were also prepared in the clinical manufacturing facility at the Division of Pharmaceutical Service at the College of Pharmacy at the University of Iowa (Iowa City, Iowa, U.S.A.). The raw materials used in the preparation of the placebo were microcrystalline cellulose, magnesium stearate, and a white opaque gelatin capsule (size 00).

Patients

After written informed consent was obtained, 35 therapy-naïve HIV-infected adult patients were screened for study eligibility. Patients had to fulfill all of the following inclusion criteria within 15 days prior to enrollment, unless specified otherwise, to be eligible for study entry:

- Documented laboratory diagnosis of HIV infection with Plasma HIV-1 RNA level $>1,000$ and $<50,000$ copies/mL on at least two occasions within 2 weeks prior to study entry using the Roche Amplicor HIV- 1 Monitor™ Ultrasensitive Test.
- CD4⁺ T-cell count of >250 cells/mm³, obtained within 30 days before study entry.
- No previous anti-retroviral therapy
- Ambulatory performance status of $> 80\%$ on the Karnofsky Performance Scale
- Negative serum pregnancy test (females of childbearing potential only).
- Willingness to use effective contraception (barrier method) by both males and females while on study treatment and for 3 months following Stampidine treatment.
- Age 21-65 years.
- Life expectancy > 1 year.
- The ability to understand and sign a written informed consent form, which must be obtained prior to initiation of study procedures.
- Adequate renal function defined as Serum creatinine ≤ 1.5 x normal, or Creatinine clearance or radioisotope GFR ≥ 70 ml/min/1.73 m²
- Adequate liver function defined as: Total bilirubin ≤ 1.5 mg/dL and hepatic transaminases (AST and ALT) < 3 x upper limit of normal (ULN).
- No evidence of pancreatitis: Total serum amylase ≤ 1.5 x normal (if amylase is > 1.5 x N, pancreatic amylase or lipase must be ≤ 1.5 x N).
- Adequate cardiac function defined as: No clinical signs or

symptoms of cardiac failure; Normal vital signs; Normal cardiac size by chest x-ray, normal EKG, and ejection fraction of $>50\%$ by either echocardiogram or gated radionuclide study if there is clinical indication for determination.

- Adequate pulmonary function defined as: No evidence of dyspnea at rest, no exercise intolerance, and a pulse oximetry $> 94\%$ if there is clinical indication for determination.
- Adequate central nervous system function defined as: CNS toxicity \leq grade 1.
- Adequate hematologic function (absolute neutrophil count $> 1000/\mu\text{L}$; platelets $>50,000/\mu\text{L}$, hemoglobin > 8.0 g/dL) in the absence of erythropoietin, granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor GM-CSF, or other hematologic growth factors.
- Patients who presented with *any* of the following exclusion criteria were not enrolled in this study:
- Immunization within 30 days of baseline.
- A new AIDS-defining event diagnosed within 30 days of baseline.
- Patients with lymphoma.
- Patients with malignancy which requires systemic therapy, other than minimal Kaposi's sarcoma.
- Patients with a history of \geq Grade 2 bilateral peripheral neuropathy
- Patients with history of acute or chronic pancreatitis.
- Patients with active uncontrolled opportunistic infection or other uncontrolled infections within 14 days prior to study entry. Unexplained temperature $> 38.5^\circ\text{C}$ (101.3°F) for any 7 days within 30 days prior to study entry.
- Patients with severe chronic diarrhea defined as $>$ than 3 liquid stools per day persisting for 15 days, within the last 30 days prior to study entry.
- Uncontrolled diabetes mellitus, or other serious medical condition(s) within 14 days prior to study entry.
- Pregnant or lactating patients.
- Evidence of a gastrointestinal malabsorption syndrome or chronic nausea or vomiting which may confer an inability to receive an orally administered medication.
- Current alcohol or substance abuse judged by the investigator to potentially interfere with patient compliance
- Active, serious infections (other than HIV- 1 infection) requiring parenteral antibiotic therapy within the past 15 days.
- Any other clinical condition or prior therapy that, in the opinion of the investigator, would make the patient unsuitable for study or unable to comply with the dosing requirements.
- Pregnant or nursing patients
- Any experimental therapy within 30 days prior to study entry.
- Erythropoietin, G-CSF or GM-CSF treatments within 30 days prior to study entry.
- Interferons, interleukins or HIV vaccines within 30 days prior to study entry.
- Rifampin or rifabutin within 14 days prior to study entry or at any time while on study
- On-going therapy with nephrotoxic agents, systemic chemotherapeutic agents, Systemic corticosteroids, Investigational agents, or anti-retroviral drugs - Administration

of any of the above medications must be discontinued at least 30 days prior to study entry and for the duration of the study period. Patients must have never received an anti-retroviral drug to be eligible for this study.

Four patients were excluded because of not meeting the eligibility criteria and one patient withdrew consent. A total of eligible 30 patients were enrolled in the study, including 6 females and 24 males. The median age was 34.5 years (Range: 23-44 years).

Clinical study

The clinical safety of Stampidine was examined in a placebo-controlled first-in-human Phase I clinical study in compliance with ICH Guidelines and Good Clinical Practice (GCP) conditions (Argentinian Ministry of Health and Environment, ANMAT Record # 1-0047-0000-0025930-06-9, ANMAT Approval # 2455). The study and its amendments were reviewed and approved by the Comité de Bioética of the Fundación Huesped and the Comité de Docencia e Investigación of the Hospital General de Agudos Juan A. Fernandez. Laboratorio de Bioequivalencia y Biología Molecular (LBBM), Universidad Barceló. French 2464 – Ciudad Autónoma de Buenos Aires, Argentina served as the CRO for regulatory Affairs and local monitoring. The study was conducted according to the ICH/GCP guidelines, regulatory requirements, and the provisions of the Helsinki Declaration (version 2004). A total of 30 evaluable patients in cohorts of 6 patients/group were included. Of 30 patients who were enrolled in the study, 5 were treated with placebo and 25 were treated with Stampidine at one of the 5 different dose levels (5 patients/dose level). In each cohort, the number of placebo pills matched the number of study drug pill. On the morning of Day 1, after an overnight fasting, patients took a single dose of the study medication or placebo under site staff supervision. They had to remain NPO (nothing per mouth) for 4 hours after drug intake. Patients were observed in an inpatient setting for 48 hours and if no dose limiting toxicity (DLT) was observed at 1 week after dosing, new patients were entered at the next dose level according to a stepwise dose escalation scheme. Each cohort included one patient receiving placebo and 5 receiving Stampidine (~5, 10, 15, 20 and 25 mg/kg respectively):

- Group 1 (6 patients): 1 capsule of Placebo (N=1) or Stampidine (N=5)
- Group 2 (6 patients): 2 capsules of Placebo (N=1) or Stampidine (N=5)
- Group 3 (6 patients): 3 capsules of Placebo (N=1) or Stampidine (N=5)
- Group 4 (6 patients): 4 capsules of Placebo (N=1) or Stampidine (N=5)
- Group 5 (6 patients): 5 capsules of Placebo (N=1) or Stampidine (N=5)

No intra-patient dose-escalation was permitted. For the purpose of dose escalation, a patient who experienced no DLT was evaluable for toxicity if the patient received Stampidine and was followed for a minimum of 4 weeks after completion of therapy. A patient who started the regimen and discontinued therapy due to dose limiting toxicity was considered evaluable. The maximum tolerated dose of Stampidine was defined as the dose at which fewer than 30% of patients experience dose limiting toxicity. A patient experiencing grade III or IV toxicity using the standard toxicity criteria, was considered to have a DLT.

Results

In vitro Anti-HIV potency of Stampidine against NRTI-resistant clinical HIV-1 isolates and recombinant HIV-1 clones

Stampidine (Figure 1A) inhibited the replication of each of the 30 primary clinical HIV-1 isolates with genotypic and/or phenotypic NRTI resistance as well as 6 multi-NRTI resistant recombinant HIV-1 clones containing common patterns of RT mutations responsible for

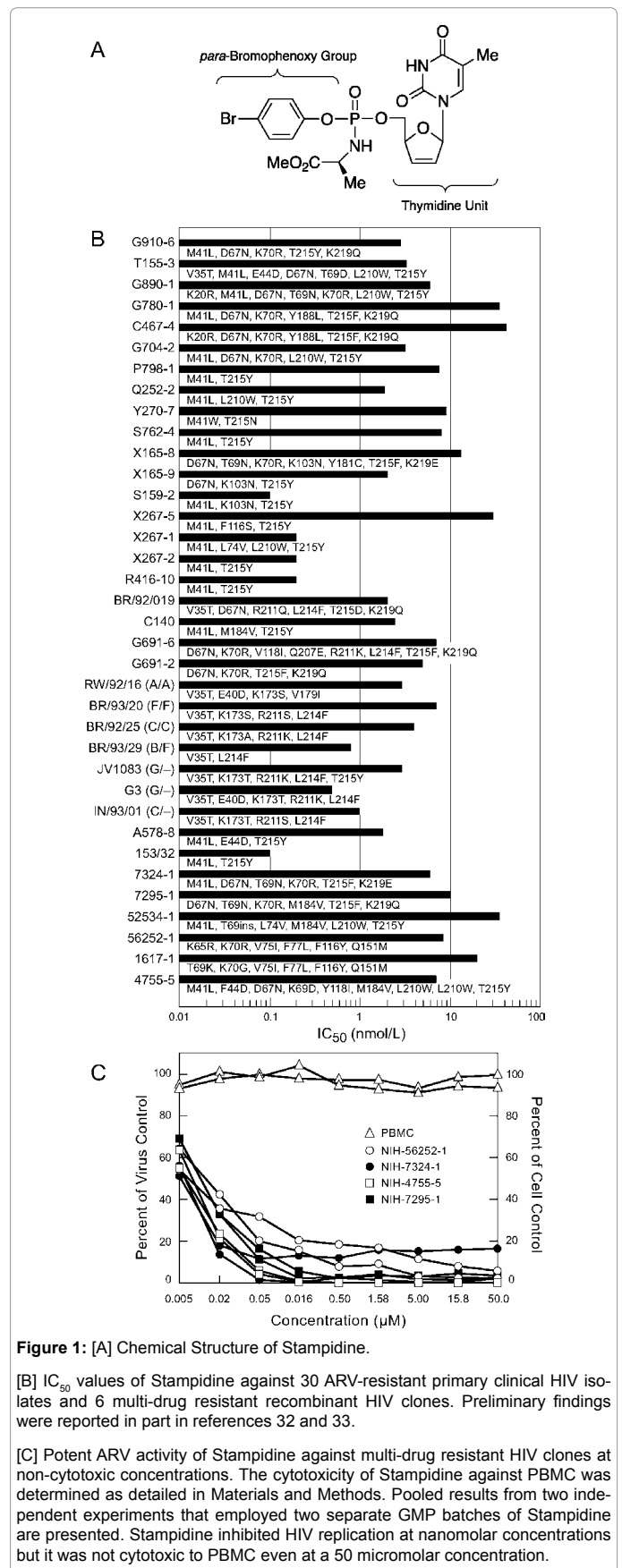


Figure 1: [A] Chemical Structure of Stampidine.

[B] IC_{50} values of Stampidine against 30 ARV-resistant primary clinical HIV isolates and 6 multi-drug resistant recombinant HIV clones. Preliminary findings were reported in part in references 32 and 33.

[C] Potent ARV activity of Stampidine against multi-drug resistant HIV clones at non-cytotoxic concentrations. The cytotoxicity of Stampidine against PBMC was determined as detailed in Materials and Methods. Pooled results from two independent experiments that employed two separate GMP batches of Stampidine are presented. Stampidine inhibited HIV replication at nanomolar concentrations but it was not cytotoxic to PBMC even at a 50 micromolar concentration.

Animal Species	N	Treatment	Daily Dose mg/kg	Highest Cumulative Dose (mg/kg)	Wt Loss	Toxicities Observed							Toxic Organ Lesions
						HE	G	LI	PA	RE	ME	PUL CV	
Mice	75	po bolus x1	25-100	100	-	-	-	-	-	-	-	-	0/22 (N=75)
Mice	165	po qd x 7d-56d	50-100	5,600	-	-	-	-	-	-	-	-	0/22 (N=105)
Rats	30	po qd x 14d	50-200	1,400	-	-	-	-	-	-	-	-	0/21 (N=30)
Dogs	9	po bid x 5d-30d	50-100	3,000	-	-	-	-	-	-	-	-	0/37 (N=5)
Cats AI	20	po bid x 28d-49d	50-100	4,900	-	-	-	-	-	-	-	-	ND
Cats CI*	10	po bid x 28d	50-200	5,600	+	-	+	+	-	-	+	-	0/31 (N=7)
Cats CI	6	po bolus x 1	50-100	100	-	-	-	-	-	-	-	-	0/31 (N=2)

Preliminary results were reported in part in reference 34. AI, acutely FIV-infected; CI, chronically FIV-infected; HE, hematologic; G, gastrointestinal; LI, liver; PA, pancreatic; RE, renal; ME, metabolic; PUL, pulmonary; CV, cardiovascular; See Materials and Methods for experimental details for the toxicity studies presented.

*Three of these cats had two months earlier been treated at either 1.4 g/kg (N=1) or 2.8 g/kg (N=2) dose levels, so that their total cumulative doses were 7.0 g/kg (N=1) and 8.4 g/kg (N=2), respectively.

Table 1: Preclinical Toxicity Profile of Orally Administered Stampidine in Small and Large Animal Species.

resistance to each of the currently available NRTI at subnanomolar to low nanomolar concentrations with a mean IC_{50} value of 8.0 ± 1.8 nM (Figure 1B). Notably, HIV-1 isolates with the MDR Q151M complex (1617-1, 56252-1), 69 insertion complex (52534-1), the non-thymidine mutations M184V (7295-1, 56252-1, C140, 4755-5), L74V (X267-1, 52534-1), and K65R (56252-1), or 5 different TAMs (G910-6, G890-1, G780-1, G704-2, 7324-1) were all exquisitely sensitive to Stampidine (Figure 1B). Further, Stampidine exhibited potent anti-HIV-1 activity against non-B subtype clinical isolates (A, C, F, and G) originating in Africa (Rwanda, Nigeria), South America (Brazil), and Asia (India) (Figure 1B) (RW/92/16, BR/93/20, BR/92/25, BR/93/29, JV 1083, G3, IN/93/01). Importantly, Stampidine was not toxic to the peripheral blood mononuclear cells even at 50 μ M (Figure 1C).

Preclinical toxicity profile of orally administered stampidine in multiple animal species

As summarized in Table 1 and presented in detail in the Supplemental Material, orally administered Stampidine was well tolerated in mice, rats, dogs, and cats with a single dose “no observable adverse effect level” (NOAEL) of 100 mg/kg. Repeated daily administration of Stampidine at this daily dose level did not cause clinical or subclinical toxicity detectable by laboratory tests at cumulative dose levels as high as 5.6 g/kg in BALB/c mice, 1.4 g/kg in Wistar Albino rats, 3.0 g/kg in Beagle dogs, or 4.9 g/kg in domestic shorthair cats. No toxic lesions were found in any of the organs from Stampidine-treated animals. In dogs, a 5-day treatment course with Stampidine did not cause any alterations in blood pressure or heart rate. There were no significant differences between the pre-treatment, on-treatment, and post-treatment values for systolic arterial pressure (SAP), diastolic arterial pressure (DAP), mean arterial pressure (MAP) and pulse rate (PR). Electrocardiogram (ECG) readings of lead II at 50 mm/sec were evaluated in both high dose (~50 mg/kg bid) and low dose (~25 mg/kg bid) Stampidine treated dogs. Stampidine did not cause any ST segment changes suggestive of myocardial ischemia, intraventricular conduction delays with widening of the QRS complex, RBBB, or LBBB, atrioventricular conduction delays with prolonged PR interval or AV block, bradycardia or tachycardia, PVC's, SVT or VT. We examined the toxicity profile of Stampidine in acutely and chronically FIV-infected shorthair domestic cats. Twenty acutely infected cats tolerated their twice daily (bid) treatment with 25 mg/kg/dose-50 mg/kg/dose Stampidine x 28d-49d administered alone or in combination with AZT(ZDV)/3TC very well without any clinical signs of morbidity. Likewise, six chronically FIV-infected cats treated with a 28-d course of Stampidine at daily dose levels ranging from 50 mg/kg (n=3) to 100 mg/kg (n=3) tolerated their treatments without

any immediate adverse reactions. In these acutely or chronically FIV-infected cats, blood tests done during therapy or post-therapy confirmed that Stampidine did not cause (a) anemia, thrombocytopenia, neutropenia, or lymphopenia suggestive of hematologic toxicity, (b) elevations of BUN or creatinine or electrolyte disturbances suggestive of renal toxicity or metabolic abnormalities, (c) elevations of ALT, AST, Alk. Ptase, or bilirubin suggestive of hepatotoxicity, (d) decreases in serum albumin or total protein suggestive of protein losing renal or gut toxicity, or (e) hyperglycemia, hypoglycemia or hypercholesterolemia suggestive of abnormalities in carbohydrate or fat metabolism. No toxic lesions were found in any of the 31 organs examined (Table 1).

Stampidine caused gastrointestinal and hepatic toxicity in chronically FIV-infected cats when used at a daily dose level of 200 mg/kg. Four chronically FIV-infected cats were treated with 100 mg/kg/dose stampidine in hard gelatin capsules twice daily (200 mg/kg/day) for 28 consecutive days (cumulative dose = 5.6 g). Three of these cats had two months earlier been treated at either 1.4 g/kg (N=1) or 2.8 g/kg (N=2) dose levels, so that their total cumulative doses were 7.0 g/kg (N=1) and 8.4 g/kg (N=2), respectively. In these cats, stampidine caused nausea/vomiting for 2-3 days during the first week of treatment. All cats exhibited a decreased appetite for at least 3 weeks during the 4-week treatment course and lost weight during the first 1-3 weeks of treatment. Diarrhea was observed in 3 of the 4 cats and only on 1-3 days of the 4-week treatment period (Table 1). Otherwise, the treatments were well tolerated and did not cause any significant toxicity. Blood tests done at 2, 4, 8, or 10 weeks after initiation of therapy showed that stampidine did not cause (a) anemia, neutropenia, lymphopenia, or thrombocytopenia suggestive of hematologic toxicity, (b) elevations of BUN or creatinine or electrolyte disturbances suggestive of renal toxicity, or (c) hyperglycemia, hypoglycemia or hypercholesterolemia suggestive of abnormalities in carbohydrate or fat metabolism. However, all 4 cats showed an elevation of the serum ALT level lasting 1-3 weeks without a concomitant elevation in total bilirubin or Alk Ptase levels. The average ALT level was 1.8-fold higher than the upper normal level (Normal range: 8.3-100.0 U/L; mean \pm SE at 2 weeks = 183.5 \pm 77.1, P=0.06). These laboratory results are consistent with hepatotoxicity (Table 1). However, no Stampidine-related toxic lesions were found in any of the tissues examined (Table 1).

In vivo activity of stampidine against NRTI-resistant HIV-1 in a surrogate SCID mouse model of human AIDS

We next examined the *in vivo* anti-HIV activity and toxicity of Stampidine in Hu-PBL-SCID mice infected with BR/92/019, a

genotypically and phenotypically NRTI-resistant HIV-1 isolate. Treatments were initiated on the day of virus inoculation. Throughout the experimental period, mice were monitored daily for morbidity and mortality. Two weeks after infection, Hu-PBL-SCID mice were electively killed, and their peritoneal lavage cells as well as spleen cells were examined for evidence of infection by NASBA. Stampidine exhibited significant and dose-dependent anti-HIV activity in this surrogate Hu-PBL-SCID mouse model of human AIDS, when it was administered orally for 14 consecutive days (Table 2). Administration of Stampidine resulted in a dose-dependent reduction in the incidence of NASBA positivity of the spleen specimens. Whereas spleen specimens from 35 of 42 vehicle-treated control Hu-PBL-SCID mice showed NASBA evidence of HIV-1 infection at 14 days after BR/92/019 inoculation, spleen specimens from only 1 of 19 test mice treated with 400 mg/kg Stampidine showed NASBA evidence of HIV-1 infection (Chi-square value = 36.9, $P < 0.0001$; Table 2A). Similarly, whereas the peritoneal lavage specimens from 40 of 43 vehicle-treated control mice showed NASBA evidence of HIV-1 infection, peritoneal lavage specimens from only 2 of 18 test mice treated with 400 mg/kg Stampidine showed NASBA evidence of HIV-1 infection (Chi-square value = 41.3, $P < 0.0001$, Table 2A). Stampidine treatment was not associated with any morbidity or mortality. Histopathologic examination of multiple organs from 48 Hu-PBL-SCID mice treated with Stampidine at dose levels of 100 mg/kg/day (cumulative dose=1.4 g/kg/day, n=10), 200 mg/kg (cumulative dose=2.8 g/kg, n=19), or 400 mg/kg (cumulative dose = 5.6 g/kg, n=19) did not reveal any toxic lesions (Table 2A).

In vivo anti-retroviral (ARV) activity of Stampidine in chronically FIV-infected shorthair domestic cats

We next examined in chronically FIV-infected domestic cats the

in vivo ARV activity of Stampidine administered orally in hard gelatin capsules according to a twice daily treatment schedule for 28 consecutive days. Stampidine exhibited a significant ARV effect, as evidenced by a reduction in the FIV load of peripheral blood mononuclear cells (PBMC) (Table 2B). Three of 3 cats treated at the 100 mg/kg daily dose level (viz.: 50 mg/kg/dose b.i.d) showed a therapeutic response, as evidenced by a 1-2 logs (Mean±SE = 1.33±0.33 logs, $P=0.008$) reduction of the FIV load in PBMC within 2 weeks, as measured by VI-RT assays (Table 2B). Likewise, 4 of 4 cats treated at the 200 mg/kg daily dose level (viz.: 100 mg/kg/dose b.i.d) showed a 1-2 logs (Mean±SE = 1.25±0.25 logs, $P=0.002$) reduction of the PBMC FIV load (Table 2B).

Early safety and activity assessment of stampidine in a first-in-human placebo-controlled phase I clinical study in therapy-naïve HIV-infected patients

A phase I randomized, placebo controlled study to explore the safety and tolerability of Stampidine in patients infected with HIV 1 with plasma HIV-1 RNA levels > 1,000 copies/mL and < 50,000 copies/mL was performed. A single dose intake of Stampidine in the range from 5 to 25 mg/kg did not result in clinically significant adverse events or clinically significant laboratory adverse events (Table 3). A total of 24 adverse events in 13 patients occurred after taking Stampidine most of which were grade I toxicities unrelated to the study drug and 8 adverse events were reported in the 5 patients on the placebo arm. There was no dose relationship for any of the reported adverse events. No dose-limiting or severe adverse side effects were observed after Stampidine administration. In particular, no patient developed neuropathy, hyperlactatemia, lactic acidosis, or lipodystrophy/fat atrophy suggestive of mitochondrial toxicity. The laboratory evaluations, ECG, or the

A. ARV Activity of Stampidine in Acutely HIV-Infected Hu-PBL-SCID Mice*					
Stampidine Dose Level (mg/kg/d)	HIV-NASBA*				Histopathology (Toxic lesions)
	Spleen	P-value (Chi-square value)	Peritoneal Lavage	P-value (Chi-square value)	
50	4/10	0.007 (7.2)	7/10	0.06 (3.5)	ND
100	7/20	0.0002 (14.2)	12/20	0.002 (9.7)	0/10
200	10/30	<0.0001 (19.2)	17/28	0.0008 (11.2)	0/19
400	1/19	<0.0001(36.9)	2/18	<0.0001 (41.3)	0/19
Vehicle	35/42*	----	40/43*	----	ND

B. ARV Activity of Stampidine in Chronically FIV-Infected Cats**				
Stampidine Dose Level (mg/kg xbid)	Log ₁₀ FIV-load in PBMC		Log Reduction of FIV-load	Average Log Reduction Mean±SE (P-value)
	Pre	Post		
50 (#1)	1	0	1	1.33±0.33 (P=0.008)
50 (#2)	2	0	1	
50 (#3)	1	0	1	
100 (#1)	2	1	1	1.25±0.25 (P=0.002)
100 (#2)	3	2	2	
100 (#3)	2	0	1	
100 (#4)	2	1	1	
Placebo (#1)	1	2	-1	-2.0±0.6
Placebo (#2)	2	5	-3	
Placebo (#3)	2	4	-2	

*The anti-HIV activity of Stampidine against BR/92/019, a genotypically and phenotypically NRTI-resistant HIV-1 isolate was evaluated in Hu-PBL-SCID mice. Stampidine was administered via gavage or by addition to the food starting on the day of virus inoculation. Throughout the experimental period, mice were monitored daily for morbidity and mortality. Two weeks after virus inoculation, Hu-PBL-SCID mice were electively killed, and their peritoneal lavage cells as well as spleen cells were examined for evidence of HIV-1 infection by NASBA. At the time of necropsy, 18 selected tissues were submitted for standard hispathological examinations.

* Log₁₀ HIV-load (Mean±SE): 4.4±1.0

**The anti-retroviral activity of Stampidine was examined in chronically FIV-infected shorthair domestic cats, as described in Materials and Methods. The FIV load of PBMCs from Stampidine- treated vs. placebo-treated cats was determined in standard PBMC co-culture experiments, as previously reported (30).

Table 2: Anti-Retroviral Activity of Stampidine in HIV-Infected Hu-PBL-SCID Mice and FIV Infected Shorthair Domestic Cats.

Patient Random Number	Cohort	Dose mg/kg	Age (years)	Gender	DLT	Adverse Events	Day -1 HIV-Load (in logs)	Day +7 HIV Load (In logs)	Log Reduction
6	1	0/PCB	31	M	None	Odinophagia, Cough, Dysuria (CTC1, NR for all)	36403 (4.6)	46146 (4.7)	-0.1
10	2	0/PCB	44	M	None	Dizziness (CTC1, NR)	4280 (3.6)	11489 (4.1)	-0.5
16	3	0/PCB	30	M	None	Headache, OM (CTC1, NR for all)	70961 (4.9)	98908 (5.0)	-0.1
23	4	0/PCB	37	M	None	Soft tissue infection (CTC 2, NR)	31250 (4.5)	65556 (4.8)	-0.3
27	5	0/PCB	41	M	None	Vomiting (CTC1, NR)	>100000(>5.0)	>100000(>5.0)	ND
1	1	5	39	M	None	Tooth ache (CTC1, NR)	2369 (3.4)	5182 (3.7)	-0.3
2	1	5	30	M	None	None	3601 (3.6)	5393 (3.7)	-0.1
3	1	5	38	M	None	None	6820 (3.8)	10416 (4.0)	-0.2
4	1	5	42	F	None	None	9379 (4.0)	81221 (4.9)	-0.9
5	1	5	36	F	None	Headache (CTC1, NR)	5264 (3.7)	7599 (3.9)	-0.2
7*	2	10	29	M	None	Scabies, N/V (CTC1, NR for all)	>100000 (>5.0)	65933 (4.8)	>0.2
8	2	10	30	M	None	None	40425 (4.6)	37196 (4.6)	0.0
9	2	10	28	M	None	None	6771 (3.8)	69059 (4.8)	-1.0
11	2	10	41	M	None	None	25687 (4.4)	>100000 (>5.0)	<-0.6
12	2	10	23	M	None	Headache (CTC1, UNL R)	65366 (4.8)	>100000 (>5.0)	<-0.2
13	3	15	25	F	None	Gingivitis, Throat pain, Fever (CTC1, NR for all)	45462 (4.7)	48402 (4.7)	0.0
14	3	15	30	M	None	Headache, Nausea (CTC1, NR for all)	19207 (4.3)	46207 (4.7)	-0.4
15*	3	15	31	F	None	None	71108 (4.9)	34011 (4.5)	0.4
17*	3	15	42	M	None	Nasal congestion, cough, tooth ache, headache (CTC1, NR for all)	12169 (4.1)	6135 (3.8)	0.3
18*	3	15	34	M	None	None	>100000(>5.0)	81576(4.9)	>0.1
19	4	20	35	M	None	None	23380 (4.4)	23660 (4.4)	0.0
20	4	20	34	M	None	Diarrhea, Abdominal pain (CTC1, NR for all)	47932 (4.7)	>100000 (>5.0)	<-0.3
21*	4	20	33	M	None	None	6789 (3.8)	2418 (3.4)	0.4
22*	4	20	45	M	None	Tooth ache (CTC1, NR); Headache (CTC1, Pos R)	7473 (3.9)	3714 (3.6)	0.3
24	4	20	37	M	None	Nausea (CTC1, UNL R); Diarrhea (CTC1, NR)	74012 (4.9)	>100000 (>5.0)	<-0.1
25	5	25	40	F	None	None	16440 (4.2)	17366 (4.2)	0.0
26	5	25	32	M	None	Night sweats (CTC1, NR); Diarrhea (CTC1, Prob R)	47857 (4.7)	40419 (4.6)	0.1
28*	5	25	38	M	None	Headache (CTC1, NR)	41058 (4.6)	30973 (4.5)	0.12
29*	5	25	32	M	None	None	40634 (4.6)	17081 (4.2)	0.4
30*	5	25	42	F	None	Diarrhea (CTC1, Prob R)	8482 (3.9)	4026 (3.6)	0.3

CTC, common terminology criteria for toxicity grading (ctep.cancer.gov/reporting/ctc.html). PCB, Placebo; DLT, dose limiting toxicity

UNL, unlikely to be related; Prob. R, probably related; Pos R, possibly related; NR, not related. OM, otitis media;

N/V, nausea and vomiting. * >0.1 logs reduction of the HIV burden.

Table 3: Characteristics and Treatment Response of Patients Enrolled in The First-In-Human Placebo-Controlled Phase I Clinical Trial of Stampidine.

physical examinations did not reveal any clinically significant adverse effects suggestive of toxicity. Viral load changes were analyzed using the same time points (Day -1 and +7): Three out of the 5 patients randomized to the highest dose of Stampidine (cohort 5) showed a mild-modest >0.1 log viral load reduction (Range: 0.12-0.4 logs or 25-58%). No patient in cohort 1, one in cohort 2, 3 in cohort 3 and 2 in cohort 4 also showed >0.1 log reduction in viral load. No patient in the placebo arm showed any level of viral load reduction (Table 3).

Discussion

Our lead anti-HIV drug candidate Stampidine exhibits (a) remarkable subnanomolar to low nanomolar *in vitro* ARV potency against genotypically and phenotypically NRTI-resistant primary clinical HIV isolates, non-nucleoside reverse transcriptase inhibitor (NNRTI)-resistant HIV-1 isolates, clinical non-B subtype HIV-1 isolates (subtypes A, C, E, and G) originating from South America, Asia, and sub-Saharan Africa with resistance to stavudine, adefovir and tenofovir, as well as recombinant HIV clones containing common patterns of RT mutations responsible for NRTI resistance such as multiple TAMs plus M184V, multiple TAMs plus T69 insertion, and Q151 complex (b) favorable, safety profile in mice, rats, dogs, and cats,

and (c) *in vivo* anti-retroviral activity in Hu-PBL-SCID mice as well as FIV-infected domestic cats. Except for the sporadic occurrence of nausea and vomiting after its administration and elevation of serum ALT levels in some of the cats, Stampidine therapy was not associated with any clinical or laboratory evidence of significant toxicity. No Stampidine-related toxic lesions were found in any of the organs from Stampidine-treated mice, rats, cats or dogs. Stampidine exhibited dose-dependent *in vivo* anti-HIV activity in Hu-PBL-SCID mice against a genotypically and phenotypically NRTI-resistant clinical HIV-1 isolate at nontoxic dose levels. Likewise, Stampidine reduced the FIV load in PBMC of chronically FIV-infected domestic cats. The *in vitro* potency of Stampidine against primary clinical HIV-1 isolates with genotypic and/or phenotypic NRTI- or NNRTI-resistance as well as non-B envelope subtype along with its prophylactic *in vivo* anti-retroviral activity in HIV-infected Hu-PBL SCID mice as well as therapeutic *in vivo* anti-retroviral activity in FIV-infected cats [29] prompted its further development as a new NRTI candidate for clinical use in HIV-1 infected individuals. Notably, in a placebo-controlled Phase I study involving 30 therapy-naïve adult HIV-infected adult patients, formulated GMP-grade oral Stampidine capsules did not cause dose-limiting toxicity at single dose levels ranging from 5 to 25 mg/kg. Taken together, the presented favorable preclinical and early clinical safety/

activity profile of Stampidine warrants its further development as a new anti-HIV drug candidate against ARV-resistant HIV strains. Our immediate future plans include a 14 day dose-ranging study designed to collect more safety and activity data for Stampidine as well as a non-human primate study to obtain preclinical proof-of-principle for using Stampidine as a PrEP agent.

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