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Zidampidine, an aryl phosphate derivative of AZT: in vivo pharmacokinetics, metabolism, toxicity, and anti-viral efficacy against hemorrhagic fever caused by Lassa virus

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Abstract—The pharmacokinetics, metabolism, and toxicity of Zidampidine, an aryl phosphate derivative of AZT, 3′-azidothymidine-5′-[*p*-bromophenyl methoxyalaninyl phosphate] were investigated in CD-1 mice. Following iv injection, Zidampidine was rapidly converted to its metabolites Ala-AZT-MP and AZT. Zidampidine was not toxic to mice at doses up to 250 mg/kg. We next examined the therapeutic effect of Zidampidine in CBA mice challenged with intracerebral injections of the Josiah strain of Lassa virus. Mice were treated either with vehicle or non-toxic doses of Zidampidine administered intraperitoneally 24 h prior, 1 h prior, and 24, 48, 72, and 96 h after virus inoculation. The probability of survival following the Lassa challenge was significantly improved for Zidampidine-treated mice (Kaplan Meier, Log-Rank p value < 0.0001). This pilot study provides the basis for future preclinical evaluation of Zidampidine and its potential as a new agent for the treatment of viral hemorrhagic fevers caused by Lassa virus.

1. Introduction

Lassa virus is the causative agent of Lassa fever, an acute viral disease found throughout West Africa, Europe, Asia, and the United States, which causes considerable morbidity and mortality.[1–12] Severe multi-organ involvement occurs in 5–10% of infections and case-fatality rates for hospitalized patients range from 15% to 25%.[13,14] The virus is transmitted by the respiratory route and by direct contact with contaminated materials. The potential use of hemorrhagic fever (HF) viruses as agents of biological warfare (BW) is a growing concern. Because of the ability of Lassa virus to spread from person to person, risk of its importation by international travel, and renewed threats about the potential use of HF viruses for BW, Lassa fever has emerged as a worldwide concern among public health officials especially among children.[15–17]

We and others have synthesized a number of phenyl phosphate derivatives of AZT (3-azido-3′-deoxythymidine) as potential anti-viral agents.[18–35] The purpose of the present study was to examine the effects of the novel AZT derivative Zidampidine (3′-azidothymidine-5′-[*p*-bromophenyl methoxyalaninyl phosphate]) on the survival outcome of mice challenged with fatal amounts of Lassa virus.

2. Results and discussion

2.1. Pharmacokinetic profiles of Zidampidine in mice following intravenous administration

Zidampidine was immediately converted to metabolite 1 and metabolite 2 after a single bolus intravenous injection. The plasma metabolite concentration–time curves are presented in Figure 1A and B. The estimated pharmacokinetic parameter values are presented in Table 1. Following iv administration of Zidampidine (240 mg/kg) to CD-1 mice, metabolite 1 (herein known as Zidampidine-M1) was formed almost immediately (t_{max} of 5.4 min) and showed rapid elimination ($t_{1/2}$ of

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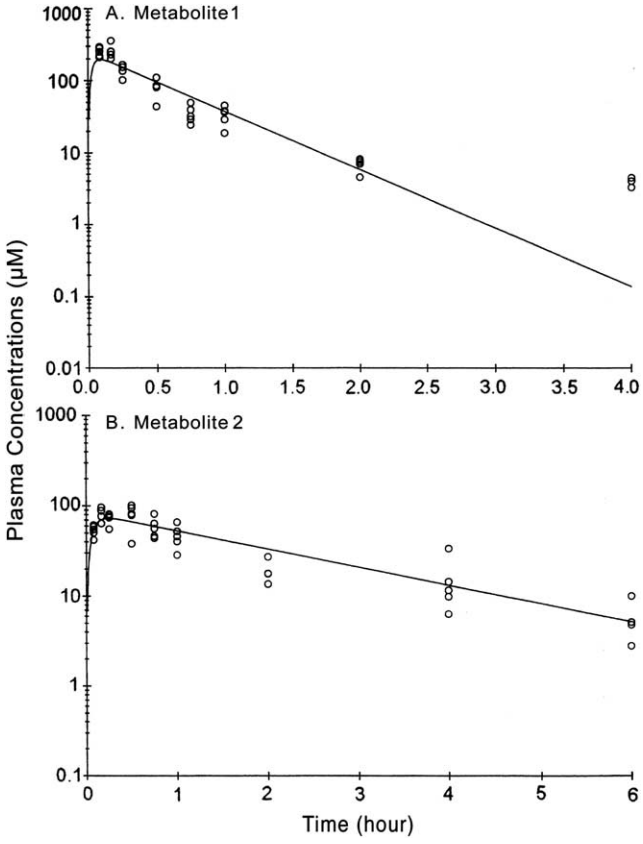


Figure 1. Plasma concentration–time profiles of Zidampidine-M1 (A) and Zidampidine-M2 (B) in CD-1 mice following iv bolus injection of Zidampidine at dose of 240/kg (five mice per time point).

22.3 min). The estimated AUC and C_{max} values were 196.0 μM h and 124.2 μM, respectively.

When Zidampidine was further metabolized to form metabolite 2 (herein known as Zidampidine-M2) the time to reach the maximum plasma concentration was 14.7 min. The elimination half-life of Zidampidine-M2 following iv injection of Zidampidine was 90.3 min. The estimated AUC and C_{max} values of metabolite 2 were 177.3 μM h and 73.0 μM, respectively.

2.2. Identification of Zidampidine metabolites

The first Zidampidine metabolite (Zidampidine-M1) was extracted from the plasma of CD-1 mice following iv injection of Zidampidine. The LC–MS spectrum showed peaks corresponding to a mass value of 417 along with additional mass peaks (Fig. 2A). In contrast, the synthetic compound, (3′-azido-3′-deoxythymidine-5′-methoxyalaninyl phosphate) under the same LC–MS conditions (Fig. 2B) showed a clean spectrum with a mass value of 417. Based on this result we propose that Zidampidine-M1 was formed in the plasma samples along with other unknown compounds.

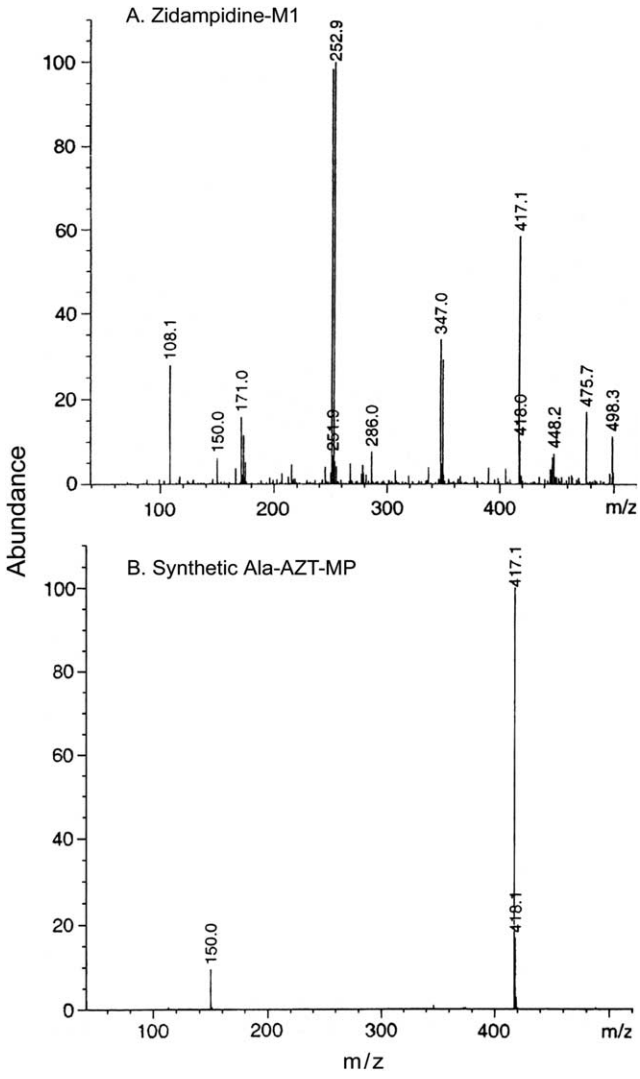


Figure 2. Mass spectra of Zidampidine-M1 obtained from plasma extraction (A) and synthetic Ala-AZT-MP: (A) corresponds to the material extracted from the plasma after iv injection of Zidampidine and (B) represents the authentic synthetic sample of Ala-AZT-MP. The material obtained from the plasma shows complexity in the pattern perhaps due to the presence of other unknown material that eluted with Zidampidine-M1. Our intent was not to identify all the peaks from the plasma sample, but was instead to compare the peaks in the spectrum corresponding to the authentic sample of Ala-AZT-MP.

Table 1. Estimated pharmacokinetic parameter values of Zidampidine metabolites in CD-1 mice

Measured	AUC (μM h)	C_{max} (μM)	$t_{1/2}$ (min)	t_{max} (min)
Ala-AZT-MP	124.2 (149.6 ± 10.7)	196.0 (226.0 ± 16.5)	22.3 (23.1 ± 2.5)	5.4 (6.6 ± 0.9)
AZT	177.3 (165.4 ± 13.5)	73.0 (75.9 ± 2.9)	90.3 (78.9 ± 6.1)	14.7 (15.3 ± 1.7)

Pharmacokinetic parameters in CD-1 mice following intravenous injection of 240 mg/kg are present as the average values estimated from composite plasma concentration–time curves of pooled data. The mean ± SE values are indicated in the parentheses ($N = 5$ mice per time point). Abbreviations: $t_{1/2}$ is terminal elimination half-life; t_{max} is the time required to reach the maximum plasma drug concentration following injection.

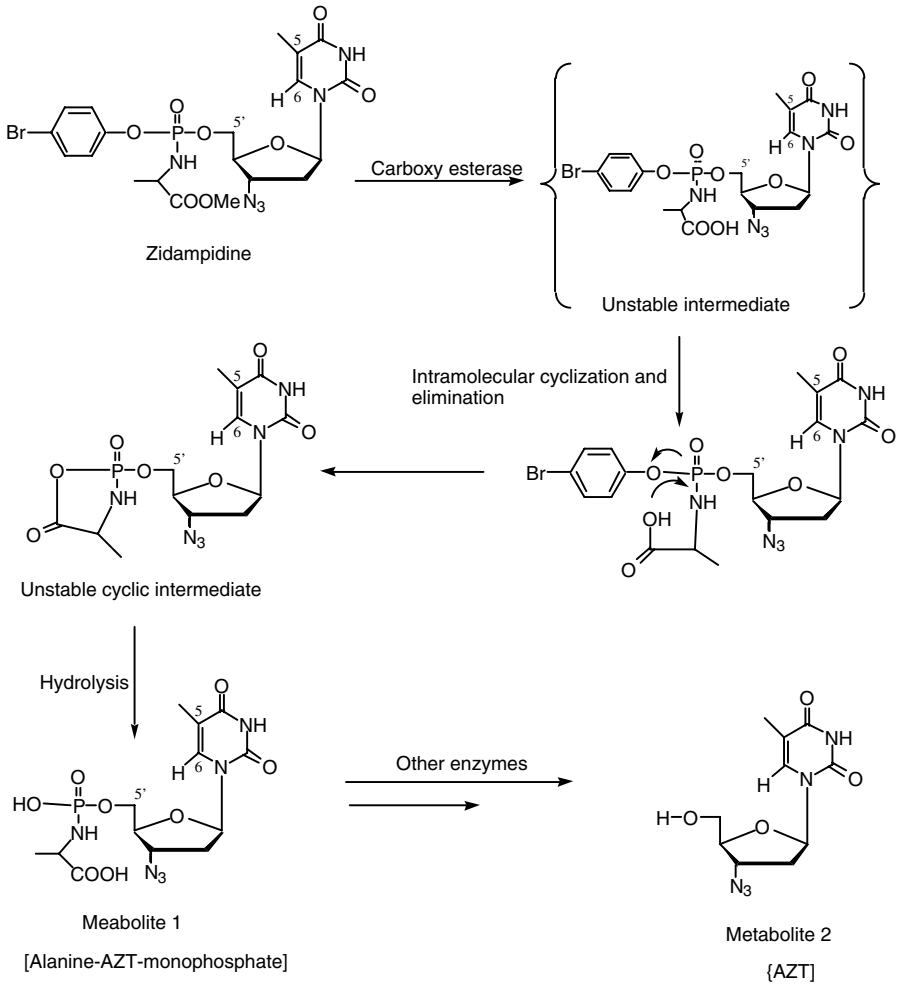
The second metabolite (Zidampidine-M2) was extracted directly with ethyl acetate from the urine of Zidampidine-treated mice. The NMR spectrum of Zidampidine-M2 was found to be identical to that of pure AZT. Therefore, Zidampidine-M2 was identified as AZT.

Zidampidine-M2 (AZT) may be produced from Zidampidine-M1 (Ala-AZT-MP) through enzymatic metabolism in vivo. Hence there are three pathways through which AZT could be formed in vivo. In the first pathway, Zidampidine enzymatically converts into Zidampidine-M1 by hydrolysis of the methyl ester side chain of the compound. The carboxylic acid cyclizes intramolecularly by eliminating the bromophenoxy group thus forming an unstable cyclic intermediate (see Scheme 1). This intermediate is further hydrolyzed by water to form Zidampidine-M1(Ala-AZT-MP), that can further metabolize in the presence of other enzymes to form Zidampidine-M2 (AZT).

Alternatively, direct enzymatic cleavage of the nucleoside can break the nucleoside moiety thus forming Zidampidine-M2 (AZT). In a third pathway, simultaneous attack on both the ester side chain in combination with direct attack on the phosphorus center may form both the metabolites. However, when we compared the HPLC profiles of the plasma samples, we observed formation of both Zidampidine-M1 (Ala-AZT-MP) as well as Zidampidine-M2 (AZT). If cleavage occurred via the second pathway, then we would have observed formation of Zidampidine-M2 (AZT) only, rather than Zidampidine-M1 and Zidampidine-M2 (Fig. 3). In the case of the third proposed pathway, formation of only Zidampidine-M2 would be visualized as Zidampidine-M1 would lack the AZT moiety due to the reasons explained earlier and hence we would not be able to observe the formation of Zidampidine-M1. From these results, we propose that formation of both the metabolites demonstrates that the methyl ester side chain of Zidampidine undergoes hydrolysis via the first proposed pathway by first forming Ala-AZT-MP.

2.3. In vivo toxicity of Zidampidine in mice

Six weeks old female CD-1 mice were administered an intraperitoneal bolus injection of Zidampidine in 0.2 mL 10–20% DMSO/PBS solution, or 20% DMSO/PBS alone (control mice). Groups of 10 mice received a single treatment at one of the three different dose levels of Zidampidine (100 μg, 1 mg, and 5 mg). Ten control mice were injected intraperitoneally with 20% DMSO/PBS solution in accordance with the experimental



Scheme 1. Proposed hydrolysis pathway of Zidampidine in vivo.

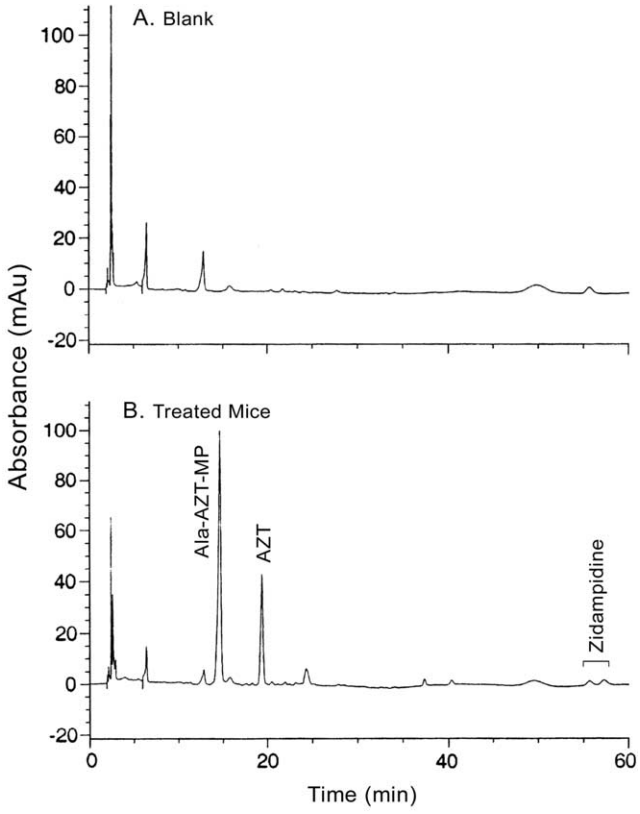


Figure 3. Representative HPLC chromatograms of blank plasma (A) and plasma sample at 10 min following iv injection 240 mg/kg Zidampidine.

protocol. There were no immediate adverse clinical effects on the test mice following Zidampidine administration. All mice were electively sacrificed healthy without any weight loss on day 30 (Table 2). No significant gross organ pathology was observed at the time of necropsy. Histopathologic examination of multiple tissues revealed no toxic lesions.

2.4. In vivo anti-Lassa activity of Zidampidine

In order to evaluate the anti-Lassa activity of Zidampidine, CBA mice were inoculated with intracerebral injections of the Josiah strain of Lassa[36] at a 1000 PFU dose level, which is lethal to 70–100% of mice within 7–12 days.[37] Mice were treated either with vehicle or Zidampidine administered intraperitoneally 24 h prior, 1 h prior, and 24, 48, 72, and 96 h (total number of doses administered to each mouse = 6) after virus inoculation. Mice were then observed twice daily for 21 days for morbidity and mortality. Of the 20 control mice, 2 died on day 1 immediately after intracerebral injection due to accidental brain injury and are not evaluable. All of the remaining 18 vehicle-treated control mice developed decreased mobility and scruffy fur as the clinical signs of Lassa infection between days 6 and 10 (Table 3). Fourteen of the 18 control mice developed seizures between days 7 and 11. Thirteen mice experienced 4–10% weight loss and died between days 8 and 11 (Table 3, Fig. 4). Of the 10 mice treated with Zidampidine at the 25 mg/kg dose level, 1 died accidentally immediately after intracerebral Lassa virus inoculation. All of the remaining nine mice developed decreased mobility and scruffy fur as the clinical signs of Lassa infection between days 8 and 10. All nine mice survived the Lassa challenge beyond the 21-day observation period and did not experience any weight loss or seizures (Table 3, Fig. 4). The probability of survival following the Lassa challenge was significantly improved for Zidampidine-treated mice (Kaplan Meier, Log-Rank p value $\ll 0.001$). The probability of survival at 21 days was 28% (7–48%, 95% confidence limits) for vehicle-treated mice (median survival = 9 days), and 100% for mice treated with Zidampidine at the 25 mg/kg dose level (median survival >21 days).

These results provide unprecedented evidence that Zidampidine exhibits a potent prophylactic effect against Lassa fever and therefore shows clinical potential as a new agent for the treatment of viral hemorrhagic fevers caused by Lassa virus.

3. Experimental

3.1. Chemicals

All the reagents used in this study were HPLC grade. Deionized distilled water was prepared using Milli-Q purification system (Millipore, Medford, MA). Methanol, acetonitrile, acetic acids were purchased from Fisher Chemicals (Fair Lawn, NJ). AZT (3′-azido-3′-deoxythymidine) was from Toronto Research Chemicals (Ontario, Canada).

3.2. Synthesis and characterization of Zidampidine derivatives

Zidampidine [3′-azidothymidine-5′-[*p*-bromophenyl methoxyalaninyl phosphate]] and Ala-AZT-MP [AZT-5′-

Table 2. Life-table analysis of survival data and statistical analysis of weight change

Treatment group	# of mice	Mean weight change (g) ± SEM	Weight change p value[a]			
			20% DMSO	Zidampidine		
				100 μg	1 mg	5 mg
20% DMSO	10	6.17 ± 1.017		0.7886	0.6201	0.9284
100 μg Zidampidine	10	6.49 ± 0.591	0.7886		0.7280	0.7035
1 mg Zidampidine	10	6.96 ± 1.192	0.6201	0.7280		0.5620
5 mg Zidampidine	10	6.04 ± 1.002	0.9284	0.7035	0.5620	

[a] Weight p value determined by unpaired t-test analysis. A p value of <0.05 was considered statistically significant.

Table 3. Anti-Lassa activity of Zidampidine in CBA mice

	Disease onset (days after inoculation with Lassa virus)				
	Decreased mobility	Scruffy fur	Convulsions	Weight loss (%)	Survival (days)
Group A—vehicle					
Mouse #1[a]	NA	NA	NA	NA	⩽1
Mouse #2[a]	NA	NA	NA	NA	⩽1
Mouse #3	6.0	6.0	7.0	4.5	8
Mouse #4	6.0	6.5	8.0	5.0	9
Mouse #5	7.5	7.0	8.0	9.0	9.5
Mouse #6	7.5	7.5	8.0	4.3	9.0
Mouse #7	7.0	7.0	8.0	4.3	9.0
Mouse #8	7.0	7.0	8.5	4.5	9.5
Mouse #9	7.0	7.0	8.5	8.3	9.5
Mouse #10	7.0	7.0	8.5	4.8	9.5
Mouse #11	8.5	8.0	9.0	5.3	10.5
Mouse #12	8.5	8.0	9.0	4.5	10.0
Mouse #13	8.0	8.5	9.5	4.5	10.0
Mouse #14	9.0	9.0	10.5	4.1	11.0
Mouse #15	9.0	9.5	11.0	4.3	11.5
Mouse #16	9.0	10.0	NO	NO	>21
Mouse #17	9.0	10.0	NO	NO	>21
Mouse #18	9.0	10.0	11.0	NO	>21
Mouse #19	9.0	10.0	NO	NO	>21
Mouse #20	9.5	10.0	NO	NO	>21
Group B—Zidampidine 25 mg/kg					
Mouse #1[a]	NA	NA	NA	NA	⩽1
Mouse #2	8.5	8.0	NO	NO	>21
Mouse #3	8.0	8.0	NO	NO	>21
Mouse #4	8.0	8.0	NO	NO	>21
Mouse #5	8.0	8.0	NO	NO	>21
Mouse #6	8.0	8.0	NO	NO	>21
Mouse #7	8.0	8.0	NO	NO	>21
Mouse #8	8.5	8.0	NO	NO	>21
Mouse #9	9.0	8.0	NO	NO	>21
Mouse #10	9.0	8.5	NO	NO	>21

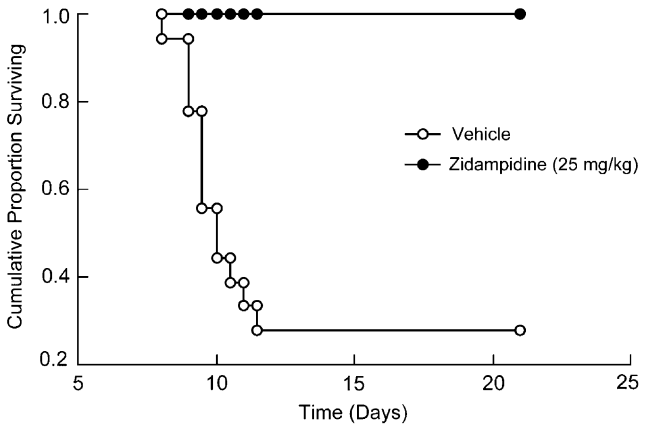
NA = not applicable; NO = not observed.
[a] Mouse died after traumatic intracerebral injection.

(methoxyalaninyl phosphase)] were synthesized as described previously[19,24–35] and as follows.

3.2.1. Synthetic scheme for aryl phosphoramidates. Scheme 2 illustrates the route followed for the synthesis of aryl phosphoramidate derivatives of AZT.[24–30]

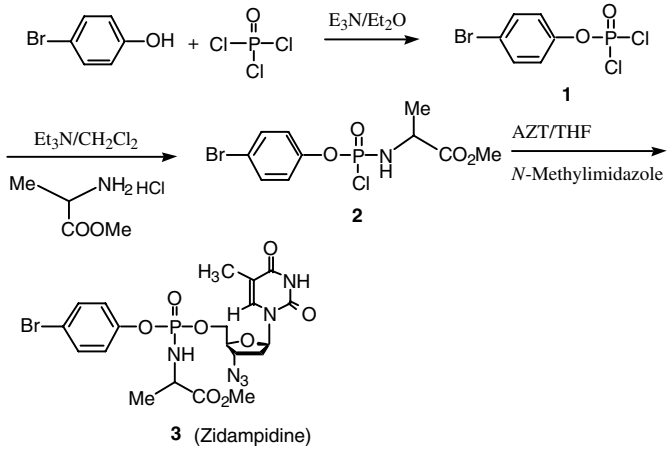
3.2.1.1. *p*-Bromophenyl phosphorodichloridate (1). Phosphorus oxychloride (17.0 g, 111 mmol) in diethylether (100 mL) was placed in a three-neck RB flask equipped with an additional funnel. The contents were cooled to 0 °C using an external ice bath. A solution of *p*-bromophenol (17.3 g, 100 mmol) and triethylamine (10.1 g, 100 mmol) in anhydrous diethyl ether (250 mL) was added dropwise while maintaining the temperature of the mixture at 0 °C throughout the addition (ca: 2–3 h). After this period, the ice bath was removed and the mixture was allowed to gradually attain room temperature and was stirred vigorously overnight. The additional funnel was replaced by a condenser and the reaction mixture was heated to reflux for an additional 2 h to ensure completion of the reaction. The reaction mixture was cooled to room temperature and the precipitated triethylammonium salt was filtered under vacuum and the precipitate was washed with additional anhydrous ether. The layers were combined and the solvent was evaporated under vacuum using a rotary evaporator to yield crude phosphorodichloridate (**1**) as a pale yellow viscous oil. Vacuum distillation of the crude product gave *p*-bromophenyl phosphorodichloridate (**1**) as a colorless, viscous oil. Bp 98–102 °C/0.1 mm, UV: λ_{max}: 242, 271 nm, IR: 3878, 3095, 2358, 1888, 1712, 1483, 1187, 831 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.46–7.44 (m, 2H), 7.50–7.47 (m, 2H), ^{31}P NMR ($CDCl_3$): δ 3.12.

3.2.1.2. *p*-Bromophenyl methoxyalaninyl phosphorochloridate (2). *p*-Bromophenyl phosphorodichloridate (**1**) (5.8 g, 20.02 mmol) and L-alaninemethylester hydrochloride (2.8 g, 20.08 mmol) were placed into a RB flask under nitrogen atmosphere. Using a dry syringe, anhydrous methylene chloride (160 mL) was added and the mixture was cooled to −70 °C using a dry ice/acetone bath. A solution of triethylamine (4.0 g, 40.0 mmol) in anhydrous methylene chloride (120 mL) was added dropwise with vigorous stirring over a period of 3 h. After completion of the addition, the reaction mixture was allowed to gradually warm to room temperature and stirred for 48–72 h until the reaction was complete as evidenced from TLC. The contents were concentrated in vacuum, anhydrous ether (60 mL) was added and the precipitated triethylammonium hydrochloride salt was filtered. The precipitate was further washed with



Treatment	# of mice	Proportion Surviving 6 days	9 days	13 days	21 days	Median Survival (days)	p-value
Vehicle	18	100 ± 0	94.4 ± 5.6	27.8 ± 10.6	27.8 ± 10.6	9	
zidampidine 25 mg/kg	9	100 ± 0	100 ± 0	100 ± 0	100 ± 0	>21	<0.0001

Figure 4. Protective activity of Zidampidine in CBA mice challenged with Lassa virus. CBA mice were inoculated with intracerebral injections of the Josiah strain of Lassa at a 1000 PFU dose level. Mice were treated either with vehicle or Zidampidine (25 mg/kg) administered intraperitoneally 24 h prior, 1 h prior, and 24, 48, 72, and 96 h after virus inoculation. Mice were then observed twice daily for 21 days for morbidity and mortality. Results are presented as the cumulative proportion of mice surviving after virus inoculation. See Table 3 for more detailed information of the treatment outcome.



Scheme 2. Synthetic scheme for Zidampidine.

additional ether (2 × 20 mL). The combined ether extracts were pooled together and rotary evaporated under vacuum to yield (**2**) as a viscous oil. UV: λ_{max}: 241, 273 nm, IR: 3212, 2989, 1747, 1483, 1270, 1209, 1147, 1010, 927, 831 cm^{-1}; ^{1}HNMR ($CDCl_3$): δ 8.70 (br s, 1H), 7.16 (d, 2H, J = 9.0 Hz), 7.48 (d, 2H, J = 9.0 Hz), 3.79 (s, 1H), 3.77 (s, 1H), 1.51 (m, 3H), 1.40 (m, 3H); MS (CI, *m/e*): 357.9 (M^++2), 355.9 (M^+), 322.0 (M^++2−Cl), 320 (M^+−Cl), 295.9 (M^+−$COOCH_3$), 186.0 (M^++2−Br−PhO), 184.0 (M^+−Br−PhO).

3.2.2. 3′-Azidothymidine-5′-[(*p*-bromophenyl)methoxyalaninyl phosphate] (3) (Zidampidine). *p*-Bromophenyl methoxyalaninyl phosphorochloridate (**2**) (4.27 g, 12.0 mmol) was placed in a dry RB flask under nitrogen atmosphere. Using a dry syringe, anhydrous tetrahydrofuran (15 mL) was introduced and the contents stirred to dissolve the phosphorochloridate. Using another dry syringe, *N*-methyl imidazole (2.0 g, 24.5 mmol) was added and the contents were stirred for additional 20 min. After this period, a solution of AZT (1.25 g, 4.1 mmol) in anhydrous tetrahydrofuran (40 mL) was added and the reaction mixture was stirred vigorously at room temperature under a nitrogen atmosphere for 100–120 h. Throughout the reaction, the flask was covered with an aluminum foil to avoid light during the reaction. After the reaction was complete as evidenced by TLC, the mixture was concentrated to remove tetrahydrofuran under vacuum and the residue was dissolved in chloroform (100 mL). The chloroform solution was

washed consecutively with a 1 N aqueous HCl solution (2×100 mL) saturated aqueous sodium bicarbonate (2×75 mL), water (3×75 mL). The separated organic layer was dried over anhydrous sodium sulfate, filtered, and evaporated to dryness to afford (**3**) as a pale yellow viscous oil. The crude product was further purified by column chromatography using 2–5% MeOH/$CHCl_3$ to give analytically pure (**3**).

The structures of the resulting compounds were identified by ^{1}H and ^{13}C NMR (Varian Mercury 300 instrument), mass spectra [MALDI-TOF spectrometer (Model G2025A LD-TOF)], and IR [Nicolet Protege 460 spectrometer] and are shown in Figure 5.

3.3. Physico-chemical data of Zidampidine

White foamy solid; yield (83%); IR (neat): ν 3205, 2109, 1745, 1691 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 8.69 (br s, 1H), 7.45 (m, 2H), 7.33 (s, 1H), 7.14 (m, 2H), 6.18 (t, 1H) 6.13 (t, 1H), 4.44–3.77 (m, 4H), 3.73*, 3.72* (s, 3H), 2.18 (s, 3H), 1.39 (m, 3H), 1.41 (m, 3H); ^{13}C NMR ($CDCl_3$): δ 173.8, 163.6, 150.1, 149.2, 149.1, 135.4, 132.4, 121.8, 121.7, 117.8, 111.1, 85.0, 84.7, 81.9, 81.8, 82.2, 65.5, 60.1, 59.4, 52.4, 50.0, 49.9, 36.9, 20.6, 12.2; GC/MS (CI) m/z 587.1 ($C_{20}H_{24}{}^{79}BrN_6O_8P^+$), 589.1 ($C_{20}H_{24}{}^{81}BrN_6O_8P^+$); HPLC t_R 36.10, 37.51 min (acetonitrile/water).

3.4. Statistical analysis

Statistical significance was determined using the Kaplan Meier Log-Rank test. Cumulative survival proportions obtained from survival curves at 6, 9, 13, and 21-day time points were compared for all treatments. *Z*-Test was performed to calculate significant differences in the proportions that survived using the cumulative proportion surviving and the standard error values (z-score = (cumulative proportion 1 − cumulative proportion 2)/square root(standard error 1^2 + standard error 2^2); $p < 0.05$ two tailed were deemed significant).

3.5. Animal housing

All mice were housed and cared for in accordance with guidelines established by the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals, following approval of the Parker Hughes Institute Animal Care and Use Committee.

3.5.1. Toxicity studies in CD-1 mice. All CD-1 mice used in this toxicity study were obtained from the specific pathogen free (SPF) breeding facilities of Charles River Laboratories (Wilmington, MA) at 5 weeks of age. The mice were housed in the animal housing facility of the Parker Hughes Institute. All husbandry and experimental contact made with the mice maintained SPF conditions. The mice were kept in microisolator cages (Lab Products, Inc., Maywood, NJ) containing autoclaved food, water, and bedding.

In this toxicity study, 40 weighed 6 weeks old female CD-1 mice averaging 21.5 g were administered an intraperitoneal bolus injection of Zidampidine in 0.2 mL 10–20% DMSO/PBS solution, or 20% DMSO/PBS alone (control mice). Groups of 10 mice received a single treatment of 1 of 3 different dose levels of Zidampidine (100 μg, 1 mg, and 5 mg). Ten control mice were injected intraperitoneally with 20% DMSO/PBS solution in accordance with the experimental protocol. No sedation or anesthesia was used throughout the treatment period. Mice were monitored daily for mortality for determination of day 30 LD_{50} values. Mice surviving 30 days post-treatment were sacrificed and the tissues were immediately collected and preserved in 10% neutral phosphate buffered formalin. Mice were weighed at the time of sacrifice or death. At the time of necropsy the selected tissues (bone, bone marrow, brain, spinal cord, cecum, heart, kidney, large intestine, liver, lung, lymph node, ovary, pancreas, skeletal muscle, skin, small intestine, spleen, stomach, thymus, thyroid gland, urinary bladder, and uterus, as available) were immediately collected from mice for histopathologic examination.

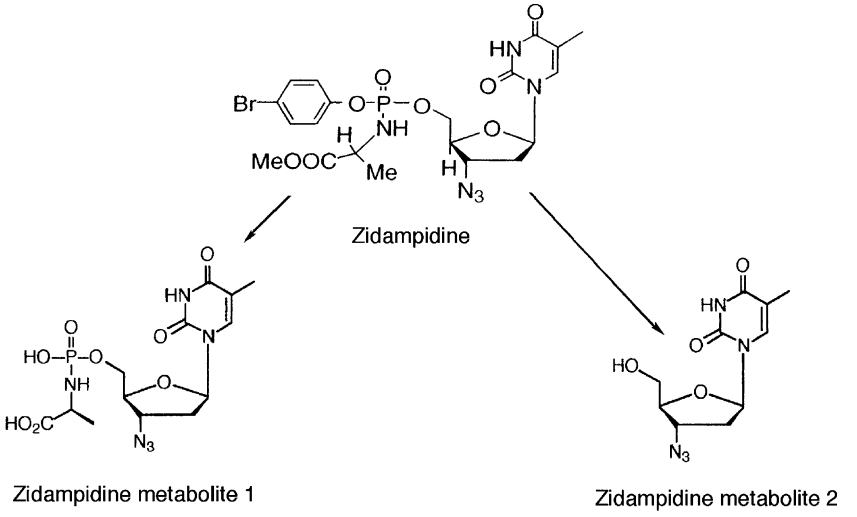


Figure 5. Metabolic pathway of Zidampidine (3′-azidothymidine-5′-[*p*-bromophenyl methoxyalaninyl phosphate]). Zidampidine-M1 (Ala-AZT-MP = 3′-azido-3′-deoxythymidine-5′-methoxyalaninyl phosphate) and Zidampidine-M2 (AZT = 3′-azido-3′-deoxythymidine).

For histopathologic studies, tissues were fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin by routine methods. Glass slides with affixed 4–5 μm tissue sections were prepared and stained with Hemotoxylin and Eosin (H&E).

3.6. HPLC determination of Zidampidine and its metabolites

Zidampidine and its metabolites were determined by adding 75 μL of methanol to 25 μL of plasma and vortexing for at least 30 s. Following centrifugation (1500 rpm, 5 min), the supernatant was transferred into a clean HPLC vial and 50 μL was injected for HPLC analysis. The HPLC system was a Hewlett Packard (Palo Alto, CA) series 1100 instrument consisting of a quaternary pump, an auto sampler, an auto electronic degasser, an automatic thermostatic column compartment, diode array detector, and a computer having a Chem Station software program for data analysis.

The analytical column was a Hewlett Packard, 250 × 4 mm Lichrospher 100, RP-18 (5 μM) column and the guard column was a 4 × 4 mm Lichrospher 100, RP-18 (5 μM). The mobile phase was degassed automatically by the electronic degasser system. Before the analysis, the column was equilibrated and a gradient program was used for analysis of samples. The flow rate was maintained at 1 mL/min and the column was maintained at room temperature. The linear gradient mobile phase was 100% 10 mM ammonium phosphate buffer (pH 3.7) at 0 min; 20% acetonitrile, 80% ammonium phosphate buffer (pH 3.7) at 20 min; 32% acetonitrile, 68% water (0.1% HAC) at 30–60 min. The wavelength of detection was set at 270 nm. Peak width, response time, and slit were set at >0.03 min, 0.5 s, and 8 nm, respectively.

3.7. In vivo metabolism

3.7.1. Isolation and identification of plasma metabolite (Zidampidine-M1). CD-1 mice were injected intravenously with Zidampidine at dose of 240 mg/kg. After 10 min, all blood was withdrawn from the ocular venous plexus by retro-orbital veni-puncture, and the plasma samples were collected. Acetonitrile was added to the pooled plasma to precipitate the plasma protein. Subsequently, acetonitrile was evaporated under a steady stream of nitrogen and the residue was further treated with ethyl acetate (to remove other components from the plasma samples). The above procedure was repeated twice and the aqueous layer was separated from the ethyl acetate layer. The aqueous layer, which contained Zidampidine-M1, was further lyophilized and the residue was reconstituted in methanol. The reconstituted solution was analyzed using a Hewlett Packard LC–MS instrument.

The experimental conditions for mass spectrum analysis were as follows: fragmentor of 75, gas flow of 10 L/min, nebulizer pressure of 25 psi, drying gas temperature of 350 °C, peak width and gain were set at 0.03 s and 1.0, respectively.

3.7.2. Isolation of urinary metabolite (Zidampidine-M2). Ten CD-1 mice were placed into a Nalgene metabolic cage after being injected intravenously with 260 mg/kg Zidampidine. Urine was collected at 0–24 h. The urine was centrifuged and stored at −20 °C until further analysis.

An aliquot of urine sample was extracted with ethyl acetate and the ethyl acetate phase was transferred to a clean tube. The ethyl acetate was evaporated using a steady stream of nitrogen and the residue was dissolved in deuterated chloroform and used directly for NMR analysis.

3.7.2.1. NMR analysis. ^{1}H NMR spectra were run on a Model Varian XL-400 NMR spectrometer operating at 399.9 MHz, and employing a deuterium field-lock frequency in the normal manner. The samples were dissolved in $CDCl_3$ for NMR analysis.

3.8. Evaluation of pharmacokinetics of Zidampidine

Female CD-1 mice (7–9 weeks old) from Charles River Laboratories (Wilmington, MA) were housed in a controlled environment (12-h light/12-h dark photoperiod, 22 ± 1 °C, 60 ± 10% relative humidity), which is fully accredited by the USDA (United States Department of Agriculture). All rodents were housed in microisolator caging systems (Lab Products, Inc., NJ) containing autoclaved bedding. The mice were allowed free access to autoclaved pellet food and tap water throughout the experiments. Animal studies were approved by the Parker Hughes Institute Animal Care and Use Committee, and all animal care procedures conformed to the Principles of Laboratory Animal Care (NIH publication #85-23, revised 1985).

Zidampidine was dissolved in DMSO and 50 μL of drug solution was injected intravenously into each mouse. This amount of DMSO was well tolerated by mice when administrated by rapid intravenously (iv) or extravascular injection.[20,21] In pharmacokinetic studies, mice were injected iv via the tail vein with a bolus dose of 240 mg/kg Zidampidine. Blood samples (∼200 μL) were obtained from the ocular venous plexus by retro-orbital veni-puncture at 0, 5, 10, 15, 30, 45 min and 1, 1.5, 2, 4, and 6 h following iv injection.

All collected blood samples were heparinized and centrifuged at 7000*g* for 5 min in a microcentrifuge to obtain plasma. Aliquots of plasma were used for extraction within 2 h after withdrawal from the treated mice and the extract was subjected for HPLC analysis as described above.

3.9. Pharmacokinetic analysis

Pharmacokinetic modeling and pharmacokinetic parameter calculations were carried out using the pharmacokinetic software, WinNonlin program, Professional Version 3.0. (Pharsight Inc., Mountain View, CA).[22,23] An appropriate pharmacokinetic model was chosen on the basis of the lowest sum of weighted

squared residuals, lowest Schwartz criterion (SC), lowest Akaike's information criterion (AIC) value, lowest standard errors of the fitted parameters, and dispersion of the residuals. The half-life was estimated by linear regression analysis of the terminal phase of the plasma concentration profile. The time (t_{max}) taken to achieve peak concentration (C_{max}) was calculated using differential calculus.

3.10. Linearity and sensitivity of HPLC-based detection method

Under the chromatographic separation conditions described in Section 3.1, the retention times for Zidampidine, Ala-AZT-MP and AZT were ~56, 14.5, and 19.2 min, respectively. At the retention time of the Zidampidine and the metabolites, no significant interference peaks from blank plasma were observed (Fig. 3A and B). The standard curve was linear over the concentration–dose ranges tested. The linearity was statistically confirmed using the Instat Program V3.0. The lowest limit of detection of Zidampidine, Ala-AZT-MP, and AZT was 0.5, 1.0, 0.5 μM at a signal-to-noise ratio of ~4.

3.11. Animal infection

Animal infection was performed as described[37] in an appropriate Animal BioSafety Level-3 Laboratory (ABL-3) at BRIEM (Research Institute for Epidemiology and Microbiology, MINSK, Belarus) with the technician wearing appropriate facility clothing. The culture was thawed in a water bath at 37 °C and then diluted in normal saline to achieve the required concentration. In this study, all mice were challenged with 1000 PFU, which is 100-times higher than the LD_{50} dose. Each group of animals was placed in a separate cage.

3.12. Lassa virus model

CBA strain mice were intracerebrally infected with 1000 PFU of Lassa virus (Josiah strain) that resulted in lethality of 80–100% of control (non-treated) animals in 7–9 days after infection. Control animals were given physiologic salt solution as a placebo instead of the compound. In general, for non-treated animals, clinical signs of the disease manifested on the fifth and seventh day by presenting: weight loss, immobility, disheveled hair, convulsions, severe decubitus paralysis, and death. All subjective measurement of decreased mobility and scruffy fur were done in a blinded fashion as not to influence the results. The protective properties of the experimental anti-viral agent were assessed by using the following treatment-preventative regimen: mice were treated either with vehicle or Zidampidine (25 mg/kg) administered intraperitoneally 24 h prior, 1 h prior, and 24, 48, 72, and 96 h after virus inoculation. Mice were then observed for 21-days post infection. The protective effect of the experimental anti-viral drugs was evaluated according to the rise of the survival rate and prolongation of mean life of the experimental animals as compared with the control animals.[38]

4. Conclusion

In summary, treatment with Zidampidine at non-toxic doses significantly improved the probability of survival following Lassa challenge. Therefore, Zidampidine shows clinical potential as a new agent for treatment of viral hemorrhagic fevers caused by Lassa virus. The elucidation of optimized prophylactic as well as post-exposure treatment regimens will be the focus of our future studies. It will also be important to determine if Zidampidine is active against other viruses associated with lethal viral hemorrhagic fevers and/or encephelomyelitis, such as the Ebola viruses of the Filoviridae family. This is the first report of pharmacokinetic and metabolism profiles of Zidampidine and its metabolites in mice. These pilot pharmacokinetic and metabolism studies as well as the preliminary toxicity studies of this aryl phosphate derivative of AZT combined with the availability of the described quantitative HPLC method for its detection in plasma provide the basis for future preclinical evaluation of Zidampidine and its potential as an anti-viral agent.

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