DNA Binding Affinity of Bisguanidine and Bis(2-aminoimidazoline) Derivatives with in Vivo Antitrypanosomal Activity

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A new antitrypanosomal hit compound that cures an acute (STIB 900) mouse model of *Trypanosoma brucei rhodesiense* trypanosomiasis is described. This bis(2-aminoimidazolinium) dicationic compound proved to be an excellent DNA minor groove binder, suggesting a possible mechanism for its trypanocidal activity. From these studies, the 4,4′-diaminodiphenylamine skeleton emerged as a good scaffold for antitrypanosomal drugs.

Introduction

Human African trypanosomiasis, HAT, caused by subspecies of the parasitic protozoan *Trypanosoma brucei*, is a daily threat to millions of people in sub-Saharan Africa and results in great human and social cost in affected areas.[1] However, it is one of the most neglected diseases in the world and no currently available drug to treat this condition is satisfactory (high cost, unacceptable side effects, intravenous mode of administration, and drug resistance are all issues restricting use).[2] The arsenic-based drug melarsoprol, which is the only medicine available to treat the central nervous system (CNS) stage of the disease caused by *T. b. rhodesiense* and is still the principal drug used for late-stage disease, is highly toxic. Treatment failures with melarsoprol are reaching up to 30% in some foci.[3] With no new drugs to treat late-stage disease in the pipeline, the prospect of being unable to treat sleeping sickness has emerged as an alarming threat. Hence, the discovery of new lead compounds that kill trypanosomes but also penetrate into the CNS and cure the second-stage HAT is a priority in international health.[4]

We previously reported aromatic and aliphatic families of bisguanidine and bis(2-aminoimidazoline) derivatives structurally related to pentamidine and synthalin with great potential as antitrypanosomal agents.[5] Some of these compounds (Figure 1) showed excellent activity and selectivity (>3000 for **2a**) toward the parasite in vitro, making them promising new trypanocidal hit compounds. Since pentamidine (Figure 2) is only effective in the treatment of first-stage *T. b. gambiense* sleeping sickness (the chronic form of the disease), new safe compounds active against the acute and the chronic CNS stage are of particular interest. Encouraged by the excellent in vitro activity displayed by some compounds, we selected a series of diphenyl (**1**−**5**) and aliphatic (**6**−**9**) derivatives for in vivo testing in two acute mouse models (STIB 795 and STIB 900). The STIB 795 model has proven to be relatively easy to cure. STIB 900, on the other hand, has proven to be relatively difficult to cure. For example, pentamidine and diminazene cure the STIB 795 model but not the STIB 900 model possibly because in this latter model the parasites invade extravascular sites early in infection. The most active compound, **2a**, that was able to cure 100% of the mice in these models was also evaluated in the second-stage CNS mouse model (GVR 35 strain), a model that is cured only by a minority of trypanocides that are able to cross the blood−brain barrier (BBB).

Many agents of the aromatic diamidine[6] or diguanidine class have strong binding affinity for the DNA at AT-rich sites.[7] Some evidence suggests that this interaction may contribute to the antiprotozoal activity frequently associated with this class of compound. Transcription and activity of other DNA-dependent enzymes may all be inhibited by dicationic drugs.[8] To gain insight into the mechanism of action of a series of dicationic compounds that we have shown to have profound trypanocidal activity, the DNA binding affinity (i.e., ΔT_m) of the diphenyl (**1**−**5**) and aliphatic (**6**−**9**) derivatives was measured.[9]

Another factor that is essential to the efficacy and selectivity of antitrypanosomal drugs is their effective uptake by the parasite. Dicationic drugs have very slow rates of diffusion across biological membranes, and their uptake is dependent on different transporters.[10] Three different transporters have been implicated in the transport of pentamidine: the high- and low-affinity pentamidine transporters (HAPT and LAPT, respectively, whose endogenous substrates are not known) and the P2 aminopurine transporter.[11] The P2 transporter also participates in the uptake of other diamidines (e.g., diminazene, propamidine) and the melaminophenyl arsenicals (e.g., melarsoprol) that bear the common recognition motifs (i.e., amidine moiety, aromatic ring, and electronegative heteroatom; Figure 2).[12−14] Diphenyl bisguanidines too carry this P2 recognition motif. The loss of the P2 transporter alone appears to be sufficient to render trypanosomes highly resistant to some drugs, for example, diminazene.[15] The loss of the transporter also correlates with resistance to melarsoprol, although in this instance additional transporters too must be lost to achieve high-level resistance.[16] A number of other melamine-based[17] and benzamidine-based[18] compounds designed for entry via the P2 transporter also appear to have additional routes into the cell. With regard to understanding the likelihood of resistance arising from compounds that appear to be substrates for the P2 transporter, it is important to know whether uptake is exclusively

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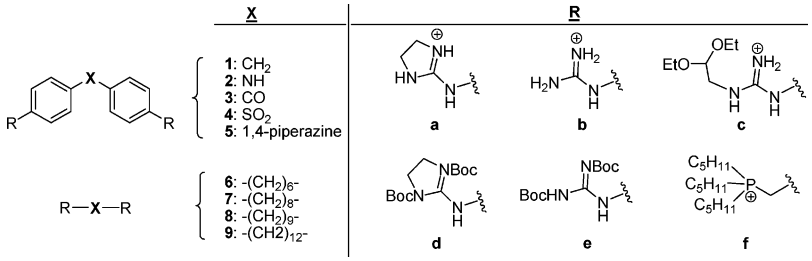


Figure 1. Bisguanidine and bis(2-aminoimidazoline) diphenyl and aliphatic derivatives with activity and selectivity against *T. brucei rhodesiense*.

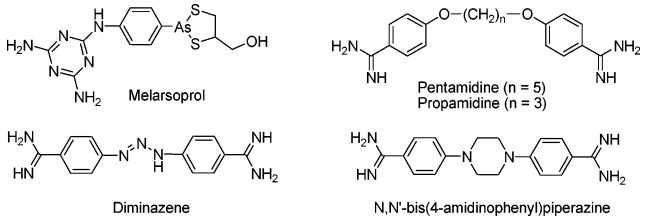


Figure 2. Structure of antitrypanosomal drugs that bear the P2 transporter recognition motifs.

via this transporter or whether alternative routes into the cell also exist. Thus, the most active compounds, **2a** and **2b**, were also evaluated against a cell line defective in P2 mediated transport to gain information about their possible mechanism of uptake.

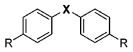
Results and Discussion

DNA Binding Affinity at AT-Rich Sites. Table 1 presents the results of DNA binding as estimated by ΔT_m measurements (i.e., the difference between the T_m of DNA and the T_m observed in the presence of drug[9]) with a nonalternating AT sequence DNA polymer. In general, the diphenyl dicationic derivatives showed a strong DNA binding with ΔT_m values in the range of those for pentamidine for unsubstituted guanidines and 2-aminoimidazolines (**1a**, **1b**, **2a**, **2b**, **3b**, **5a**, and **5b**) whereas substituted guanidines **1c** and **2c** had lower values. Differences in binding affinity were observed depending on the bridge linking both phenyl rings; **2**, with a NH bridge, had a higher ΔT_m than **1** (CH_2 bridge) and **4** (SO_2 bridge). This result could be explained by a better fit of **2** to the minor groove in contrast to **1** and **4**, the dihedral angle of which would not be expected to fit the width of the groove. By contrast, **3b** (CO bridge) bound strongly (27.6 °C), as expected on the basis of a better fit to the groove. In those series, a good correlation was observed between in vitro activity and DNA binding affinity for the 2-aminoimidazoline compounds (**2a** > **1a** ≫ **4a**) whereas a rough correlation was observed for the guanidine analogues (**2b** > **3b** ∼ **1b** ≫ **4b**). Indeed, the best trypanocides in vitro, **2a** and **2b**, were also the best DNA binders of the series (38.5 and 29.6 °C, respectively).

Moreover, plotting T_m versus IC_{50} against trypanosomes revealed the two to be correlated for two sets of compounds as shown in Figure 3. Compounds **4a** and **4b** were omitted because they showed no activity that might conceivably relate to their inability to enter cells. Further experiments would be required to test this. In addition, the alkyl dicationic derivatives (**6a**, **6b**−**9b**) were omitted because there was no correlation between activity and T_m increase. It is likely that there are multiple modes of activity for dications, each of which contributes to the overall IC_{50}. In the case of the alkyl group sets, an alternative mode of action might mask activity that results from DNA binding.

The nature of the cationic moiety appeared to play a role in the DNA binding affinity of these diphenyl compounds.

Table 1. DNA Binding Affinity and in Vitro Trypanocidal Activity of Diphenyl and Aliphatic Derivatives

Structure	Compound	X	*T. b. rhodesiense* STIB 900 IC_{50} (nM)[a]	ΔTm (°C)[b] poly(dA.dT)$_2$
	Pentamidine	-O(CH$_2$)$_5$O-	2.2	32.3
	N,N'-bis(4-amidinophenyl)piperazine		13.5[c]	19.4
	**1a**		897	18.8
	1b	CH$_2$	161	15.0
	1c		316	5.3
	2a		69	38.5
	2b	NH	22	29.6
	2c		228	13.6
	3a		2050	nd
	3b	CO	206	27.6
	3f		414	1.2
	4a	SO$_2$	32 x 10^3	12.1
	4b		4.3 x 10^3	12.8
	5a		118	38.8
	5b		270	27.8
	5e		13.8 x 10^3	0.2
R—X—R	**6a**	-(CH$_2$)$_6$-	19.3 × 10^3	14.0
	6b		8.05 × 10^3	13.1
	7b	-(CH$_2$)$_8$-	251	13.1
	8b	-(CH$_2$)$_9$-	49	12.2
	9b	-(CH$_2$)$_{12}$-	47	9.1

[a] Average of duplicate determinations (values taken from ref 5). [b] Buffer: MES (0.01 M) + EDTA (0.001M). Ratio compound/DNA is 0.3 (see ref 9). [c] Value for drug-resistant clinical isolate of *T. b. rhodesiense* KETRI 243 (taken from ref 19).

Compounds with 2-aminoimidazolinium cations had stronger binding than their guanidinium counterparts (compare **1a**/**1b**, **2a**/**2b**, and **5a**/**5b**), and two molecules (**2a** and **5a**) bound to DNA with higher affinity than pentamidine. The more bulky and flexible bis(*n*-pentylphosphonium) derivative **3f** had negligible DNA binding in contrast to its guanidine counterpart. Interestingly, derivative **5a** with the 2-aminoimidazolinium cation bind more strongly than its amidine counterpart (*N*,*N*′-bis(4-amidinophenyl)piperazine, Figure 2) described by Donkor et al.[19] This could relate to the higher basicity of this cation compared to the amidinium or the capacity to form hydrogen bonds. As expected, on the basis of the greater negative potential at the floor of the minor groove in AT-rich regions,[20] the uncharged Boc-protected analogue (**5e**) had insignificant binding. A good correlation between DNA binding affinity and trypanocidal activity was observed with those derivatives (**5a**> **5b** ≫ **5e**), which confirmed the findings of Donkor et al. for

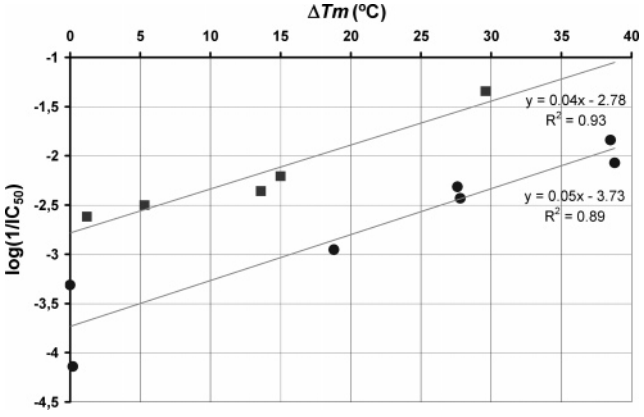


Figure 3. Plot of $\log(1/IC_{50})$ vs T_m for the diphenyl series showing good correlation between in vitro antitrypanosomal activity and T_m increase for two sets of compounds: (■) **1b**, **1c**, **2b**, **2c**, and **3f**; (●) **1a**, **2a**, **3b**, **5a**, **5b**, and **5e**.

this family of conformationally restricted pentamidine congeners.[19] Hence, the mechanism of action of the diphenyl compounds that have strong DNA interaction could be due in part to the formation of a DNA complex.

On the other hand, the alkyl dicationic compounds had much lower affinity than pentamidine, in the range 9.1−14 °C. This is probably because these derivatives lack the aromatic rings that help to position the cations to interact with A and T bases at the floor of the minor groove. Despite the low DNA binding, some of these aliphatic molecules (**7b**, **8b**, and **9b**) showed excellent antitrypanosomal activity in vitro. This could indicate mechanisms of action other than binding to DNA, although it is also noteworthy that dications, including pentamidine and diminazene, accumulate to very high levels within trypanosomes, with the mitochondrion in particular concentrating the drug. Thus, even low affinity binding may lead to high overall interactions with kinetoplast DNA at these greatly elevated concentrations.

In Vivo Activity. The eight compounds displaying the best in vitro activities and selectivity were then administered intraperitoneally to infected mice to determine whether activity was retained in vivo (Table 2). Two diphenyl compounds (**2a** and **2b**) cured all mice infected with *T. b. brucei* STIB 795 at 20 mg/kg. The Boc-protected analogue **2d** had little effect on survival (10.25 days), and the 1,4-piperazine compound **5a** was toxic after the first treatment at 20 mg/kg. The four alkyl derivatives tested did not result in cures. Two of them (**9a** and **9b**) were toxic at 20 mg/kg, whereas the other two (**7b** and **8a**) only extended the survival rate at 10 mg/kg (> 37.5 and 13.5 days, respectively).

This first screening against the acute STIB 795 model revealed that the most promising compounds were the 2-aminoimidazoline (**2a**) and guanidine (**2b**) diphenyl analogues with a NH bridge. Hence, these compounds were tested by ip administration in the difficult *T. b. rhodesiense* STIB 900 mouse model. At 20 mg/kg, the guanidine derivative **2b** cured 2/4 mice, whereas **2a** cured 4/4 mice. Lower doses of **2a** (5 and 10 mg/kg) achieved only 1/4 cure. On the basis of these excellent in vivo results, **2a** was assayed in the chronic CNS mouse model (GVR 35) of sleeping sickness. Unfortunately, no cures were obtained probably because of poor BBB penetration of this hit compound. Indeed, this kind of guanidine-like compound has very basic nitrogen atoms that are charged at physiological pH and thus is potentially poorly liposoluble and not able to cross the BBB by passive diffusion. Different synthetic strategies exist to improve the pharmacokinetics of cationic compounds such as amidines[21] and guanidines.[22] These prodrug approaches are currently being studied in our laboratory.

Table 2. In Vivo Antitrypanosomal Activities for Diphenyl and Aliphatic Derivatives in the Acute (STIB 795 and STIB 900) and Late-Stage (GVR 35) Mouse Models

compd	mouse model[a]	dosage route[b]	dosage (mg/kg)	cured[c]/ infected	survival (days)[d]
control	STIB 795			0/4	6.25
control	STIB 900			0/4	7.5
control	GVR 35			0/5	38
diminazene diaceturate	GVR 35	ip	1 × 40	0/5	71
2a	STIB 795	ip	4 × 20	4/4	>60
2a	STIB 900	ip	4 × 20	4/4	>60
2a	STIB 900	ip	4 × 10	1/4	>40
2a	STIB 900	ip	4 × 5	1/4	>42.5
2a	GVR 35	ip	5 × 20	0/5	40.8
2b	STIB 795	ip	4 × 20	4/4	>60
2b	STIB 900	ip	4 × 20	2/4	>43.3
2d	STIB 795	ip	4 × 20	0/4	10.25
5a	STIB 795	ip	4 × 20	toxic	*e*
7b	STIB 795	ip	4 × 10	0/4	>37.5
8a	STIB 795	ip	4 × 10	0/4	13.5
9a	STIB 795	ip	4 × 20	toxic	*f*
9b	STIB 795	ip	4 × 20	toxic	*f*

[a] See Experimental Section for details of STIB 795 (*T. b. brucei*) and STIB 900 (*T. b. rhodesiense*) models. For GVR 35, see ref 24. [b] ip = intraperitoneal. [c] Number of mice that survive and are parasite-free for 60 days. [d] Average days of survival. [e] All mice died after the first treatment. [f] All mice died after the third treatment.

Table 3. In Vitro Activities against Bloodstream *T. brucei*

compd	IC_{50} (nM) *T. brucei brucei* AT1 wild type	IC_{50} (nM) *T. brucei brucei* AT1 knockout[a]
2a	63.7	162
2b	3.0	2.2
pentamidine[b]	11	26
diminazene[b]	301	5773

[a] Mutant with a nonfunctional P2 transporter. [b] Data taken from ref 16.

Roles for the P2 Transporter in Uptake and Resistance. Since these compounds carry a motif typical of substrates of the P2 aminopurine transporter and since the loss of this transporter can contribute to the development of resistance to drugs that enter via this route, we evaluated the in vitro activity of **2a** and **2b** against both the wild type and the TbAT1 knockout line that lacks the P2 transporter.[16] The difference in activity between the two lines for both compounds was small or insignificant (Table 3). This is similar to results obtained with pentamidine, known to enter via other transporters,[11] but different from diminazene, which enters predominantly via the P2 transporter.[15] It has recently been shown that a number of other melamine-based[17] and benzamidine-based[18] trypanocides also enter via other transporters in addition to P2. This is significant because it indicates that parasites selected for resistance to drugs through loss of the P2 transporter alone will not show cross-resistance to these compounds.

Conclusion

We have described a correlation between the DNA binding affinity and trypanocidal activity of two series of diphenyl dicationic derivatives previously found by screening our in-house library.[5] Two hit compounds (**2a** and **2b**), which demonstrated excellent DNA binding affinity and in vivo activity

in two models (STIB 795 and STIB 900) of acute *T. brucei* infections in mice, emerged from this study. The bis(2-aminoimidazolinium) compound **2a** was particularly active and, upon ip injection of 20 (mg/kg)/day for 4 days, cured 100% of treated mice in the difficult to cure *T. b. rhodesiens*e STIB 900 model, without overt toxicity. Although this compound could not cure the rodent model of CNS infection, probably because of poor BBB penetration, it has the potential to be modified (e.g., using a prodrug approach) to improve its CNS delivery. Hence, the 4,4′-diphenylamine scaffold, and **2a** in particular, represents a promising dicationic hit compound for the treatment of sleeping sickness.

In this series of bis(2-aminoimidazolinium)diphenyl derivatives, a good correlation between in vitro antitrypanosomal activity and DNA binding was observed, suggesting that this binding could be part of their mechanism of action. Finally, we showed that even though the P2-aminopurine transporter may be involved in the uptake of these hit compounds, this was not the sole route of entry of these guanidine-like compounds into trypanosomes, and thus, parasites that have lost the P2 transporter in selection of resistance to other drugs will not be cross-resistant to these compounds.

Experimental Section

DNA Binding Assays. Poly(dA)·poly(dT) was obtained from Pharmacia Corp. and characterized as previously described.[9] Thermal melting experiments were conducted with Cary 300 spectrophotometers interfaced to microcomputers as previously described.[9] A thermistor fixed into a reference cuvette was used to monitor the cell temperature. A 1 mL sample of poly(dA)·poly(dT) DNA in buffer (MES buffer contained 0.01 M MES and 10^{-3} M EDTA with the pH adjusted to 6.2 with NaOH) was placed in a 1 cm path length reduced volume quartz cell, and the concentration was checked by measuring the absorbance at 260 nm. Experiments were conducted at 50 μM DNA bases, and T_m values were determined from first-derivative plots. The free polydA·polydT duplex had a T_m of 46 °C. Compounds were compared by the increase in T_m [T_m(complex) − T_m(free DNA)] they produced at a molar ratio of 0.3 of compound to nucleic acid bases (saturating amounts of the compound). Ratios greater than 0.3 did not affect the T_m. The estimated errors in the ΔT_m are 0.5 °C.

In Vitro Activity against *T. brucei* spp. IC_{50} determinations for *T. b. rhodesiense* STIB 900 were done using the Alamar blue assay.[5,23] Bloodstream forms of *T. brucei brucei* (strain 427) were cultivated in HMI-9 medium containing 20% fetal calf serum at 37 °C in a humidified CO_2 environment. To investigate whether transport of these compounds through the P2 transporter is necessary for activity, compounds were assayed against the *T. brucei brucei* trypomastigotes, either wild type or P2 knockout mutants ($TbAT1^{-/-}$).[16] The Alamar blue assay[23] was used to determine IC_{50} against wild type lines and the $TbAT1^{-/-}$ derivative.

In Vivo Activity against *T. brucei*. Female NMRI mice weighing 22−25 g were infected with cryopreserved stabilates of *T. brucei brucei* STIB 795 (derivate of strain 427) or *T. brucei rhodesiense* STIB 900. Each mouse was infected intraperitoneally with 1×10^5 (STIB 795) or 2×10^4 (STIB 900) bloodstream forms. Groups of four mice were treated intraperitoneally with the compounds on days 3−6. A control group remained untreated. The parasitemia of all animals was checked every second day up to day 14 postinfection and two times a week thereafter until 60 days. Death of animals was recorded to calculate the mean survival time. Surviving and aparasitemic mice were considered cured at 60 days and then euthanized.

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