Trypanocidal Activity of Conformationally Restricted Pentamidine Congeners

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A series of conformationally restricted congeners of pentamidine in which the flexible pentyl bridge of pentamidine was replaced by *trans*-1,2-bismethylenecyclopropyl, phenyl, pyridinyl, piperazinyl, homopiperazinyl, and piperidinyl groups were synthesized. The compounds were evaluated for trypanocidal activity in vitro and in vivo against one drug-sensitive and three drug-resistant trypanosome isolates. The DNA binding affinity of the compounds was also studied using calf thymus DNA and poly(dA-dT). The nature of the linker influenced the DNA binding affinity as well as the trypanocidal activity of the compounds. *trans*-1,2-Bis(4-amidinophenoxymethylene)cyclopropane (**1**) was over 25-fold more potent than pentamidine against the drug-resistant isolate KETRI 243As-10-3, albeit with comparable DNA binding affinity. *N*,*N*′-Bis(4-amidinophenyl)homopiperazine (**8**) was the most potent trypanocide in vitro against all four trypanosome isolates studied, but *N*,*N*′-bis(4-amidinophenyl)piperazine (**6**) was the most effective agent in vivo against both drug-sensitive and drug-resistant trypanosomes.

Introduction

Human African trypanosomiasis or sleeping sickness and the related cattle disease Nagana are caused by subspecies of the parasitic hemoflagellate *Trypanosoma brucei*. Each year between 300000−500000 cases of the human disease are reported in sub-Saharan Africa[1] and the disease is resurgent.[2] *Trypanosoma brucei* exhibits a complex, digenetic life cycle that alternates between the tsetse fly (*Glossina spp*.) vector and the mammalian host. The life cycle is characterized by a complex series of cell-type differentiations and variations in metabolism. Infection with African trypanosomiasis starts with the bite of an infected tsetse fly.[3] The parasites move from the site of infection to the draining lymphatic vessels and the bloodstream, where they proliferate and later invade other tissues including the central nervous system (CNS). Once the parasites have established residence in the CNS, there is progressive breakdown of neurological function ending with death, which may be preceded by coma. There are two forms of African trypanosomiasis. One form is caused by *Trypanosoma brucei rhodesiense* and is endemic in Eastern and Southern Africa, in which parasites rapidly invade the CNS, causing death within weeks if untreated. The other form is caused by *T. b. gambiense*, which was originally designated West African trypanosomiasis but is also widespread in Central Africa. This form of the disease proliferates more slowly and can take several years before spreading to the CNS. Four drugs are generally used to treat African trypanosomiasis, two (pentamidine and suramin) of which are used prior to CNS infection. The arsenic-based drug, melarsoprol is used once the parasites have penetrated the CNS. The fourth drug, eflornithine, is effective against the late-stage disease caused by *T. b. gambiense* but is ineffective against *T. b. rhodesiense*. Another drug, nifurtimox is licensed for South American trypanosomiasis but has also been used in trials against melarsoprol-refractory late-stage disease. These therapies for African trypanosomiasis are plagued by drawbacks such as toxicity and the emergence of resistance;[4−7] hence, the need for new less-toxic agents is widely accepted.[1]

Pentamidine, an aromatic diamidine, is used to treat early-stage African trypanosomiasis, but the mechanism of action of the drug is not well understood. It is, however, known that pentamidine binds to the minor groove of double helix DNA, and this has been suggested to play a role in the mechanism of action of the drug. Indeed, it has been postulated that aromatic diamidines such as pentamidine may exert their biological activity by first binding to DNA, which eventually leads to inhibition of one or more of several DNA-dependent enzymes (e.g., topoisomerases and nucleases) or by direct inhibition of transcription.[8−14] In support of this hypothesis, Agbe and Yielding[15] recently demonstrated that an intact kinetoplast is required for the trypanocidal action of aromatic diamidines. We[16] and others[17] have recently reported the trypanocidal action of a series of pentamidine-related aromatic diamidines. We[18,19] have also reported the anti-*Pneumocystis carinii* activity and DNA binding affinity of the pentamidine-related aromatic dicationic compounds shown in Table 1. Because the interaction of aromatic diamidines with the minor groove of double helix DNA[8−14] and selective inhibition of kinetoplast DNA synthesis[20,21] is believed to play a significant role in the antimicrobial action of aromatic diamidines, we were interested in investigating the trypanocidal activity of the aromatic dicationic

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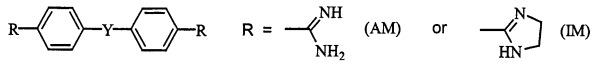
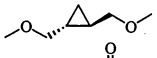
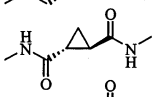
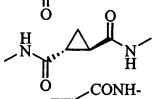
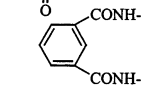
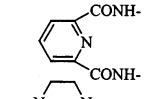
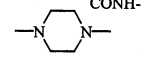
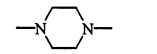
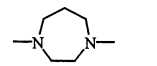
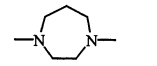
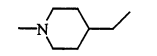
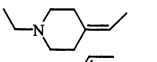
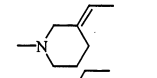
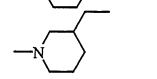
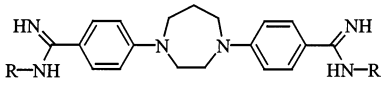
Table 1. Structure, in Vitro Trypanocidal Activity, and DNA Binding Affinity of Conformationally Restricted Pentamidine Congeners **1**−**13**



			IC_{50} (nM) [a]				DNA binding [a] (ΔT_m, °C)	
			Lab 110	KETRI				
Cpd. #	R	Y	EATRO	243	269	243As10-3	CT[b]	AT[c]
Pent[d]	AM	$-(CH_2)_5-$	0.80	3.10	3.20	5.00	11.1	20.6
1	AM		9.40	45.0	15.5	0.17	11.1	20.3
2	AM		>1,000	>1,000	>1,000	>1,000	7.1	11.1
3	IM		>1,000	440	>1,000	810	6.0	9.6
4	AM		70.0	70.0	12.0	28.5	8.0	9.9
5	AM		>1,000	>1,000	>1,000	>1,000	7.1	15.9
6	AM		58.0	16.0	18.0	16.5	17.0	23.8
7	IM		18.5	13.5	13.0	100	15.0	19.4
8	AM		2.15	2.70	7.10	3.30	15.0	23.1
9	IM		27.5	52.0	155	23.0	15.0	24.0
10	IM		169	185	155	88.0	21.0	33.9
11	IM		540	465	265	90.0	17.0	19.4
12	IM		71.0	84.0	88.0	84.0	12.0	15.5
13	IM		86.0	85.0	15.0	79.0	9.9	14.1

[a] Duplicate determinations with values within ±10% of each other. [b] CT is sonicated calf thymus DNA. [c] AT is sonicated poly(dA-dT). [d] Pent is pentamidine.

Table 2. Structure, in Vitro Trypanocidal Activity, and DNA Binding affinity of Amidinoalkyl derivatives of **8**



		IC_{50} (nM) [a]			DNA binding [a] (ΔT_m, °C)	
		Lab 110	KETRI			
Cpd. #	R	EATRO	243	243As10-3	CT[b]	AT[c]
8	H	2.15	2.70	3.30	15.0	23.1
14		>1,000	900	125	13.8	20.0
15		>1,000	>1,000	>1,000	4.1	7.4
16	$HOCH_2CH_2-$	>1,000	>1,000	>1,000	11.1	14.7
17	$CH_3(CH_2)_3-$	180	79.0	78.0	11.8	13.3
18		870	810	850	15.3	23.6

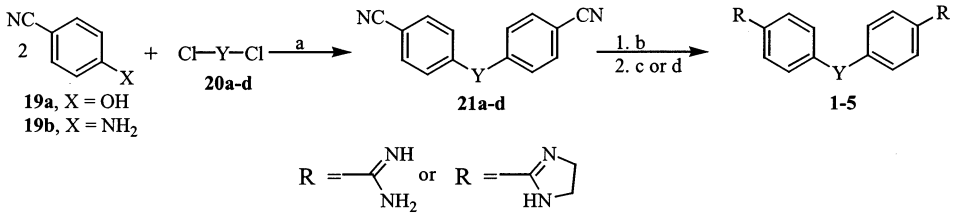
[a] Duplicate determinations with values within ±10% of each other. [b] CT is sonicated calf thymus DNA. [c] AT is sonicated poly(dA-dT).

compounds shown in Tables 1 and 2. In this report we describe the synthesis, DNA binding affinity (calf thymus DNA and poly(dA-dT)), and the trypanocidal activity of the aromatic dicationic agents **1**−**18** against one drug-sensitive (*Trypanosoma brucei brucei* Lab 110 EATRO) and three drug-resistant (*Trypanosoma brucei rhodesiense*) trypanosome isolates in vitro and in mouse model infections.

Chemistry

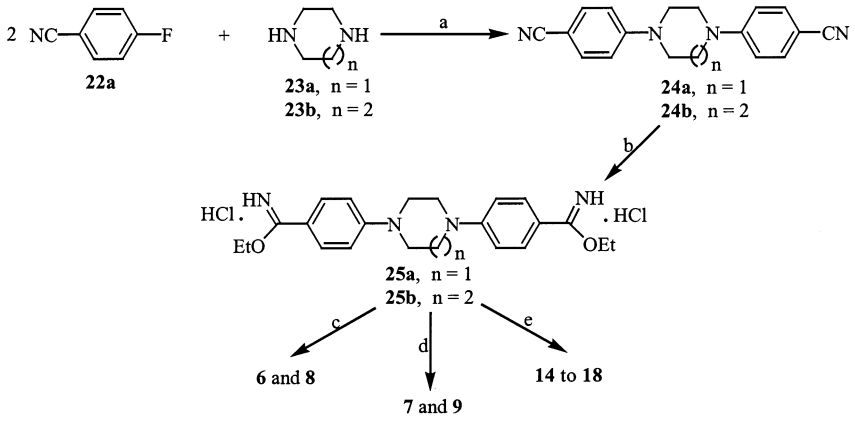
The compounds were synthesized by following our previously described procedures as summarized below.[18,19,22] Compounds **1**−**5** were synthesized as shown in Scheme 1. Reaction of either 4-cyanophenol **19a** or 4-cyanoaniline **19b** with the appropriate dichlorides **20a**−**d** gave the corresponding dinitriles **21a**−**d**. Transformation of the dinitriles to the corresponding imidate ester followed by treatment with either ethanolic ammonia or ethylenediamine afforded the appropriate diamidines and diimidazolines **1**−**5** in 58−66% yield. Compounds **6**−**9** and **14**−**18** were synthesized as outlined in Scheme 2. This involved aromatic nucleophilic displacement reaction between 4-fluorobenzonitrile **22a** and piperazine **23a** (or homopiperazine **23b**) to give dinitriles **24a** and **24b**, respectively. The dinitriles were readily transformed into the corresponding imidate

Scheme 1[a]



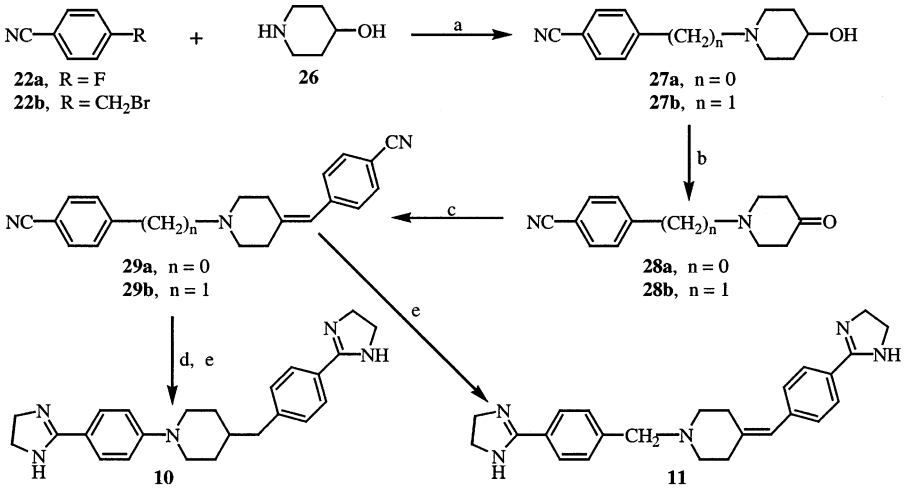
[a] Reagents: (a) Na/EtOH when X = OH or DIEA/THF when X = NH_2; (b) HClg/EtOH/THF/$CHCl_3$; (c) EtOH/NH_3, reflux to give the diamidine or (d) ethylenediamine/EtOH, reflux to give the diimidazoline. See Table 1 for the structure of Y.

Scheme 2[a]



[a] Reagents: (a) K_2CO_3/DMSO, 120 °C; (b) HCl(g)/EtOH/$CHCl_3$/THF; (c) NH_3(g)/EtOH, reflux; (d) ethylenediamine/EtOH, reflux; (e) EtOH/appropriate alkylamine.

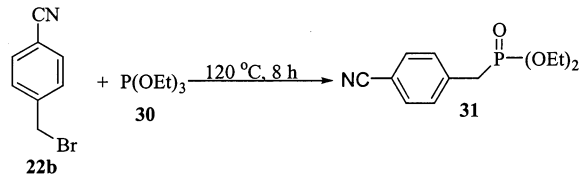
Scheme 3[a]



[a] Reagents: (a) K_2CO_3/DMSO, 120 °C, 6.5 h for **22a** or DIEA, THF for **22b**; (b) TFA, pyridine, DCC, DMSO/CH_2Cl_2 (1:2); (c) **31**, 60% NaH, THF; (d) 5% Pd/C, MeOH, H_2, 2 h; (e) THF/EtOH, HCl gas, ethyleendiamine/EtOH reflux, 4.5 h.

ester hydrochlorides **25a** and **25b**, which upon reaction with the appropriate amine gave target compounds **6**−**9** (56−81% yield) and **14**−**18** (69−81% yield). The synthesis of **10** and **11** is shown in Scheme 3. Briefly, nucleophilic aromatic displacement reaction between 4-fluorobenzonitrile **22a** or 4-cyanobenzyl bromide **22b** and 4-hydroxypiperidine **26** afforded **27a** (94% yield) and **27b** (97% yield), respectively. The hydroxyl groups of these compounds were oxidized with DDC to give the corresponding ketones **28a** and **28b**. Wittig reaction between the ketones and phosphonate ester **31** gave dinitriles **29a** (70% yield) and **29b** (68% yield), respectively. Phosphonate ester **31** was obtained as a colorless liquid in 97% yield by reacting 4-cyanobenzyl bromide **22b** with triethyl phosphite **30** as shown in Scheme 4.[23]

Scheme 4



The double bond of **29a** was reduced with H_2 over 5% Pd/C, and the reduction product and **29b** were transformed into diimidazoles **10** and **11**, respectively, as described for the synthesis of **7**. Compounds **12** and **13** were synthesized using 3-hydroxypiperidine and 4-fluorobenzonitrile as the starting materials and following similar procedures described for the synthesis of **10** and

11. The *E* configuration of **12** was established by nuclear Overhauser effect (NOE) experiments as described in the Experimental Section. ^{1}H NMR spectral data and elemental analyses of all the compounds were satisfactory.

Results and Discussion

Aromatic diamidines incorporating conformationally restricted linkers between the benzamidine groups were synthesized and screened in vitro for trypanocidal activity against one *Trypanosoma brucei brucei* pentamidine-sensitive strain (EATRO Lab 110) and three drug-resistant clinical isolates of *Trypanosoma brucei rhodesiense* (KETRI 243, KETRI 269, and KETRI 243As-10-3). The results of the study are shown in Tables 1 and 2. Generally, the nature of the linker between the dicationic groups influenced the activity of the compounds. Compound **1** and pentamidine had similar DNA binding affinities, but they exhibited different trypanocidal activity. Compound **1** was between 11-fold to 15-fold less potent than pentamidine versus Lab 110 EATRO, KETRI 243, and KETRI 269, but it was over 25-fold more potent than pentamidine against the drug-resistant isolate KETRI 243As-10-3. Compounds incorporating amide linkers (excluding **4**) were generally less effective against all of the trypanosome isolates studied. For example, **2** and **3** with *trans*-1,2-cyclopropane dicarboxamide linkers were virtually inactive compared to **1** with a *trans*-1,2-bismethylenecyclopropyl ether linker. The planar and/or polar nature of the linker between **2** and **3** compared to that of **1** might account for the observed difference in the trypanocidal action of the compounds. This is consistent with our previous report,[16] which demonstrated that introduction of polar groups in the linker between aromatic diamidines is detrimental to trypanocidal action. Alternatively, the difference in trypanocidal action between **1** and **2** or **1** and **3** could be due to the difference in the electron-rich phenoxy groups of **1** compared to the electron-poor acylated anilinyl groups of **2** and **3**. This interpretation can also explain the poor trypanocidal action of **5** compared to **4** because the pridine ring of **5** is π-deficient compared to the phenyl ring of **4**.[24]

Compounds **6**−**9** displayed good in vitro trypanocidal action versus Lab 110 EATRO and the drug-resistant clinical isolates of *Trypanosoma brucei rhodesiense* (KETRI 243, KETRI 269, and KETRI 243As-10-3). Compound **8** with a homopiperazine linker between the benzamidine groups was the most potent agent of the series versus most of the trypanosome isolates studied. The compound was as effective as pentamidine against KETRI 243 and about 2-fold more effective than pentamidine against the drug-resistant isolate KETRI 243As-10-3. Also, **8** was a better trypanocidal agent compared to **9**, corroborating our previous observation[16] that amidines are more effective trypanocidal agents than imidazolines. Derivatives of **7** (i.e., **10**−**13**) in which the piperazine linker was replaced by substituted piperidines were also studied. The compounds showed moderate trypanocidal activity (IC_{50} = 15−540 nM) compared to **7** (IC_{50} = 13−100 nM) depending on the trypanosome isolate. Compound **12**, which is a 3-piperidone derivative, was as effective (versus KETRI 243As10-3) or more effective than **11**, which is a 4-piperidone derivative. Reduction of the double bond of **12** gave **13**, which was not resolved into its stereoisomers but was tested as a mixture of isomers. Compound **13** (a 3-piperidone derivative) was also as effective or more effective than **10** (a 4-piperidone derivative) depending on the trypanosome isolate. Thus, the aromatic diimiadzolines (**12** and **13**) derived from 3-piperidone were better trypanocidal agents compared to aromatic diimidazolines (**10** and **11**) derived from 4-piperidone. Compound **13** was also as effective as **12** versus all the trypanosome isolates except KETRI 269, where it was about 6-fold more effective than **12**.

Boykin et al.[25] used a series of furamidine analogues to demonstrate that increasing the DNA binding affinity of aromatic diamidines by substituting alkyl residues on the amidines enhances the biological action of the compounds. The increased DNA binding affinity of the alkylfuramidines was attributed to enhanced van der Waals interactions between the *N*-alkyl groups and the floor of the minor groove of double helix DNA. On the basis of this precedence, **14**−**18** (Table 2) were synthesized with the objective of enhancing the trypanocidal potency of **8** via increased DNA binding affinity. The in vitro trypanocidal action of these derivatives of **8** (i.e., **14**−**16**) was disappointing. None of the compounds was more potent than **8**. Compound **17** was the best member of this set with IC_{50} values between 78.0 and 180 nM. With the exception of **18**, which displayed DNA binding affinity similar to that of **8**, all of the other derivatives were weaker DNA binders compared to **8**. Unlike the furamidine series, introduction of alkyl residues on the amidine groups of **8** did not enhance DNA binding, and as a result, no improvement in the trypanocidal potency of the compounds was observed. This discrepancy could be due in part to the conformations that the *N*-alkyl derivatives of **8** adopt when bound to the minor groove of DNA compared to the *N*-alkylfuramidines. The radius of curvature of compounds that bind to the minor groove of DNA has been shown to play a significant role in the effectiveness of ligand binding to DNA.[26] The central homopiperazine ring, unlike the furan ring of the furamidines, may not provide the required curvature for locating the *N*-alkyl substituents in proximity to the floor of the minor groove of double helix DNA.

It has been postulated that aromatic diamidines may exert their biological action by first binding to double helix DNA and causing inhibition of one or more of several DNA-dependent enzymes (e.g., topoisomerases and nucleases) or by direct inhibition of transcription.[8−14] We therefore studied the affinity of the compounds in Tables 1 and 2 for binding to sonicated calf thymus DNA and poly(dA-dT) with the objective of investigating if there is a correlation between the DNA affinity and the trypanocidal activity of this series of compounds. All 18 compounds were found to bind to both calf thymus DNA and poly(dA-dT). The compounds consistently displayed stronger affinity for poly(dA-dT) compared to calf thymus DNA, which is in agreement with the observation that pentamidine and its analogues prefer AT-rich DNA sequences.[25,27,28] The most potent trypanocidal agents, **1** and **6**−**8**, were also the strongest DNA binders in this series, and the weakly active compounds, **2**−**5**, were also the weakest DNA binders. However, a direct correlation

Table 3. Activity of Conformationally Restricted Pentamidine Congeners **6**−**9** versus *T. b. brucei* Lab 110 EATRO Mouse Model Infection[a]

dose (mg/kg)	number cured/total (MSD)[b]				
	pent.	**6**	**7**	**8**	**9**
1.0	5/5 (−)	2/3 (16.0)	0/3 (10.3)	0/3 (8.3)	0/3 (4.7)
2.5	5/5 (−)	3/3 (−)	3/3 (−)	1/3 (16.5)	0/3 (4.3)
5.0	5/5 (−)	3/3 (−)	3/3 (−)	2/3 (12.0)	0/3 (7.7)
10.0	*c*	3/3 (−)	3/3 (−)	3/3 (−)	1/3 (14.0)
25.0	*c*	3/3 (−)	0/3 (19.7*)	3/3 (−)	3/3 (−)

[a] Mice were infected (2.5×10^5 parasites), and the infections were allowed to progress 24 h before treatment began. Compounds were dissolved in distilled water (vehicle) and injected ip once a day for 3 days. Mice treated with vehicle as control all died with MSD values ranging between 5 and 13 days. [b] MSD is the mean survival time (in days) of animals dying of trypanosomiasis exclusive of cured animals. Animals surviving >30 days beyond the death of the last control with no parasites in tail blood smears were considered cured. Three mice were studied at each drug dose with MSD values for each study group shown in parentheses. For pentamidine (pent.), five mice were studied at each drug dose. The dash (−) means all the animals were cured as defined above. The asterisk (*) indicates that the compound was toxic. [c] Not determined.

Table 4. Effect of Delayed Dosing of **6** on *T. b. brucei* Lab 110 EATRO Toxicity[a]

dose (mg/kg)	24 h after infection		48 h after infection		72 h after infection	
	MSD	no. cured/ total	MSD	no. cured/ total	MSD	no. cured/ total
2.5	>35	3/3	>35	3/3	4−5	0/3
5.0	>35	3/3	5	2/3	4−5	1/3
10.0	>35	3/3	7	2/3	4−5	0/3

[a] Dosing was once daily for 3 days, starting at 24, 48, or 72 h after infection. Other methods were as in Table 3.

between the trypanocidal activity and the DNA binding affinity of the compounds in this series was not observed.

Compounds **6**−**9** displayed good in vitro trypanocidal activity, so they were selected for in vivo evaluation versus *T. b. brucei* Lab 110 EATRO and *T. b. rhodesiense* KETRI 243 isolates in mouse model infections. The compounds were administered intraperitoneally as single daily injections over 3 days except where indicated otherwise. The results of this study are shown in Table 3. Compounds **6** and **7** were as effective as pentamidine in curing infections due to *T. b. brucei* Lab 110 EATRO at doses ranging from 2.5 to 25 mg/kg per day, but **7** was toxic at 25 mg/kg per day. Compound **8** was protective at doses between 1.0 and 5.0 mg/kg per day and was curative at doses of 10.0 or 25 mg/kg per day. Compound **9** was the least effective member. It was curative only at a dose of 25 mg/kg per day. In contrast to the results of the in vitro studies, **6** was a more effective trypanocide in vivo compared to **8**, which was the best trypanocidal agent of the series in vitro. The lack of correlation between the in vitro and in vivo activities of the compounds may be attributed to differences in their pharmacokinetic properties; however, this awaits experimental confirmation.

The effect of delayed dosing of **6** on infection due to *T. b. brucei* Lab 110 EATRO at doses ranging from 2.5 to 10 mg/kg per day was studied. The data (Table 4) indicated that **6** effectively cured the mice at all of the doses studied if the drug was administered 24 h after infection. Compound **6** also cured 67−100% of the animals when administered 48 h after infection, but the mean survival times in days varied from 5 days to over 35 days depending on the dose of the drug. Compound **6** was, however, not effective when it was administered 72 h after infection. In a second study, **6** and **8** were protective but not curative versus mice infected with the drug-resistant isolate *T. b. rhodesiense* KETRI 243 (data not shown). For example, when mice were infected with KETRI 243 and treated with 10 mg/kg of either **6** or **8**, they survived for an average period of 20 and 15 days, respectively, compared to vehicle-treated mice, which had an average life span of 9 days after infection. Compound **6** was further studied versus infection due to the drug-resistant isolate *T. b. rhodesiense* KETRI 243. In this study, the dose and the frequency of administration of **6** were increased. As shown in Table 5, when mice infected with the drug-resistant isolate *T. b. rhodesiense* KETRI 243 were treated with 25 mg/kg of **6**, three times daily for 3 days, 67% of the mice were cured. When the frequency of drug administration was increased from three times daily to four or five times daily for 3 days, all of the mice were cured. The required multiple daily dosing of **6** to achieve effective trypanocidal activity may be due to rapid elimination of the compound. Derivatives of **6** that do not require multiple daily dosing may be clinically useful trypanocides. Our laboratory is currently investigating such agents.

Table 5. Activity of Multiple Daily Dosing of **6** versus *T. b. rhodesiense* KETRI 243 Mouse Model Infection[a]

dose (mg/kg)	dosed three times daily		dosed four times daily		dosed five times daily	
	MSD	no. cured/ total	MSD	no. cured/ total	MSD	no. cured/ total
5	14.0	0/3	14.7	0/3	14.7	0/3
10	18.0	0/3	22.0	0/3	21.3	0/3
25	26.0	2/3	>39	3/3	>39	3/3

[a] Conditions were the same as in Table 3 except for the dosing frequency.

In summary, a series of aromatic amidines and imidazolines incorporating conformationally restricted linkers between the cationic groups were synthesized as constrained congeners of pentamidine. The DNA binding affinity and the in vitro and in vivo trypanocidal activity of the compounds were studied. The nature of the linker between the amidine or imidazoline groups influenced the DNA binding affinity as well as the trypanocidal activity of the compounds. Generally, compounds with strong affinity for DNA also showed good trypanocidal activity. Compounds with dicarboxamide linkers were not good DNA binders and were also generally ineffective trypanocides compared to the compounds without such linkers (e.g., **1** versus **2**). However, a clear correlation between in vitro trypanocidal activity and in vitro DNA binding affinity was not observed.

Compound **1** was the most effective agent against the drug-resistant isolate KETRI 243As-10-3. It was over 25-fold more potent than pentamidine versus this isolate. Compound **8** with a homopiperazine linker showed a broad spectrum of trypanocidal activity. It was also the most potent agent in vitro. However, **6** with a piperazine linker emerged as the most effective agent in vivo. Compound **6** cured infections due to the drug-

resistant isolate *T. b. rhodesiense* KETRI 243, but its effectiveness was dependent on the dosage and the frequency of administration. Derivatives of **6** that do not require multiple daily dosing may be clinically useful trypanocides.

Experimental Section

Chemistry. Melting points were determined on a Haake-Buchler melting point apparatus and are uncorrected. The ^{1}H NMR spectra were recorded on Varian Gemini-300 and GE Omega-500 spectrometers, and DMSO-d_6 was used as the solvent unless otherwise noted. The chemical shifts (δ) are reported in ppm relative to TMS, 0.00 ppm. Elemental analyses (C, H, N) were performed by M-H-W Laboratories, Phoenix, AZ, and are within ±0.4% of the theoretical values. All chemicals and solvents were purchased from Aldrich Chemical Co. THF was distilled from Na and benzophenone before use, and other chemicals were used as received. Anhydrous ethanol and methanol were used throughout this study unless stated otherwise.

***trans*-1,2-Bis(4-cyanophenoxymethylene)cyclopropane (21a).** This dinitrile precursor to the synthesis of **1** was prepared as previously reported.[18] Yield, 76%; mp 150−152 °C; ^{1}H NMR ($CDCl_3$) δ 7.58−7.55 (m, 4H), 6.92−6.90 (m, 4H), 4.07−4.05 (m, 4H), 2.00−1.98 (m, 4H).

General Procedure for the Synthesis of Dinitriles Required for the Synthesis of 2−5. Following a known procedure,[29] a solution of the appropriate acid dichloride in THF was added to a stirred solution of 4-aminobenzonitrile (2 equiv) and DIEA (2 equiv) in THF at 0 °C under a N_2 atmosphere. After 30 min, another portion of DIEA (2 equiv) in THF was added and the mixture was stirred overnight. The solvent was removed under reduced pressure followed by recrystallization of the residue to give the corresponding dinitrile.

***N,N′*-Bis(4-cyanophenyl)-*trans*-1,2-cyclopropanediamide (21b, dinitrile precursor for the synthesis of 2 and 3):** yield 62%; mp >305 °C; ^{1}H NMR δ 10.80 (s, 2H), 7.75−7.71 (m, 8H), 2.31 (t, 2H), 1.34 (t, 2H).

***N,N′*-Bis(4-cyanophenyl)-1,3-benzodiamide (21c, dinitrile precursor for the synthesis of 4):** yield 77%; mp 298−299 °C; ^{1}H NMR δ 10.80 (s, 2H), 8.51(s, 1H), 8.16 (dd, 2H), 7.98 (d, 4H), 7.82 (d, 4H), 7.72 (t, 1H).

***N,N′*-Bis(4-cyanophenyl)-2,6-pyridinediamide (21d, dinitrile precursor for the synthesis of 5):** yield 72%; mp 304−306 °C; ^{1}H NMR δ 11.30 (s, 2H), 8.41 (d, 2H), 8.32 (t, 1H), 8.16 (d, 4H), 7.91 (d, 4H).

General Procedure for the Synthesis of Dinitriles Required for the Synthesis of 6−9. Under a N_2 atmosphere, a mixture of either piperazine or homopiperazine (1 equiv), 4-fluorobenzonitrile (2 equiv), and K_2CO_3 (3 equiv) in dry DMSO (100 mL) was heated at 120 °C for 6 h. After the mixture was cooled to room temperature, an excess of water was added and the precipitated solid was filtered, washed with water, and dried to give the appropriate dinitrile.

***N,N′*-Bis(4-cyanophenyl)piperazine (24a, dinitrile precursor for the synthesis of 6 and 7):** yield 83%; mp 271−273 °C; ^{1}H NMR ($CDCl_3$) δ 7.53(d, 4H), 6.87(d, 4H), 3.52 (s, 8H).

***N,N′*-Bis(4-cyanophenyl)homopiperazine (24b, dinitrile precursor for the synthesis of 8 and 9):** yield 80%; mp 208−209 °C; ^{1}H NMR ($CDCl_3$) δ 7.48 (d, 4H), 6.72 (d, 4H), 3.71 (s, 4H), 3.49 (t, 4H), 2.13−2.10 (m, 2H).

General Procedure for the Synthesis of Dinitriles Required for the Synthesis of 10−13. Under a N_2 atmosphere, a mixture of either 3-hydroxypiperidine or 4-hydroxypiperidine (1 equiv), 4-fluorobenzonitrile (1 equiv) or 4-cyanobenzyl bromide (1 equiv), and K_2CO_3 (1.5 equiv) in dry DMSO (100 mL) was heated at 120 °C for 6 h. After the reaction mixture was cooled to room temperature, it was poured into excess water and the precipitate that separated out was filtered, washed with water, dried, and recrystallized from acetone to give the corresponding N-substituted hydroxypiperidine. The product was dissolved in DMSO/benzene mixture (1:2), and DCC and pyridine were added under a nitrogen atmosphere. The mixture was cooled to 5 °C, and TFA was added dropwise. Following this, the mixture was allowed to warm to room temperature and stirring was continued for 24 h. EtOAc was added to precipitate *N,N′*-dicyclohexylurea, which was filtered off. The filtrate was washed with brine (3 × 100 mL), and the organic layer was left at room temperature overnight. The piperidone that separated out was collected, dried, and reacted with 4-cyanobenzyl phosphonate (**31**), which was obtained by heating a mixture of 4-cyanobenzyl bromide (1 equiv) and triethyl phosphite (1 equiv) at 160−170 °C for 8 h followed by distillation to give **31** (bp 165 °C/0.1 mmHg). The piperidone was then reacted with **31** as follows. NaH (1 equiv) was added to dry THF, the slurry was cooled to 20 °C, and **31** (1 equiv) was added dropwise with stirring. After the addition, the mixture was stirred at room temperature for 30 min. The resulting yellow-brown solution was maintained below 25 °C, and a solution of the piperidone (1 equiv) in dry THF was added dropwise. The mixture was stirred at room temperature for 30 min and refluxed overnight. After the mixture was cooled, the THF layer was recovered and concentrated to dryness and the residue was recrystallized from a hexanes/acetone mixture (1:2) to give the corresponding dinitrile.

***N*-(4-Cyanophenyl)-4-(4-cyanobenzylidinyl)piperidine (29a, dinitrile precursor for the synthesis of 10):** yield 70%; mp 124−125 °C; ^{1}H NMR ($CDCl_3$) δ 7.62 (d, 2H), 7.50 (d, 2H), 7.30 (d, 2H), 6.85 (d, 2H), 6.40 (s, 1H), 3.51 (t, 2H), 3.42 (t, 2H), 2.59 (m, 2H), 2.52 (t, 2H).

***N*-(4-Cyanobenzyl)-4-(4-cyanobenzylidinyl)piperidine (29b, dinitrile precursor for the synthesis of 11):** yield 68%; ^{1}H NMR ($CDCl_3$) δ 7.58 (d, 2H), 7.42 (d, 2H), 7.31 (d, 2H), 6.83 (d, 2H), 6.38 (s, 1H), 3.50 (s, 2H), 2.66 (m, 4H), 2.20 (m, 4H).

***N*-(4-Cyanophenyl)-3-(4-cyanobenzylidinyl)piperidine (dinitrile precursor for the synthesis of 12 and 13):** ^{1}H NMR ($CDCl_3$) δ 7.56 (d, 2H), 7.44 (d, 2H), 7.34 (d, 2H), 6.81 (d, 2H), 6.40 (s, 1H), 3.98 (s, 2H), 3.43 (t, 2H), 2.82 (t, 2H), 1.97 (m, 2H).

General Procedure for the Synthesis of the Diamidines, *N*-Alkylamidines, and Diimidazolines. Anhydrous HCl gas was bubbled through an ice-cooled solution of the dinitrile in EtOH, dry THF, and/or $CHCl_3$ for 1 h. The solution was allowed to warm to room temperature and stirred for 3−5 days. The solvent was concentrated to near-dryness below 40 °C under reduced pressure, and ether was added to precipitate the imidate ester as the hydrochloride salt. The solid was immediately dissolved in MeOH or EtOH after filtration. For the synthesis of amidines, anhydrous ammonia gas was bubbled through the solution for 1 h, during which time the solution was gently refluxed for 4 h. After the mixture was cooled, the solvent was removed under reduced pressure and ether was added to precipitate the crude product, which was purified by recrystallization to give the amidine as the dihydrochloride salt. For the synthesis of imidazolines, excess ethylenediamine was added to a solution of the imidate ester hydrochloride in MeOH or EtOH. The mixture was refluxed for 2−4 h before the solvent was evaporated. Ether was added to the residue to afford the imidazoline free base, which was refluxed in saturated HCl/EtOH solution for 2−4 h. The solvent was removed under reduced pressure, and the residue was purified by recrystallization to give the desired imidazoline as the dihydrochloride salt, with the exception of **2**, which was obtained as a free base without refluxing in HCl/EtOH. For the synthesis of *N*-alkyl-substituted amidines **14**−**18**, the imidate ester of *N,N′*-bis(4-cyanophenyl)homopiperazine was reacted with the appropriate alkylamine (2 equiv) to give the desired products.

***trans*-1,2-Bis(4-amidinophenoxymethylene)cyclopropane (1):** yield 66%; mp 257−259 °C; ^{1}H NMR δ 9.23 (s, 4H), 9.02 (s, 4H), 7.83 (d, 4H), 7.12 (d, 4H), 4.14 (m, 4H), 1.88 (m, 4H). Anal. ($C_{19}H_{22}N_4O_2 \cdot 2HCl \cdot 2H_2O$) C, H, N.

***N,N′*-Bis(4-amidinophenyl)-*trans*-1,2-cyclopropane dicarboxamide (2):** yield 64%; mp >300 °C; ^{1}H NMR δ 11.10 (s, 2H), 9.15 (s, 8H), 7.79 (m, 8H), 2.46 (m, 2H), 1.38 (m, 2H). Anal. ($C_{19}H_{20}N_6O_2$·2HCl) C, H, N.

***N,N′*-Bis(4-imidazolinophenyl)-*trans*-1,2-cyclopropane dicarboxamide (3):** yield 63%; mp >360 °C; ^{1}H NMR δ 11.16 (s, 2H), 10.55 (bs, 4H), 8.00 (d, 4H), 7.87 (d, 4H), 3.97 (s, 8H), 2.45 (m, 2H), 1.40 (m, 2H). Anal. ($C_{23}H_{24}N_6O_2$·2HCl) C, H, N.

***N,N′*-Bis(4-amidinophenyl)-1,3-benzodicarboxamide (4):** yield 63%; mp >360 °C; ^{1}H NMR δ 11.23 (s, 2H), 9.30 (s, 4H), 9.02 (s, 4H), 8.94 (s, 1H), 8.22 (d, 2H), 8.20 (d, 4H), 7.88 (d, 4H), 7.74 (t, 1H). Anal. ($C_{22}H_{20}N_6O_2$·2HCl·2H_2O) C, H, N.

***N,N′*-Bis(4-amidinophenyl)-2,6-pyridinedicarboxamide (5):** yield 58%; mp 318 °C; ^{1}H NMR δ 11.72 (s, 2H), 9.33 (s, 4H), 9.04 (s, 4H), 8.44 (d, 4H), 8.32 (m, 3H), 7.93 (d, 4H). Anal. ($C_{21}H_{19}N_7O_2$·2HCl) C, H, N.

***N,N′*-Bis(4-amidinophenyl)piperazine (6):** yield 81%; mp 356 °C; ^{1}H NMR δ 9.02 (s, 4H), 8.69 (s, 4H), 7.79 (d, 4H), 7.15 (d, 4H), 3.58 (s, 8H). Anal. ($C_{18}H_{22}N_6$·2HCl) C, H, N.

***N,N′*-Bis(4-imidazolinophenyl)piperazine (7):** yield 65%; mp >360 °C; ^{1}H NMR δ 10.17 (s, 4H), 7.87 (d, 4H), 7.09 (d, 4H), 3.91 (s, 8H), 3.61 (s, 8H). Anal. ($C_{22}H_{26}N_6$·2HCl·2H_2O) C, H, N.

***N,N′*-Bis(4-amidinophenyl)homopiperazine (8):** yield 56%; mp 340 °C; ^{1}H NMR δ 8.91 (s, 4H), 8.61 (s, 4H), 7.73 (d, 4H), 6.92 (d, 4H), 3.76 (s, 4H), 3.53 (m, 4H), 1.96 (m, 2H). Anal. ($C_{19}H_{24}N_6$·2HCl·2H_2O) C, H, N.

***N,N′*-Bis(4-imidazolinophenyl)homopiperazine (9):** yield 71%; mp 326 °C; ^{1}H NMR δ 10.14 (s, 4H), 7.85 (d, 4H), 6.94 (d, 4H), 3.89 (s, 8H), 3.78 (s, 4H), 3.55 (m, 4H), 1.93 (m, 2H). Anal. ($C_{23}H_{28}N_6$·2HCl·H_2O) C, H, N.

***N*-(4-Imidazolinophenyl)-4-(4-imidazolinobenzyl)piperidine (10):** yield 32%; mp 240 °C; ^{1}H NMR δ 10.80 (s, 2H), 10.26 (s, 2H), 8.04 (d, 2H), 7.90 (d, 2H), 7.50 (d, 2H), 7.07 (d, 2H), 4.22 (t, 2H), 4.01 (s, 4H), 3.93 (s, 4H), 2.89 (t, 2H), 2.69 (d, 2H), 1.91 (m, 1H), 1.65 q, 2H), 1.24 (q, 2H). Anal. ($C_{24}H_{29}N_5$·2HCl) C, H, N.

***N*-(4-Imidazolinobenzyl)-4-(4-imidazolinobenzylidinyl)-piperidine (11):** yield 40%; mp 150−151 °C; ^{1}H NMR δ 11.00 (s, 2H), 10.82 (s, 2H), 8.15 (d 2H), 8.04 (d, 2H), 7.94 (d, 2H), 7.50 (d, 2H), 6.53 (s, 1H), 4.43 (s, 2H), 3.99 (s, 4H), 3.96 (s, 4H), 2.48−3.01 (m, 8H). Anal. ($C_{25}H_{29}N_5$·2HCl) C, H, N.

***N*-(4-Imidazolinophenyl)-3-(4-imidazolinobenzylidinyl)-piperidine (12):** yield 21%; mp 180−181 °C; ^{1}H NMR δ 10.71 (s, 2H), 10.17 (s, 2H), 8.00 (d, 2H), 7.87 (d, 2H), 7.50 (d, 2H), 7.10 (d, 2H), 6.67 (s, 1H), 4.20 (s, 2H), 3.97 (s, 4H), 3.89 (s, 4H), 3.58 (t, 2H), 2.60 (t, 2H), 1.73 (q, 2H). NOE experiments showed that **12** assumed the *E* configuration. The free base of **12** in CD_3OD was used in these experiments. Irradiation of the alkene proton (δ 6.53) of **12** caused enhancement of the methylene protons (δ 3.98) adjacent to the nitrogen atom of the piperidine ring. Similarly, irradiation of the methylene protons caused enhancement of the alkene proton. Anal. ($C_{24}H_{27}N_5$·2HCl) C, H, N.

***N*-(4-Imidazolinophenyl)-3-(4-imidazobenzyl)piperidine (13):** yield 38%; mp 260−261 °C; ^{1}H NMR δ 10.89 (s, 2H), 10.37 (s, 2H), 8.04 (d, 2H), 7.79 (d, 2H), 7.48 (d, 2H), 6.89 (d, 2H), 3.93 (s, 4H), 3.81 (s, 4H), 2.58−2.85 (m, 6H), 1.80 (m, 1H), 1.19−1.60 (m, 4H). Anal. ($C_{24}H_{29}N_5$·2HCl) C, H, N.

***N,N′*-Bis[4-(*N*-isopropylamidino)phenyl]homopiperazine (14):** yield, 71%; mp >300 °C; ^{1}H NMR δ 9.09 (m, 4H), 8.66 (s, 2H), 7.60 (d, 4H), 6.90 (d, 4H), 4.02 (m, 2H), 3.73 (s, 4H), 3.49 (m, 4H), 1.93 (m, 2H), 1.22 (d, 12H). Anal. ($C_{25}H_{36}N_6$·2HCl·H_2O) C,H, N.

***N,N′*-Bis[4-(*N*-*tert*-butylamidino)phenyl]homopiperazine (15):** yield, 78%; mp 271−273 °C; ^{1}H NMR δ 8.40 (bs, 2H), 7.97 (s, 4H), 7.64 (d, 4H), 6.76 (q, 4H), 3.67 (s, 4H), 3.43 (m, 4H), 1.98 (m, 2H), 1.26 (m, 18H). Anal. ($C_{27}H_{40}N_6$·2HCl·H_2O) C, H, N.

***N,N′*-Bis[4-(*N*-2-hydroxyethylamidino)phenyl]homopiperazine (16):** yield, 73%; mp 281−283 °C; ^{1}H NMR δ 9.23 (s, 2H), 8.90 (s, 2H), 8.58 (s, 2H), 7.65 (d, 4H), 6.90 (d, 4H), 5.16 (bs, 2H), 3.74 (s, 4H), 3.61 (t, 4H), 3.51 (m, 4H), 3.44 (t, 4H), 1.93 (m, 2H). Anal. ($C_{23}H_{32}N_6O_2$·2HCl·0.5H_2O) C, H, N.

***N,N′*-Bis[4-(*N*-*n*-butylamidino)phenyl]homopiperazine (17):** yield, 81%; mp >300 °C; ^{1}H NMR δ 9.14 (s, 2H), 8.84 (s, 2H), 8.46 (s, 2H), 7.50 (d, 4H), 6.82 (d, 4H), 3.68 (s, 4H), 3.45 (s, 4H), 3.26−3.28 (m, 4H), 1.88 (m, 2H), 1.51−1.55 (m, 4H), 1.27−1.32 (m, 4H), 0.82−0.87 (m, 6H). Anal. ($C_{27}H_{40}N_6$·2HCl·0.6H_2O) C, H, N.

***N,N′*-Bis[4-(*N*-cyclopropylamidino)phenyl]homopiperazine (18):** yield, 69%; mp >300 °C; ^{1}H NMR δ 9.56 (bs, 2H), 9.31 (bs, 2H), 8.72 (bs, 2H), 7.67 (d, 4H), 6.87 (d, 4H), 3.73 (s, 4H), 3.50 (s, 4H), 2.71 (m, 2H), 1.91 (m, 2H), 0.89 (m, 4H), 0.76 (m, 4H). Anal. ($C_{25}H_{32}N_6$·2HCl·0.8H_2O) C, H, N.

Thermal Denaturation Studies. Pentamidine, EDTA, Tris-HCl, calf thymus DNA, and poly(dA-dT) used in this study were purchased from Sigma Chemical Company. The method used for the determination of the binding affinity (ΔT_m) of **1**−**18** to calf thymus DNA and the nucleic acid homopolymer poly(dA-dT) has been described previously.[18,30,31] The binding affinity was measured by determining the change in the midpoint (T_m) of the thermal denaturation curves of calf thymus DNA as well as poly(dA-dT) at a 1:5 compound to base pair ratio. Each ΔT_m value reported in Table 1 represents the mean of at least two experimental determinations.

Pharmacology. *Trypanosoma brucei brucei* Lab 110 EATRO strain (pentamidine-sensitive) and clinical isolates of *Trypanosoma brucei rhodesiense* were used in this study. *T. b. rhodesiense* isolates were obtained from A. R. Njogu of the Kenya Trypanosomiasis Research Institute (KETRI, Muguga, Kenya). These included KETRI 243 (DFMO, melarsoprol, pentamidine, and berenil resistant), KETRI 243As-10-3, which is a cloned subpopulation of KETRI 243 and is refractory to arsenicals and aromatic diamidines such as berenil and pentamidine, and KETRI 269 (DFMO and pentamidine resistant).[32]

In Vitro Studies. The compounds were tested against trypanosome isolates grown as blood forms in HMI-18 medium[33] containing 20% horse serum in 24-well microplates at 37 °C. Wells were inoculated with 1×10^5 trypanosomes. The compounds were diluted in the medium at the appropriate concentration and replaced daily. Cell counts were made daily with a Coulter counter, model Z1 (Beckman Coulter, Miami, FL). Cells were diluted with Isoton I buffer (Beckman Coulter), and the aperture was standardized at 5.14 μm. Background checks were performed daily on 1:10 dilutions of medium. Counts, normalization, and coincident counts were accounted for by the standardized Coulter analysis program. Occasionally, hemocytometer counts were performed as a check on the validity of the Coulter program. IC_{50} values were determined after 48 h from semilog plots, and the values are the result of duplicate determinations. Initially a broad concentration curve was used and then a close concentration curve from which IC_{50} values were determined. Assays were done in duplicate, and each point was the average of the two. Control cells grew to 5×10^6/mL.

In Vivo Studies. Female Swiss-Webster mice (weight, 20 g) were infected (intraperitoneally) with 2.5×10^5 trypanosomes from rat blood, and the infection was allowed to develop for 24 h before drug treatment was begun. Groups of three mice each were injected (intraperitoneally) with each drug concentration. All of the experiments included a group of untreated controls. Untreated mice died 5−13 days after infection, depending on the isolate. Parasitemia of animals dying of infection averaged $(0.5-1.0) \times 10^9$/mL of blood. These infections are very predictable, and daily counts were not done because of the labor involved. The procedures used are standard for our laboratory.[32] Animals were monitored weekly for parasites in tail vein blood smears. Mice were considered cured if they survived more than 30 days after the death of the last control with no parasites in tail vein blood smears. MSD (mean survival in days) was also recorded for each group. It is the average time of survival of the animals in the group, exclusive of cured animals.

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