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Novel Alkylpolyamine Analogues That Possess Both Antitrypanosomal and Antimicrosporidial Activity

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Abstract—A novel series of alkyl- or aralkyl-substituted polyamine analogues was synthesized containing a 3-7-3 polyamine backbone. These analogues were evaluated in vitro, and in one case in vivo, for activity as antitrypanosomal agents, and for activity against opportunistic infection caused by *Microsporidia*. Compound **21** inhibits trypanosomal growth with an IC_{50} as low as 31 nM, while compound **24** shows promising activity in vitro against trypanosomes, and against *Microsporidia* in vitro and in vivo. © 2001 Published by Elsevier Science Ltd.

There is no doubt that significant advancements in anti-infective therapy have improved the quality of life in developed nations. However, in underdeveloped countries, there exist major infectious diseases that account for a large portion of global morbidity. Some of these diseases have the potential to become a threat to those living in North America. Tuberculosis claims an estimated 2 million lives each year, and drug-resistant strains originally found in New York and Russia are now being identified in other locations. Malaria, African trypanosomiasis and Leishmaniasis accounted for an additional 1,210,000 deaths in 1999.[1] Also of concern is the increasing incidence of opportunistic infections such as those caused by *Pneumocystis carinii* and strains of *Microsporidia*. Drug discovery efforts against the diseases mentioned above are limited because infected persons in underdeveloped areas cannot afford even a single course of therapy, or because the infected population represents too small of a drug market. Clearly, there is a need for new antiinfective agents that are potent, nontoxic and inexpensive to manufacture. We recently described the synthesis and evaluation of a small series of (bis)alkylated polyamines that possess promising antitrypanosomal effects in vitro.[2] Analogues possessing a 3-3-3 carbon skeleton (Fig. 1), such as BENSpm **1**, CPENSpm **2**, and CHENSpm **3**[2,3] have significant antitumor effects in vitro and in vivo, but are inactive against cultured trypanosomes. By contrast, analogues with a 3-7-3-carbon skeleton, typified by MDL 27695 **4**,[4] CHE-3-7-3 **5**,[2,3] and bis-CH-3-7-3 **6**[2,3] are inactive as antitumor agents, but possess promising antitrypanosomal effects in vitro. We now report second generation compounds in the 3-7-3 series, their biological evaluation as antitrypanosomal agents, and their activity against *Microsporidia* in vitro and in vivo.

Chemistry

The syntheses leading to (bis)alkylated polyamines, which are outlined in Scheme 1, are facile and straightforward, and as such are suitable for the efficient and cost-effective production of a variety of active analogues. The tetraamine **7** can be readily synthesized from 1,3-bis-[(amino)methyl]cyclohexane in three steps.[2] Cyanoethylation[5] followed by Raney nickel reduction[5] of the cyano groups affords the corresponding tetraamine in high yield, which is then tetramesitylated (2-mesitylenesulfonyl chloride, dichloromethane, 10%

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aqueous NaOH)[6] to afford the protected intermediate **7**. Bis alkylation of **7** (Method A, NaH, 2.2 equiv of R-X)[2] affords the (bis)-alkylated intermediates, which are deprotected (30% HBr in AcOH)[7] to yield the pure desired target compounds **8**–**12** as the tetrahydrobromide salts following recrystallization from ethanol/water.

A second route (Scheme 1, method B) was employed for the synthesis of analogues **16**–**19**. The monoalkylated 1,3-diaminopropanes **13**–**15**[5] were appended to the appropriate *cis*-1-acetoxy-4-aminocycloalkane[8] by palladium(0) catalyzed coupling as previously described, affording the target compounds **16**–**19** in a single step. These analogues were converted to the corresponding trishydrobromide salts by anion exchange chromatography, and purified by recrystallization from ethanol/water. The absolute stereochemistry of all three *cis*-1-acetoxy-4-aminocycloalkanes used was determined by Mosher amide analysis, as previously described,[9] and this stereochemistry was retained during the palladium(0) coupling reaction.

The remaining target compounds were synthesized using method C, outlined in Scheme 1. Bis alkylation of **20** (NaH, 1.5 or 2.2 equiv of R-X)[2] afforded the (mono)- or (bis)-alkylated intermediates, respectively, which were deprotected (30% HBr in AcOH)[7] to yield the pure desired target compounds **21**–**25** as their tetrahydrobromide salts following recrystallization from ethanol/water.

Each synthetic intermediate and target compound was fully characterized by ^{1}H and ^{13}C NMR (Bruker QE 300), and by IR spectroscopy (Nicolet Magna 550). The purity of all target compounds was assessed by either combustion analysis (Atlantic Microlabs) or high resolution mass spectrometry. The alkylpolyamine analogues synthesized in this series to date are shown in Figure 2.

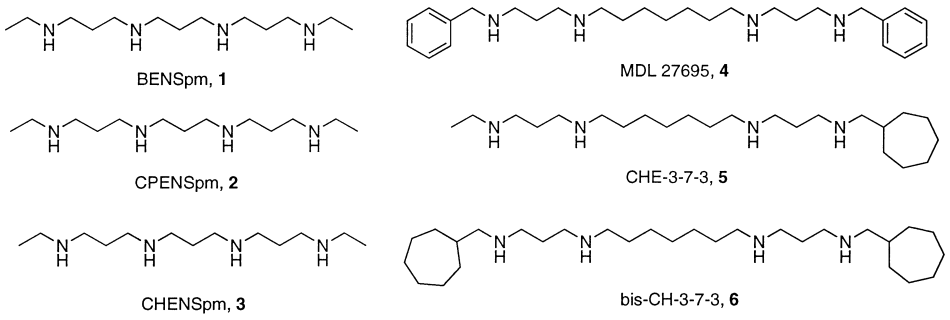
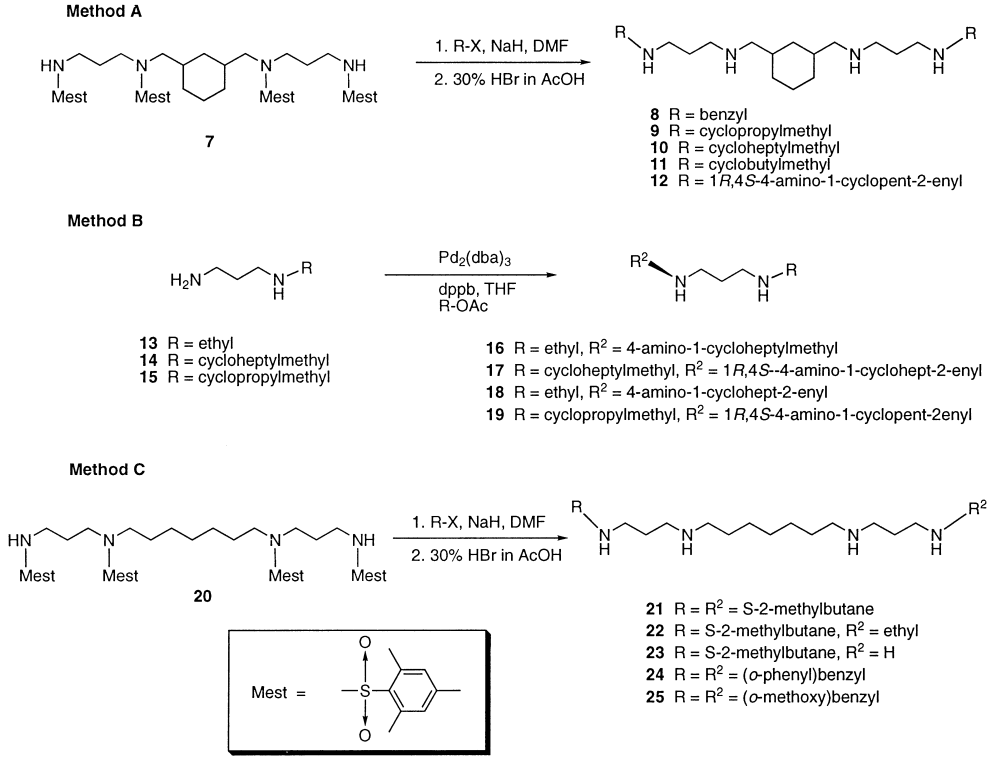


Figure 1. Structures of the biologically active alkylpolyamines **1**–**6**.



Scheme 1.

Antitrypanosomal Assay

Assays for inhibition of trypanosomal growth following treatment with compounds of general structure **4** were conducted as previously described.[2,10] Activities of the test compounds were compared to that of melarsen oxide, a known trypanocidal agent, as a positive control. Trypanosomes were grown in modified IMDM +20% horse serum in 24-well microplates at 37 °C. Wells were inoculated with 1×10^5 trypanosomes, and compounds were solubilized in the medium. One half the volume of each well was changed daily. Cell counts (Coulter counter) were made at 24 and 48 h. Control cells grew to 5×10^6/mL. IC_{50} values were determined from semi-log plots. *T.b. brucei* Lab 110 EATRO is a pentamidine and melarsen-sensitive strain and *T.b. rhodesiense* KETRI 243 As-10-3 is a melarsen and pentamidine resistant clone of a clinical isolate, KETRI 243. KETRI 269 is also a melarsen-resistant clinically isolated strain.

Antimicrosporidial Assays

Encephalitozoon cuniculi in vitro

RK-13 cells grown as a monolayer in Falcon 24-well plates were infected with *E. cuniculi* (in controls 50–80% of cells were infected).[11] Drugs were added to duplicate wells and incubated for 7 days, changing the drug containing medium at 3 and 6 days. On day 8, wells were fixed overnight, stained with Giemsa, and counted using an inverted microscope at 400× magnification. Five confluent fields (240 cells/field) were counted for control as well as for drug treated wells. The percentage of infected cells in the presence of the compound was compared to the percentage of infected control cells. IC_{50} values are given in μM.

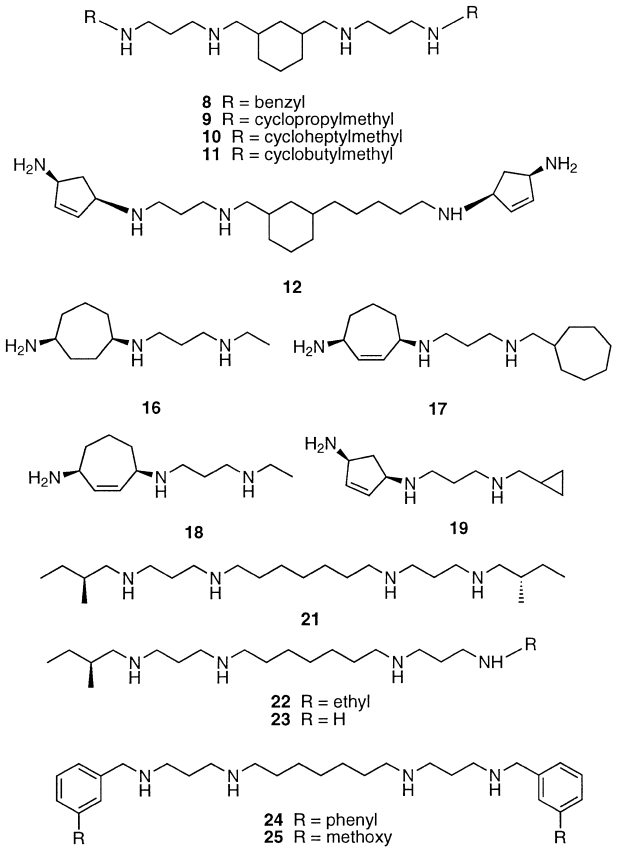


Figure 2. Structures of 'second generation' alkylpolyamines evaluated for antitrypanosomal and antimicrosporidial activity.

Effect of polyamine analogues on mice infected with *Encephalitozoon cuniculi*

CD8 knockout mice (C57B/6JDCD8)[12] were infected with 3×10^7 or 1×10^6 spores, respectively, by intraperitoneal injection 24 h before treatment.[13] Animals were treated with intraperitoneal drug for 5 days followed by 2 days without drug and then 5 more days of drug. Animals that survived >28 days post-infection with no histologic or PCR evidence of parasites were considered cured. Tissues were obtained from mice at the end of the observation periods, fixed and embedded per standard protocols. Tissue sections were stained with heamatoxylin and eosin (H&E) and tissue chromotrope stain for microsporidia. These sections were examined in a blinded fashion for pathology and presence of organisms.

Results

The results of the in vitro screening of alkylpolyamine analogues **2–6**, **8–10**, **16**, **18**, **19**, **21**, and **23–25** against four strains of trypanosomes are shown in Table 1. Consistent with our previous studies,[2] the 3-3-3 analogues **2** and **3** had no detectable activity against any of the four strains of trypanosome tested. Analogues **16**, **18**, and **19**, which contain a 1,3-diaminopropane backbone, were also devoid of activity. Marginal antitrypanosomal activity was observed in the case of analogues **8–10**. Although these analogues more closely

Table 1. In vitro IC_{50} values (μM) for growth inhibition of four strains of *Trypanosoma brucei* by bis-substituted polyamine analgues

Compound	LAB110	K243	K269	K243 As-10-3
2	>100	>100	>100	>100
3	>100	>100	>100	>100
4	14.5	15.1	12.3	13.0
5	18.0	18.4	21.0	26.2
6	0.125	0.98	0.69	0.78
8	4.05	4.15	5.1	56
9	73.0	81.5	>100	>100
10	3.25	14.0	1.71	2.15
16	>100	>100	—	—
18	>100	>100	—	—
19	>100	>100	—	—
21	0.031	0.04	—	0.165
22	0.31	0.38	—	0.79
24	0.24	0.19	0.75	0.20
25	22	18.5	16.5	5.5
Melarsen oxide	0.001	0.04	0.5	—

Each data point represents the average of two determinations which differed by less than 5% in all cases. >100 refers to the fact that there was no appreciable inhibitory activity up to the highest concentration tested.

resemble the active 3-7-3 analogues than compounds **16**, **18**, and **19**, they contain a ring in the central carbon chain which restricts their rotation. As was described previously,[2] compounds **4**, **5**, and **6** have significant activity against all four strains of trypanosomes, and are equally effective against the arsenic-resistant strain K243 As-10-3. Among the second generation analogues, compounds **21**, **23**, and **24** showed activity that was as much as an order of magnitude greater than compound **6**, the most active first generation analogue. In particular, compound **21** showed activity comparable to the currently used arsenical melarsen oxide, with the added benefit that it is active against the arsenic resistant K243 As-10-3 at a concentration of 0.165 μM.

The results of in vitro testing against the microsporidial organism *E. cuniculi* are summarized in Table 2. All of the analogues evaluated (**8**–**12**, **16**–**19**, **21**, **23**, **24**, and **25**) had some degree of activity in the micromolar range. However, the most potent analogues were **16**, **18**, **19**, and **24**, and IC_{50} values were determined for these compounds.

Because microsporidia must be grown on a feeder monolayer layer of cells, a compound with promising activity must produce potent activity against the organism without causing frank cytotoxicity to the monolayer. This requirement was satisfied by compounds **16**, **18**, and **24**. However, analogue **24** had the most dramatic effect on *E. cuniculi* in vitro ($IC_{50} = 0.47$ μM, Table 2), and as such was selected for further evaluation in a murine model for microsporidial infection. Mice that were left untreated died on day 21 (two mice), day 22 (two mice), and day 24 (one mouse). However, five of five mice treated with compound **24** at either 1, or 5 mg/kg/day survived past day 28, and as such were considered cured of the infection. In surviving animals, no evidence of microsporidia was found in tissue samples, either by microscopy or by PCR analysis.

Table 2. In vitro growth inhibition activity against *Encephalitozoon cuniculi* produced by alkylpolyamine analogues

Toxicity to compd	Activity	Monolayer
8	86% Inhibition (100 μM)	Y
9	100% Inhibition (100 μM)	Y
10	100% Inhibition (100 μM)	Y
11	100% Inhibition (250 μM)	Y
12	100% Inhibition (100 μM)	Y
16	$IC_{50} = 232$ μM	N
17	76% Inhibition (100 μM)	Y
18	$IC_{50} = 36$ μM	N
19	$IC_{50} = 204$ μM	Y
21	47% Inhibition (50 μM)	Y
23	87% Inhibition (500 μM)	Y
24	$IC_{50} = 0.47$ μM	N
25	100% Inhibition (50 μM)	Y

E. cuniculi was grown on RK-13 cells infected as a monolayer (ave 50–80% infected cells). The percentage of infected cells was determined after 7 days in the presence of the test compound, and compared to the percentage of infected control cells. All values listed are derived from three to five growth curves.

Discussion

Trypanosomes are hemoflagellates that live in the blood and tissues of their human hosts. African trypanosomiasis is caused by *Trypanosoma brucei rhodesiense* or *Trypanosoma brucei gambiense*, while South American trypanosomiasis is caused by a distinct organism, *Trypanosoma cruzi*. In these organisms, the polyamines putrescine and spermidine are synthesized,[2] but no spermine is formed. Spermidine is instead used to produce trypanothione, a disulfide intermediate analogous to glutathione, which protects the organism from oxidative stress.[14] Melaminophenyl arsenical compounds such as melarsoprol and melarsen oxide are currently the only therapy for late-stage trypanosomiasis. These compounds enter trypanosomal cells via a recently characterized adenine transporter,[15] and react with trypanothione to form an inactive, stable intermediate called melT.[16] Although arsenical drugs can be effective in the treatment of late-stage trypanosomiasis, they produce severe side effects, are extremely painful to administer, and are lethal to the patient in 5% of cases. There is a clear need to discover new antitrypanosomal agents that do not produce the adverse effects associated with arsenicals. The analogues described in Table 1, and in particular analogue **21**, show promising in vitro activity against four major strains of trypanosome, including one arsenical-resistant line, K 243 As-10-3. It has recently been reported that a series of substituted polyamines act as competitive inhibitors of trypanothione reductase, the enzyme that synthesizes trypanothione.[17] Additional experiments are required to determine whether compounds **2**–**25** exert their antitrypanosomal effects by interacting with the polyamine pathway in trypanosomes.

Microsporidia are obligate intracellular, spore-forming parasites that infect every major animal group. They form small unicellular spores that contain a coiled polar tube which facilitates transmission of infection to other cells. Microsporidiosis is a common human infection, but is self limited or asymptomatic in immunocompetent hosts. However, microsporidial infection has become a significant problem among immunocompromised patients.[18] The polyamines putrescine, spermidine and spermine, are present in mature microsporidian spores,[19] and it has recently been reported that they possess a eukaryotic-like polyamine metabolism.[18] In light of these facts, it is not surprising that polyamine analogues such as those in Table 2 exhibit significant antimicrosporidial activity. In addition, compounds **16**, **18**, and **24** were effective against the organism without producing overt cytotoxicity in the host RK-13 cell layer. The most potent of these analogues, compound **24** ($IC_{50} = 0.47$ μM) was further examined in a murine model for microsporidiosis, and was found to be curative at two different dosage levels (1 mg/kg and 5 mg/kg). Additional in vivo testing of analogue **24** is currently underway. Additional studies are required to determine the mechanism by which alkylpolyamine analogues exert their antitrypanosomal and antimicrosporidial effects. These studies, as well as the design and synthesis of additional analogues in the series, is an ongoing concern in our laboratories.

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