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Anti-plasmodial and anti-leishmanial activity of conformationally restricted pentamidine congeners

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Abstract

A library of 52 pentamidine congeners in which the flexible pentyldioxy linker in pentamidine was replaced with various restricted linkers was tested for in-vitro activity against two *Plasmodium falciparum* strains and *Leishmania donovani*. The tested compounds were generally more effective against *P. falciparum* than *L. donovani*. The most active compounds against the chloroquine-sensitive (D6, Sierra Leone) and -resistant (W2, Indochina) strains of *P. falciparum* were bisbenzamidines linked with a 1,4-piperazinediyl or 1, 4-homopiperazinediyl moiety, with IC50 values (50% inhibitory concentration, inhibiting parasite growth by 50% in relation to drug-free control) as low as 7 nM based on the parasite lactate dehydrogenase assay. Seven piperazine-linked bisbenzamidines substituted at the amidinium nitrogens with a linear alkyl group of 3–6 carbons (**22**, **25**, **27**, **31**) or cycloalkyl group of 4, 6 or 7 carbons (**26**, **32**, **34**) were more potent (IC50 < 40 nM) than chloroquine or pentamidine as anti-plasmodial agents. The most active anti-leishmanial agents were 4,4′-[1,4-phenylenebis(methyleneoxy)]bisbenzenecarboximidamide (**2**, IC50 ~ 0.290 μM) and 1,4-bis[4-(1H-benzimidazol-2-yl)phenyl] piperazine (**44**, IC50 ~ 0.410 μM), which were 10- and 7-fold more potent than pentamidine (IC50 ~ 2.90 μM). Several of the more active anti-plasmodial agents (e.g. **2**, **31**, **33**, **36**–**38**) were also potent anti-leishmanial agents, indicating broad antiprotozoal properties. However, a number of analogues that showed potent anti-plasmodial activity (**1**, **18**, **21**, **22**, **25**–**28**, **32**, **43**, **45**) were not significantly active against the *Leishmania* parasite. This indicates differential modes of anti-plasmodial and anti-leishmanial actions for this class of compounds. These compounds provide important structure–activity relationship data for the design of improved chemotherapeutic agents against parasitic infections.

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Introduction

Parasitic diseases such as malaria and leishmaniasis are serious diseases of worldwide implications. They pose serious public health and economic problems especially in developing countries. Malaria itself affects more than 6% of the global population (300 million cases) annually, resulting in the deaths of 1–2 million people (Woster 2003). In Africa alone, the mortality of children from malaria exceeds a million each year and this number is rising. Approximately 2 million new cases of leishmaniasis are reported each year, of which 500 000 are visceral leishmaniasis that is usually fatal if left untreated. The other two clinical variants of leishmaniasis are the cutaneous and mucocutaneous forms. In recent years, visceral leishmaniasis has emerged as a common and serious opportunistic infection in human immunodeficiency virus (HIV)-infected patients (Berman 1997; Calza et al 2004). About 25–70% of adult visceral leishmaniasis cases are related to HIV infection, especially in southern Europe. The high morbidity and mortality rate caused by malaria and leishmaniasis is largely due to the emergence and spread of resistance to the currently available drugs. Many of these drugs also produce serious toxicity, lack adequate efficacy and must be administered by injection.

There are four species from the genus *Plasmodium* that causes malaria in man – *Plasmodium falciparum*, *P. malariae*, *P. vivax* and *P. ovale*. *P. falciparum* is the most virulent and dangerous of the human malaria parasites. It can infect red cells of all ages and therefore produce overwhelming parasitaemia. Nearly all deaths due to malaria are because of infection caused by *P. falciparum*. It is also resistant to many drugs (Rosenthal

2003; Rathore et al 2005). The quinolines, such as chloroquine, quinine, mefloquine and primaquine, are currently available for the treatment of malaria. Chloroquine is recognized as an exceptionally safe, effective, inexpensive and orally available drug and has been used in the treatment and prophylaxis of malaria for more than 50 years. Unfortunately, its intensive use has led to the development of resistant parasites. Although some of the other quinolines, such as quinine with mefloquine, are used to treat chloroquine-resistant strains of *P. falciparum*, factors such as side effects, multidrug resistance emergence and costs have limited their use. Folate antagonists, such as pyrimethamine-sulfadoxine, have also become less effective due to the emergence of parasite resistance. The most recent additions to the drug therapy of malaria are artemisinin and its derivatives (artesunate and artemether) and atovaquone. However, concerns such as poor oral bioavailability, short plasma half-life and neurotoxicity have limited the use of artemisnin derivatives (Avery et al 2003; Ploypradith 2004). Both the artemisinin derivatives and atovaquone have had unacceptable failure rates when used as single agents, but their combination with established antimalarials are promising therapy for *P. falciparum* malaria (Rosenthal 2003; Patel & Kain 2005).

The current therapy for leishmaniasis is also inadequate. At least 20 species of *Leishmania* has been reported to infect man. Visceral leishmaniasis is caused by *L. donovani*. This is a systemic disease and is characterized by fever, diarrhoea, cough and enlarged liver and spleen. Resistance by the *Leishmania* parasite to the pentavalent antimonials (sodium stibogluconate and meglumine antimoniate), which have been considered to be the primary therapy for visceral and cutaneous leishmaniasis for over half a century, is now widespread (Singh & Sivakumar 2004; Croft et al 2005). In addition, the long course of treatment with antimonials leads to accumulation of the drug in the liver and spleen resulting in side effects (Berman 1997). Treatment of leishmaniasis with alternative drugs, such as amphotericin B and pentamidine, is hampered by severe toxic effects. New formulations of lipid-associated amphotericin B has resulted in less toxicity, but the drug is very expensive and requires close medical supervision. The most recent drug is miltifosine, an alkylphosphocholine, originally developed as an anti-cancer agent. It is the first oral drug approved for treating visceral leishmaniasis (Croft et al 2005). This drug is characterized by severe gastrointestinal side effects, high costs and cannot be given during pregnancy (Singh & Sivakumar 2004).

Despite marked improvement in the chemotherapy of leishmaniasis by miltefosine, there is a broad consensus for the need to develop new anti-parasitic agents that are more effective and less toxic than the current drugs. In addition, new drugs should preferably be effective orally and inexpensive to produce.

We have recently reported on the anti-parasitic activity of a library of bisbenzamidines connected by various conformationally restricted linkers (Donkor et al 2003; Mayence et al 2004a, b). From these earlier studies, we identified 4,4′-(1,4-piperazinediyl)bisbenzenecarboximidamide (compound **19**, Table 1) as a promising lead compound. In this report, the anti-parasitic activity of an expanded series of 1,4-diarylpiperazines (compounds **20**–**52**), based on the lead compound **19**, were studied. The terminal basic amidinium groups of **19** were substituted with various alkyl or cycloalkyl groups of variable length and sizes, or were replaced with non-basic or bulky lipophilic functionalities, and their in-vitro anti-parasitic activity was analysed. In addition, a series of bisbenzamidines connected by various linkers (compounds **1**–**19**) was tested to investigate the role of the central linker on biological activity. The anti-plasmodial and anti-leishmanial activity of the 52 pentamidine congeners was evaluated against the chloroquine-sensitive (D6, Sierra Leone) and -resistant (W2, Indochina) strains of *P. falciparum*, as well as against *Leishmania donovani* promastigotes. The cytotoxicity of these compounds was evaluated with Vero (monkey kidney fibroblast) cells.

Materials and Methods

Anti-parasitic agents

The compounds used in this study were synthesized in the laboratory of Dr Tien L. Huang in the College of Pharmacy at Xavier University of Louisiana. These compounds were synthesized according to methods that have been previously described (Huang et al 1996, 2001; Tao et al 1999; Donkor et al 2003; Vanden Eynde et al 2003, 2004; Cushion et al 2004; Mayence et al 2004a, b). The structures and purity of the compounds were confirmed with proton nuclear magnetic resonance, infrared, and elemental analyses.

Anti-plasmodial assays

The anti-plasmodial activity of the compounds was determined in-vitro against chloroquine-sensitive (D6, Sierra Leone) and -resistant (W2, Indochina) strains of *P. falciparum*. The assay is based on determining the growth of *P. falciparum* by parasitic lactate dehydrogenase (LDH) assay as described by Makler et al (1993). In brief, the appropriate dilution of the compounds (2 mg mL^{-1} stock solutions in dimethyl sulfoxide (DMSO)) were prepared in RPMI-1640 medium and added to the cultures of *P. falciparum* (2% haematocrit, 2% parasitaemia) set up in clear flat-bottomed 96-well plates. The cultures were incubated at 37°C for 72 h in a humidified chamber and flushed with a gas mixture of 90% N_2, 5% CO_2 and 5% O_2. Parasitic LDH activity was determined by using Malstat reagent (Flow Inc., Portland, OR, USA). Chloroquine and pentamidine were used as the positive controls, while DMSO was used as the negative control. IC50 values (50% inhibitory concentration, inhibiting parasite growth by 50% in relation to drug-free control) for each compound were computed from the dose–response curves generated by plotting parasitic growth against concentrations.

Anti-leishmanial assays

The compounds were dissolved in DMSO at a concentration of 2 mg mL^{-1} and diluted with the culture medium to

Table 1 Anti-plasmodial and anti-leishmanial activity of pentamidine congeners **1**–**52**

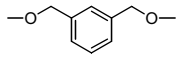
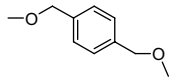
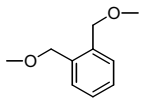
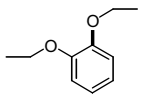
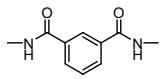
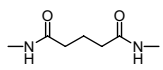
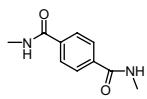
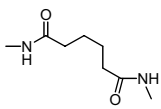
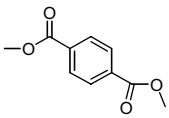
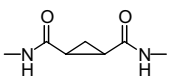
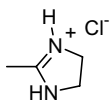
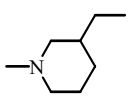
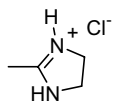
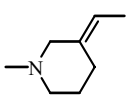
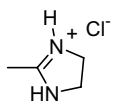
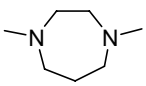
No.	Linker	R	IC50 (μM)			
			Plasmodium falciparum		*Leishmania donovani*	Vero cells
			D6	W2		
	Chloroquine		0.034 ± 0.004	0.250 ± 0.019	NT	NC
	Pentamidine		0.278 ± 0.080	0.346 ± 0.020	2.90 ± 0.40	NC
1			0.085 ± 0.000*	0.067 ± 0.007*	6.93 ± 1.72	28.5 ± 2.8
2			0.114 ± 0.040*	0.235 ± 0.060*	0.290 ± 0.150*	19.6 ± 0.5
3			0.313 ± 0.000	0.369 ± 0.140	8.94 ± 2.51	NC
4			0.671 ± 0.000	0.592 ± 0.056	> 22.4	NC
5			0.697 ± 0.060	0.507 ± 0.080	33.80 ± 4.12	NC
6			NA	NA	NA	NC
7			4.12 ± 0.11	4.85 ± 0.84	38.6 ± 4.7	NC
8			0.640 ± 0.040	0.420 ± 0.240	34.2 ± 3.1	NC
9			NA	NA	> 22.1	NT
10			NA	NA	> 102	NT
11			0.402 ± 0.010	0.337 ± 0.050	47.8 ± 5.2	NC
12			0.840 ± 0.030	0.763 ± 0.170	50.2 ± 3.4	NC
13			1.60 ± 0.04	0.953 ± 0.020	24.4 ± 2.0	NC

Table 1 *(Cont.)*

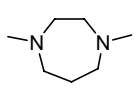
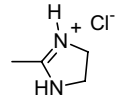
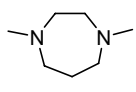
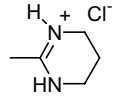
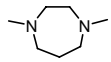
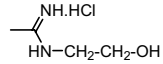
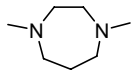
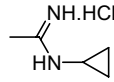
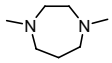
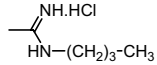
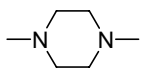
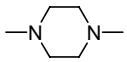
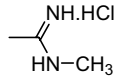
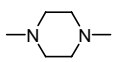
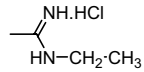
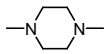
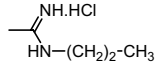
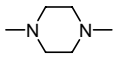
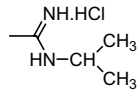
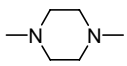
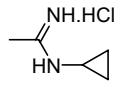
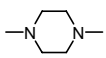
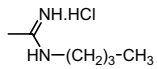
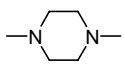
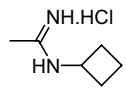
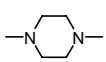
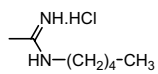
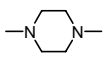
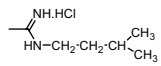
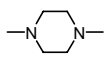
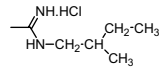
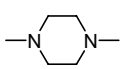
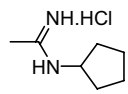
No.	Linker	R	IC50 (μM)			
			Plasmodium falciparum		*Leishmania donovani*	Vero cells
			D6	W2		
14			0.148 ± 0.050	0.145 ± 0.050	> 108	NC
15			5.11 ± 0.20	3.06 ± 0.20	NA	NT
16			NA	NA	> 101	NC
17			0.306 ± 0.040	0.190 ± 0.080	NA	NC
18			0.018 ± 0.009*	0.013 ± 0.004*	18.9 ± 2.9	NC
19			0.154 ± 0.020	0.219 ± 0.030*	21.5 ± 4.3	NC
20			0.862 ± 0.010	0.768 ± 0.010	8.57 ± 2.26	NC
21			0.124 ± 0.060*	0.152 ± 0.003*	9.53 ± 3.44	NC
22			0.029 ± 0.010*	0.016 ± 0.004*	NA	NC
23			0.245 ± 0.050	0.195 ± 0.030*	NA	NC
24			0.358 ± 0.040	0.263 ± 0.090	NA	NC
25			0.019 ± 0.004*	0.022 ± 0.005*	15.7 ± 3.2	NC
26			0.007 ± 0.001*	0.052 ± 0.000*	> 19.9	NC
27			0.021 ± 0.004*	0.021 ± 0.005*	3.73 ± 1.08	NC
28			0.042 ± 0.005*	0.050 ± 0.000*	6.16 ± 1.41	NC
29			0.069 ± 0.019*	0.068 ± 0.007*	9.34 ± 2.07	NC
30			0.297 ± 0.015	0.196 ± 0.030*	30.1 ± 4.0	NC

Table 1 *(Cont.)*

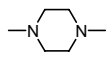
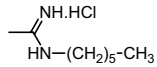
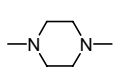
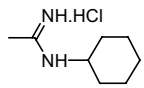
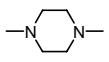
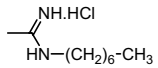
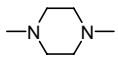
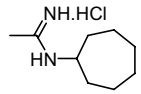
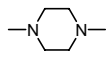
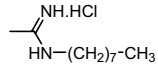
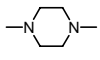
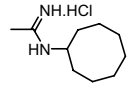
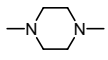
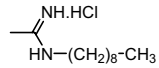
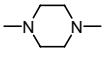
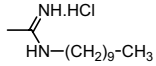
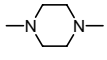
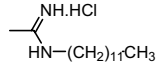
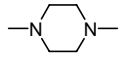
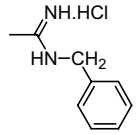
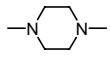
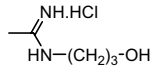
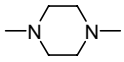
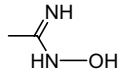
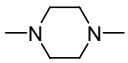
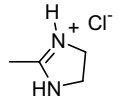
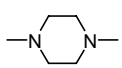
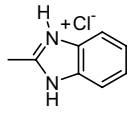
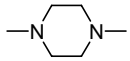
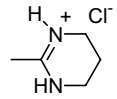
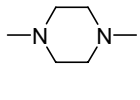
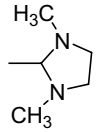
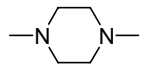
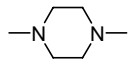
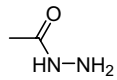
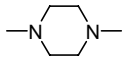
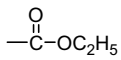
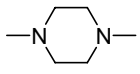
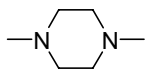
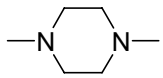
No.	Linker	R	IC50 (μM)			
			Plasmodium falciparum		*Leishmania donovani*	Vero cells
			D6	W2		
31			0.035 ± 0.005*	0.037 ± 0.009*	1.06 ± 0.91*	NC
32			0.036 ± 0.000*	0.046 ± 0.012*	11.7 ± 2.7	NC
33			0.048 ± 0.009*	0.177 ± 0.025	1.86 ± 0.92	11.0 ± 0.8
34			0.036 ± 0.002*	0.031 ± 0.009*	4.76 ± 1.34	NC
35			0.138 ± 0.002	0.274 ± 0.032*	1.42 ± 0.56*	6.10 ± 1.90
36			0.055 ± 0.008*	0.146 ± 0.016	2.27 ± 0.74	NC
37			0.088 ± 0.011*	0.286 ± 0.023	2.01 ± 0.79	18.5 ± 1.6
38			0.075 ± 0.043*	0.444 ± 0.030	1.78 ± 0.43	NC
39			3.89 ± 0.07	3.62 ± 0.20	NT	NT
40			0.101 ± 0.077	0.155 ± 0.023*	4.34 ± 0.31	NC
41			8.80 ± 1.10	4.50 ± 0.31	> 97.8	NC
42			8.32 ± 0.70	NA	56.5 ± 7.3	NC
43			0.089 ± 0.000*	0.074 ± 0.010*	6.71 ± 0.79	NC
44			4.88 ± 0.46	6.72 ± 1.93	0.410 ± 0.070*	NC
45			0.106 ± 0.016*	0.102 ± 0.012*	NA	NC

Table 1 *(Cont.)*

No.	Linker	R	IC50 (μM)			
			Plasmodium falciparum		*Leishmania donovani*	Vero cells
			D6	W2		
46			NA	NA	NA	NT
47			NA	NA	NA	NT
48			NA	NA	NA	NT
49			NA	NA	NA	NT
50			NA	NA	NA	NT
51			NA	NA	NA	NT
52			NA	NA	NA	NT

IC50 values are expressed as mean ± s.e.m. of at least three replicates. *$P < 0.05$ vs pentamidine. NT, not tested; NC, not cytotoxic at concentrations of up to 40 μM; NA, not active at the maximum dose tested (10 μM in the anti-plasmodial assay; 100 μM in the anti-leishmanial assay)

obtain the desired final concentrations. The solutions were added, in a 96-well microplate assay, to the *Leishmania donovani* promastigotes culture (2×10^6 cell/mL). Controls with corresponding dilutions of DMSO indicated that growth of the parasite was not altered under those experimental conditions. The plates were incubated at 26 °C for 72 h and growth of *Leishmania* promastigotes was determined by Alamar blue assay (Mikes & Steverding 2000). IC50 values for each compound were computed from the growth inhibition curve. Pentamidine was used as the reference drug.

Cytotoxicity

The compounds were tested for cytotoxicity on Vero cells (monkey kidney fibroblast) by a Neutral Red assay (Babich & Borenfreund 1991).

Statistical methods

All the compounds were tested at a minimum of six different concentrations. Each assay was done at least in duplicate. The mean values were used to generate the growth inhibition curves and determination of the IC50 values. Mean and standard errors of mean (s.e.m.) for the anti-plasmodial and anti-leishmanial activity of each compound were computed from at least three individual IC50 values. Analogues with IC50 values lower than the standard drug pentamidine were compared by Students's *t*-test. $P < 0.05$ denoted statistically significant improvement in the anti-parasitic activity as compared with the standard drug.

Results and Discussion

In earlier studies, we reported on the anti-plasmodial (Mayence et al 2004a) and anti-leishmanial (Mayence et al 2004b) activity of a limited series of conformationally restricted bisbenzamidines. These studies identified 4,4′-(1,4-piperazinediyl)bisbenzenecarboximidamide (compound **19**, Table 1) as a promising lead compound. To investigate the importance of the terminal amidinium groups of **19** on anti-parasitic activity, an expanded series of compounds (**20**–**52**) was synthesized and evaluated. The nature of the central

linker in pentamidine has been shown to influence its biological properties (Tao et al 1999; Brendle et al 2002; Donkor et al 2003; Mayence et al 2004a). Consequently, we decided to investigate the role of the central linker in the bisbenzamidine class of compounds by developing and testing compounds **1**–**13**, including **19**, using the assays described in this study. In this series, the flexible pentyldioxy linker in pentamidine was replaced with restricted linkers, namely 1,3-, 1,4- or 1,2-phenylenedimethyleneoxy analogues (**1**, **2** and **3**), 1,2-phenylenedioxymethylene (**4**), 1,3- or 1,4-phenylenedicarboxamides (**5** and **7**), 1,4-phenylenedicarboxylate (**9**), 1,4-homopiperazinedyl (**13**) and 1,4-piperazinedyl (**19**). Bisbenzamidines with a flexible pentanediamide (**6**) or hexanediamide (**8**) as linkers were included to compare with its restricted congeners (**5** and **7** respectively). In the case of compounds **10**–**12**, where the terminal groups are imidazolinium functions, the restricted linkers are *trans*-1,2-cyclopropane dicarboxamide and piperadine. It is to be noted that the compounds described in this study as potential anti-parasitic agents are different from previously reported analogues (Bell et al 1990; Brendle et al 2002), since the linkers that connect the bisbenzamidines (**1**–**13**, **19**) in this study are uniquely different.

Compound **1**, which is the closest conformationally restricted congener of pentamidine, was found to be the most potent anti-plasmodial agent among this series of bisbenzamidines connected with the various linkers as described above (compounds **1**–**19**). It had IC50 values of 0.085 μM and 0.067 μM against the D6 and W2 strains, respectively, which was 3- to 5-fold more potent than pentamidine. Compound **2**, which is the *para*-analogue of **1**, also displayed potent activity against both strains of *P. falciparum* and was the most potent agent against *L. donovani* in this library of 52 compounds. With an IC50 value of 0.290 μM against *L. donovani*, this compound was about 10-fold more active than pentamidine (IC50 2.90).

Compounds **1** and **2** were cytotoxic to Vero cells with IC50 values of 28.5 μM and 19.6 μM, respectively. However, compound **1** was 335- to 425-fold more toxic to the two strains of *P. falciparum* than to the Vero cells, whereas compound **2** was 68-fold more toxic to *L. donovani* than to the Vero cells. Replacing the strong electron-donating ether oxygens in **1** and **2** with poor electron-donating amide functions, as in **5** and **7**, did not improve anti-parasitic activity. Similar observations were seen with compounds **4** and **9**. Relief of conformational constraints in **5** and **7** by replacing the phenyl-enediamide linker with a pentanediamide (**6**) or hexanediamide group (**8**) led to a further decline in anti-parasitic potency for **6**, but an increase for **8**. No significant improvement in activity was seen with compounds bearing the cyclopropyldicarboxamide (**10**), piperadine (**11**, **12**) or homo-piperazinedyl (**13**) linkers. However, compound **19**, with the 1,4-piperazinedyl linker, displayed relatively potent anti-plasmodial activity against both strains (IC50 values of 0.154 μM and 0.219 μM against the D6 and W2 strains, respectively). The results suggest that the nature of the central linker in this series of compounds plays an important role in affecting their anti-parasitic activity. Similar observations were made when some of these compounds were tested against *Pneumocystis carinii* (Tao et al 1999) and *Trypanosoma brucei* (Donkor et al 2003). The piperazine linker in **19** appears to orient the molecule into the desired conformation for optimal binding to its macromolecular target in the parasite without causing toxic effects to the Vero cells at the concentrations tested. Although compound **2** showed broad anti-parasitic activity, its lack of selectivity might hinder its further development as a potential drug candidate.

The importance of the terminal amidinium groups was explored using bisbenzamidines linked with either the 1,4-homopiperazinedyl or 1,4-piperazinedyl linker. In both series of compounds, the anti-parasitic activity can be clearly modulated through the substitution of linear alkyl or cyloalkyl groups on the amidinium nitrogens. In the homopiperazine-linked bisbenzamidine series **13**–**18**, substituting an n-butyl chain to one of the nitrogens of the bisamidinium groups (**18**) resulted in an 89- or 73-fold increase in anti-plasmodial potency when compared with the unsubstituted analogue **13**. Cyclizing the amidinium nitrogens into the 5-membered imidazolinium ring (**14**) also enhanced anti-plasmodial potency by 7–11 fold. This trend was also observed in the piperazine-linked bisbenzamidines (compare **19** with **25** or **43**). On the other hand, this trend was the opposite for a series of bisbenzamidines linked by either a furan or thiophene moiety (Brendle et al 2002). The substitution of an alkyl or cycloalkyl group to one of the nitrogens of the amidinium groups consistently resulted in reduced potency when compared with the unsubstituted furan- or thiophene-linked bisbenzamidines. The results of all these studies clearly highlight the importance of not only the type of substituents attached to the terminal groups but also the geometry imposed by the central linker in the bisbenzamidine class of compounds.

The structure–activity relationships are more clearly defined in the piperazine-linked compounds (**19**–**52**). Among the linear alkyl chain analogues, maximal anti-plasmodial activity (IC50 values < 40 nM) was associated with a chain length of 3–6 carbon atoms (i.e. **22**, **25**, **27** and **31**). Shortening the chain to one carbon (**20**) or increasing the chain up to 12 carbons (**39**) led to a decline in potency. On the other hand, the anti-leishmanial activity increased with increasing chain length of the alkyl group. Maximal anti-leishmanial activity (IC50 < 2.01 μM) was associated with a chain length of 6–10 carbon atoms (i.e. **31**, **33**, **35**, **37** and **38**). However, cytotoxicity was associated with the longer alkyl chains of 7–9 carbons (i.e. **33**, **35** and **37**). Branched alkyl chains of 3 or 5 carbons were generally less potent anti-parasitic agents than their straight-chain counterparts (**22** vs **23**; **27** vs **28** or **29**). Among the cyclic alkyl groups of increasing sizes, maximal anti-plasmodial activity was observed with the cyclobutyl analogue **26**, cyclohexyl analogue **32**

and the cycloheptyl analogue **34** (IC50 7–46 nM). The most potent compound against the D6 strain in this series of 52 compounds was analogue **26**. With an IC50 value of 7 nM, this compound was 40-fold more active than pentamidine against the D6 strain. Compound **26** was not cytotoxic against *L. donovani* or the Vero cells. The anti-leishmanial activity was associated with increasing ring sizes, the highest activity being seen with the cyclooctyl analogue **36** (IC50 $\sim$ 2.27 μM). Cyclizing the amidine nitrogens into a 5-membered cyclic ring (**43** and **44**) resulted in enhanced potency against the parasites. The potency of compound **43** against the two strains of *P. falciparum* was increased by 2–3 fold, whereas the potency of compound **44** against *L. donovani* was increased by 52 fold, when compared with the parent compound **19**. Expanding the ring into a 6-membered ring, as in **45**, retained the anti-plasmodial activity, but removed anti-leishmanial activity. Bulky substituents attached to the nitrogens of the 5-membered saturated imidazolidinyl-containing compound **46** might hinder binding of the compound to the target site in the parasites resulting in lack of anti-parasitic activity. Replacing the basic terminal amidine functions with non-basic functions, as exemplified with compounds **47**–**52**, resulted in inactive compounds.

The above results indicate that there are differences in the structure–activity relationships of these compounds as anti-plasmodial or anti-leishmanial agents. This is not surprising since bisbenzamidines are known to have multiple targets depending on the organism (Brendle et al 2002; Roberts et al 2002; Bray et al 2003). Recently, it was reported that the anti-plasmodial action of pentamidine is based on its selective transport through a specific pore in the parasite and the subsequent binding to heme (ferriprotoporphyrin IX) and inhibition of hemozoin formation (Stead et al 2001). We have recently demonstrated that this class of compounds is capable of forming a complex with heme and may interfere with the formation of β-hematin in cell-free systems (Mayence et al 2004c). Furthermore, a good correlation between the binding of these compounds to heme and their in-vitro anti-plasmodial activity was observed (Mayence et al 2004a). These studies suggest that bisbenzamidines that are structurally related to pentamidine might exert their anti-plasmodial action in a manner similar to chloroquine. It is interesting to note that 35 out of 40 active compounds reported in this study inhibited both strains of *P. falciparum* with similar potency, whereas a 7-fold higher concentration of chloroquine was required to inhibit the W2 strain, based on the parasite LDH assay. This trend was found to be similar for a limited series of 12 active bisbenzamidines when they were tested against Haiti 135 (chloroquine-sensitive) and Indochina I (chloroquine-resistant) strains in which the anti-plasmodial activity was measured using the radiolabelled ^{3}H-hypoxanthine uptake assay (Mayence et al 2004a). The concentration of chloroquine required to inhibit 50% of the Indochina I strain was 33-fold higher than the Haiti 135 strain. Although several compounds, namely **26**, **33**, **36**–**38**, reported in this study were more inhibitory toward the D6 strain than the W2 strain, the remaining 35 active compounds showed similar potency for both strains. To the best of our knowledge, no cross-resistance between pentamidine with chloroquine or any other known antimalarials has been reported. These observations indicate that the anti-plasmodial activity of this class of compounds is probably not affected by the pfcrt-based efflux mechanism responsible for the chloroquine resistance in *P. falciparum* (Krogstad & De 1998).

Therefore, the bisbenzamidine class of compounds holds great promise for further development as useful antimalarials. The molecular target(s) in *L. donovani* is less clear. Bisbenzamidines are taken up into *Leishmania* cells via transporters (Basselin et al 1996; Kandpal et al 1996; Bray et al 2003). Earlier studies suggested that biologically active bisbenzamidines initially bind to the minor groove of DNA and this is followed by inhibition of some DNA-binding enzymes (e.g. topoisomerases) or simply direct inhibition of the transcription process (Bell et al 1993; Dykstra et al 1994; Henderson & Hurley 1995; Fitzgerald & Anderson 1999). However, we found that the anti-leishmanial activity of these compounds did not correlate with their DNA binding affinity (Mayence et al 2004b). This suggests that binding to DNA may not be the major mode of action. Other possible modes of action include inhibition of arginine transport into the *Leishmania* cells (Kandpal et al 1996), some toxic events in the mitochondria (Bray et al 2003; Cushion et al 2004) or inhibition of the biosynthesis or transport of polyamines in *Leishmania* (Reguera et al 1994; Calonge et al 1996; Basselin et al 1996, 1997, 2000). Pentamidine has been shown to inhibit the transport of putrescine and spermidine (Basselin et al 1996, 2000) in *Leishmania*, thereby altering the balance between the extracellular and intracellular polyamine concentrations. The activity of ornithine decarboxylase was found to be decreased in *Leishmania* cells treated with pentamidine (Basselin et al 1997). However, *S*-adenosylmethionine decarboxylase, another major enzyme in the polyamine biosynthetic pathway, was not the primary intracellular target for pentamidine (Roberts et al 2002). These observations suggest that the bisbenzamidine class of drugs, including pentamidine, probably exert their toxic effects in *L. donovani* through a multitude of potential targets.

Conclusions

Bisbenzamidines have not been extensively studied as potential antimalarials. However, pentamidine is widely used in the treatment of leishmaniasis despite its poor potency and serious toxicity. In this study we have identified several bisbenzamidines that have greater potency than chloroquine or pentamidine as potential antimalarial or anti-leishmanial agents. The

nature of the central linker between the bisbenzamidine moieties, as well as the type of substituents attached to the terminal amidinium nitrogens, can readily modulate the anti-parasitic activity of this class of compounds. Piperazine-linked bisbenzamidines emerged as a promising class of anti-parasitic agent because of their potent effects against both the D6 (chloroquine-sensitive) and W2 (chloroquine-resistant) strains of *P. falciparum*, as well as *L. donovani*. These compounds were generally more effective against *P. falciparum* than *L. donovani*. Seven piperazine-linked bisbenzamidines substituted at the amidinium nitrogens with a linear alkyl group of 3–6 carbons (**22**, **25**, **27**, **31**) or cycloalkyl group of 4, 6 or 7 carbons (**26**, **32**, **34**) were more potent than chloroquine or pentamidine as anti-plasmodial agents. The replacement of the terminal amidinium groups with benzimidazole groups resulted in the potent anti-leishmanial agent **44**. These highly active compounds were generally not toxic to mammalian Vero cells at the concentrations tested. Based on the results of this study, further in-vivo evaluation of the bisbenzamidines as potential antimalarials and anti-leishmanial agents is warranted.

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