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Azaterphenyl diamidines as antileishmanial agents

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Abstract—Eighteen diamidino azaterphenyls and analogues were evaluated as anti-leishmanials; nine of the compounds gave IC_{50} values less than 1 μM, five exhibited values less than 0.40 μM, and two gave values less than 0.10 μM in a *Leishmania donovani* axenic amastigote assay. The activity of the diamidines strongly depends on the ring *N*-atom location relative to the amidine groups and correlates with DNA affinity. Transmission electron microscopy studies showed a dramatic dilation of the mitochondrion and evidence of disintegration of the kinetoplast of the amastigotes.

Over 20 species of the *Leishmania* parasite contribute to human leishmanial disease.[1] The disease is transmitted by the bite of more than 30 different species of sand files.[1] The *Leishmania* parasite is broadly distributed in humans and animals, and recent estimates suggest that two million new human cases occur annually.[2] The disease is found in the Far East, southern Europe, and in both Americas including the United States.[3] The clinical manifestations of the disease can be generally classified into three forms: cutaneous, mucosal, and visceral.[4] The drugs currently in use, pentavalent antimonials, paromomycin, amphotericin, miltefosine, and pentamidine, all suffer from one or more of the following deficiencies: significant toxicity, variable efficacy, lack of oral bioavailability, extensive courses of parenteral administration, and problems of cost and supply.[1,2,5] Moreover, there is increasing evidence that the frequency and extent of use of these drugs is leading to selection of drug-resistant parasites.[6] The need for more effective drugs with desirable pharmacokinetic properties to treat human leishmanial disease is evident.[2]

Dicationic molecules, of which pentamidine (**I**; Fig. 1) is the best known member of the diamidine class, were first reported to have significant antiprotozoan activity in the 1930s.[7] Despite numerous studies of various structural classes of dications, pentamidine is the only compound which has seen significant human use. The primary use of pentamidine against human leishmanial disease is to treat antimony-resistant leishmaniasis.[1] The diamidine furamidine (**IIa**) and its analogues have significant activity against various parasites including *Leishmania*.[7a] Pafuramidine (**IIb**), an orally effective prodrug of furamidine, is currently in Phase III trials against both Human African Trypanosomiasis (HAT) and *Pneumocystis carinii* pneumonia.[8] Compound **I** causes swelling of the mitochondrion and destruction of mitochondrial (kinetoplast) DNA in both African trypanosomes[9] and *Leishmania*,[10] and **I** also decreases the mitochondrial membrane potential in *Leishmania donovani* promastigotes.[11] It has also been suggested that these dicationic molecules, including pentamidine, act by binding in the minor groove of DNA at AT rich sites.[7a] It has been hypothesized, based on considerable evidence, that the minor groove binding leads to inhibition of DNA dependent enzymes or possibly direct inhibition of transcription.[7a,12] Although the lethal events have not yet been determined, mitochondrial disruption and interference with kinetoplast DNA processing may together result in the death of diamidine-treated parasites.

Keywords: Leishmania; Diamidines; Azaterphenyl; DNA binding; Suzuki coupling.
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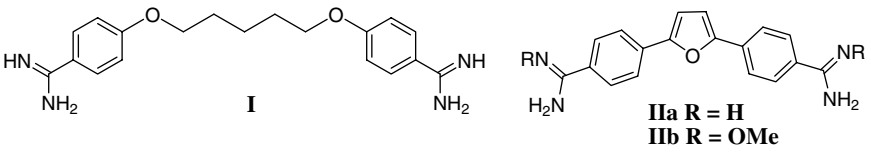
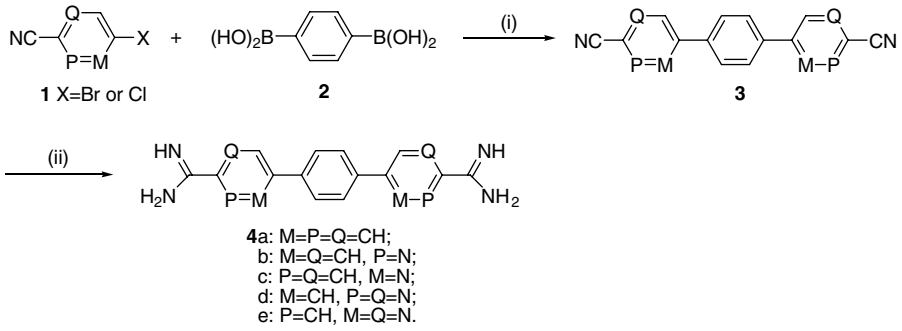


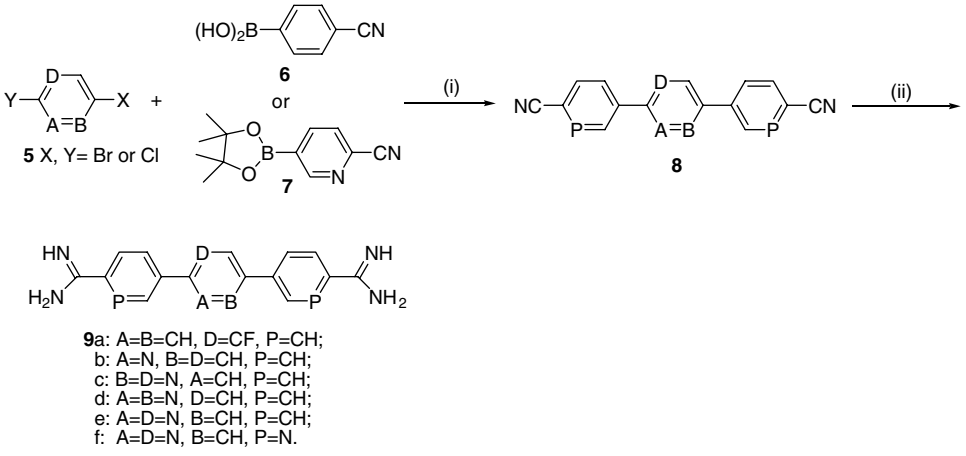
Figure 1.

Since the pioneering work of Dickerson and coworkers, the design of minor groove binders has focused on crescent shaped molecules whose molecular shape complements the DNA minor groove.[13] More recently, linear dications have been discovered which exhibit high minor groove binding affinity and show excellent antiparasitic activity.[14] X-ray structures of co-crystals of linear dications and oligonuleotides revealed that a strategically located water molecule in effect provides the curvature needed to complement the groove.[14] Based on the recognition of this new binding mode we recently prepared a series of linear terphenyl diamidines and found that they were highly active in vitro against African trypanosomes and against malaria.[15] More recently, we found that the parent molecule in this series, 4,4′-diamidinoterphenyl **4a**, was quite active against *Leishmania donovoni* (*L. d.*) in vitro. This communication reports the synthesis of new aza-terphenyl dications, their DNA affinity and evaluation against *Leishmania.*

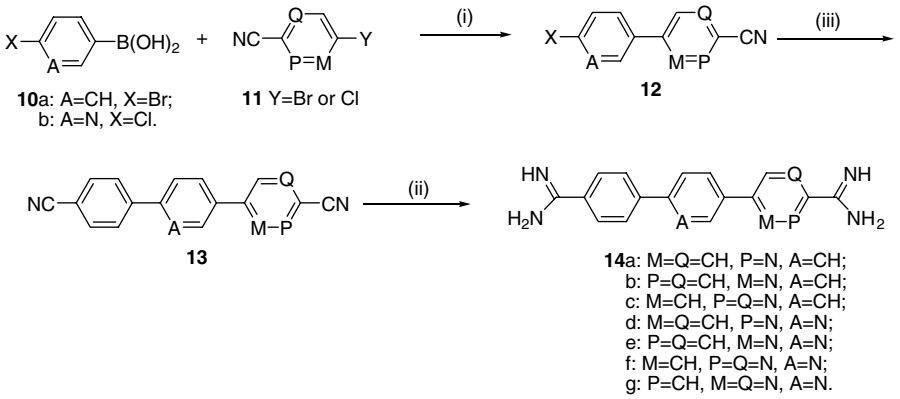
The syntheses of the azaterphenyl diamidines were achieved by Suzuki coupling of the appropriate aryl halides with the corresponding aryl boronic acids or esters to form the teraryl bis-nitriles.[15] The bis-nitriles were converted to the diamidines by the action of $LiN(TMS)_2$ followed by hydrolysis. Scheme 1 outlines the approach used to prepare azaterphenyl analogues with nitrogen atom(s) in one or both of the terminal aryl rings. Scheme 2 shows the synthesis of diamidines with nitrogen atom(s) in the central aryl ring as well as those in both the terminal aryl rings. Finally, Scheme 3 provides our approach to preparation of the compounds with nitrogen atom(s) in the central ring and one terminal aryl ring.



Scheme 1. Reagents and conditions: (i) $Pd(PPh_3)_4$, Na_2CO_3, toluene, 80 °C; (ii) a—$LiN(TMS)_2$, THF, rt, overnight; b—HCl (gas), ethanol, rt, overnight.



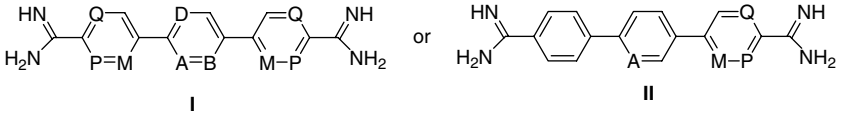
Scheme 2. Reagents and conditions: (i) $Pd(PPh_3)_4$, Na_2CO_3, toluene, 80 °C; (ii) a—$LiN(TMS)_2$, THF, rt, overnight; b—HCl (gas), ethanol, rt, overnight.



Scheme 3. Reagents and conditions: (i) $Pd(PPh_3)_4$, Na_2CO_3, toluene, 80 °C; (ii) a—$LiN(TMS)_2$, THF, rt, overnight; b—HCl (gas), ethanol, rt, overnight; (iii) —4-cyanophenylboronic acid, $Pd(PPh_3)_4$, Na_2CO_3, toluene, 80 °C.

Table 1 contains the results obtained for 18 azaterphenyl diamidines and closely related analogues using an axenic assay with *L. donovani* amastigote-like parasites. Nine of the compounds gave IC_{50}values less than 1 μM, five of those exhibited IC_{50} values less than 0.40 μM, and two of those gave values less than 0.10 μM. These results represent a marked improvement in activity over that found in our previous study[16] of 58 compounds from diverse classes of diamidines which yielded only two compounds with IC_{50} values less than 1 μM with none in the sub 0.40 μM range. The number and location of nitrogen atoms has a significant impact on the anti-leishmanial activity of this linear rigid-rod system. Compounds **14a** and **4b** in which a nitrogen atom has been placed *ortho* to one and both of the amidine groups, respectively, in the parent terphenyl system **4a** show a three- and fourfold increase in activity over the parent and represent the two most active compounds found in this study with IC_{50} values of 0.084 and 0.063 μM, respectively. The two isomeric compounds **14b** and **4c**, in which the nitrogen atom is *meta* to the amidine, show a 16- and a more than 1500-fold reduction in activity compared to **14a** and **4b**. Interestingly, introduction of two nitrogen atoms *ortho* to one amidine, **14c**, results in a modest reduction of activity compared to **4b** (IC_{50} = 0.20 μM), however if two nitrogen atoms are introduced *ortho* to both amidine units, **4d**, a significant reduction in activity is observed (IC_{50} = 1.70 μM). Introduction of one or more nitrogen atoms in the central ring (**9b**–**9e**) results in a two- to fourfold reduction in

Table 1. DNA affinities and in vitro antileishmanial activity for azaterphenyl diamidines



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Code	Aryl type	A	B	D	M	P	Q	ΔT_m[a] (°C)	*L.d.*[b] IC_{50} (μM)	Cytotoxicity[c] IC_{50} (μM)
4a	I	CH	CH	CH	CH	CH	CH	17.1	0.27	22
4b	I	CH	CH	CH	CH	N	CH	17	0.063	1.2
4c	I	CH	CH	CH	N	CH	CH	9.2	>100	26
4d	I	CH	CH	CH	CH	N	N	12.8	1.70	2.2
4e	I	CH	CH	CH	N	CH	N	3.9	$>10^5$	47
9a	I	CH	CH	CF	CH	CH	CH	15.1	0.34	6.4
9b	I	N	CH	CH	CH	CH	CH	18.7	0.82	50
9c	I	CH	N	N	CH	CH	CH	8.0	0.56	43
9d	I	N	N	CH	CH	CH	CH	16.9	0.72	31
9e	I	N	CH	N	CH	CH	CH	8.1	1.10	41
9f	I	N	CH	N	CH	N	CH	6.8	1.80	5.3
14a	II	CH	/	/	CH	N	CH	19.5	0.084	2.8
14b	II	CH	/	/	N	CH	CH	11.7	1.40	27
14c	II	CH	/	/	CH	N	N	16.0	0.20	4.7
14d	II	N	/	/	CH	N	CH	15.2	0.51	20
14e	II	N	/	/	N	CH	CH	13.8	6.8	28
14f	II	N	/	/	CH	N	N	14.9	2.6	6.4
14g	II	N	/	/	N	CH	N	12.1	16.0	93

[a] Increase in thermal melting of poly(dA-dT)$_2$; see Ref. 15.
[b] See Refs. 16 and 18; IC_{50} values are means of at least two independent assays.
[c] Cytotoxicity was evaluated using cultured L6 rat myoblast cells; see Ref. 20.

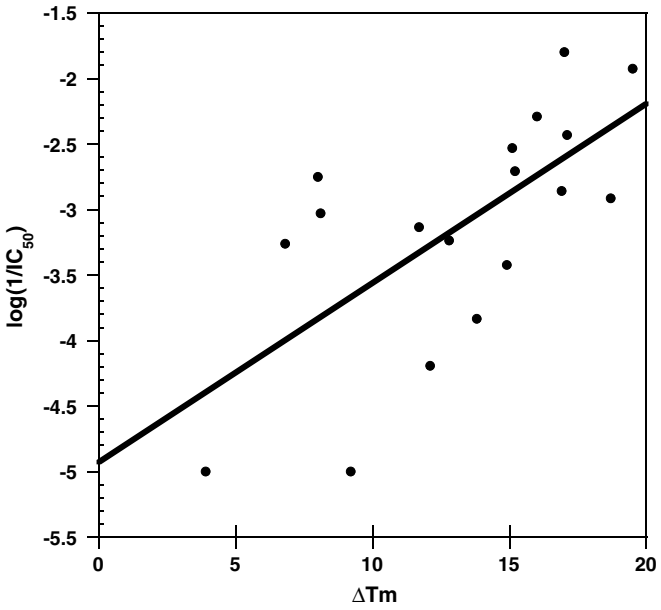

Figure 2. A plot of biological activity, on an increasing scale of $\log(1/IC_{50})$, versus DNA binding affinity, plotted as ΔT_m, is shown for all of the compounds in Table 1.

activity compared to the parent terphenyl **4a**. A diminution in activity with central ring nitrogen atom substitution also occurs when nitrogen atoms are also present either *ortho* or *meta* to the amidine functions (cf. **9f**, **14d**–**14f**). Hence, we have found a diamidine system which is highly active and shows a consistent and distinctive structure activity relationship in the *L. donovani* axenic amastigote assay. Due to their high activity in the axenic amastigote assay, evaluation of the parent molecule **4a** and the most active compound **4b** using a *Leishmania* infected macrophage assay was warranted in order to have a better predictor of the in vivo potential of this class of compounds. This assay was conducted using *L. amazonensis* parasites bearing the stably integrated β-lactamase reporter gene according to the method of Buckner and Wilson[17] with the modification that J774 murine cultured macrophages were used as the host cell rather than murine peritoneal macrophages. Neither **4a** nor **4b** was active in this assay at concentrations below 5 μM, and higher concentrations of both compounds were toxic to macrophages. In contrast, the antileishmanial drug amphotericin B possessed an IC_{50} value of 0.046 ± 0.007 μM in this assay (mean ± standard error of 13 independent measurements), while the reversed amidine 2,5-bis[3-methoxy-4-(2-pyridylimino)aminophenyl]furan[18] possessed an IC_{50} value of 0.22 ± 0.05 μM in this assay (mean ± range of two independent experiments). In contrast to reversed amidines, it would appear that the entry of typical amidines into the parasitopherous vacuole containing intracellular *Leishmania* parasites is poor. Thus, the challenge remains to develop macrophage delivery methods for the azaterphenyl diamidines.

To qualitatively estimate the DNA binding affinities of these linear diamidines, we measured melting temperatures for their complexes with poly(dA-dT) and Table 1 reports the ΔT_m values (difference in melting temperature of free DNA in solution and that of the diamidine-DNA complex). The ΔT_m range from high values of 17–19 °C to low ones of 4–6 °C. Although the high values represent strong DNA affinities for these linear mole-

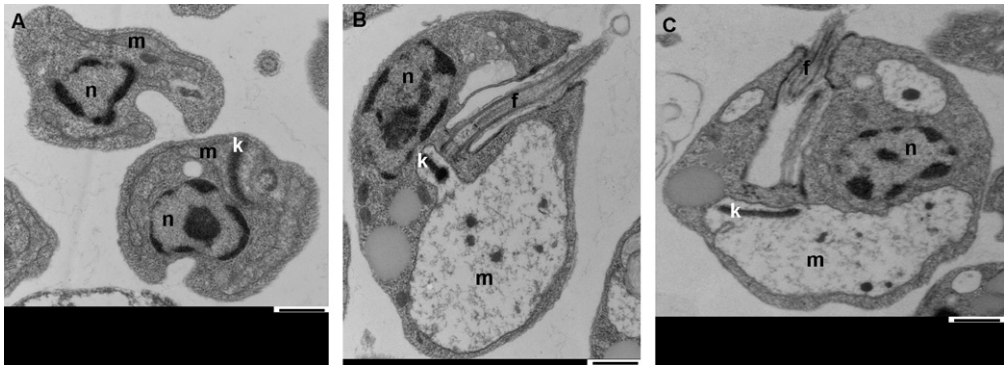

Figure 3. Ultrastructural effects of **4a** on *L. donovani* axenic amastigotes. Parasites were incubated in the absence (control, panel A) or the presence (panels B and C) of 0.68 μM **4a** and were examined by transmission electron microscopy as described in the text. f, flagellum; k, kinetoplast; m, mitochondrion; n, nucleus. The scale is the same for all of the micrographs (bar = 500 nm).

cules, they are somewhat lower than that seen for crescent shape ones which complement the DNA minor groove such as furamidine (ΔT_m = 25 °C). In general, the compounds which exhibit the higher ΔT_m values show the higher antileishmanial activity and the weaker binding compounds show low activity. Interestingly, when the ΔT_m values are plotted versus the *L. donovani* IC_{50} values a rough correlation is observed (Fig. 2). For example, all compounds with ΔT_m values above 15 °C have IC_{50} values lower than 1 μM while all except one compound with ΔT_m values below 15 have IC_{50} values above 1 μM. The compound with the highest ΔT_m has an activity below 0.10 μM, while the compound with the lowest ΔT_m has an activity of greater than 100 μM. These results are consistent with kinetoplast DNA binding playing a significant role in the antileishmanial activity of these compounds.

In an attempt to gain insight into the antileishmanial mechanism of action of these linear dications, *L. donovani* axenic amastigotes were incubated in the presence or absence of 0.68 μM **4a** (2.5× the IC_{50} value) for 24 h at 37 °C, fixed, stained, and examined by transmission electron microscopy (TEM) by methods described previously.[19] In contrast to controls (Fig. 3A), the majority of parasites exposed to **4a** displayed a dramatic dilation of the mitochondrion (m) and evidence of a disintegrating kinetoplast (k) as shown in Figure 3B and C. These observations are consistent with the ultrastructural changes observed in *Leishmania amazonensis* promastigotes exposed to pentamidine.[10]

The azaterphenyl diamidines were found to have potent activity against *L. d.* axenic amastigotes. A marked dependence on location of the ring *N*-atom(s) relative to the amidine groups was noted. A general correlation between ΔT_m and IC_{50} values was observed. TEM studies showed a dramatic dilation of the mitochondrion and evidence of disintegration of the kinetoplast of the amastigotes which is consistent with targeting DNA. Unfortunately, both **4a** and **4b** were not effective in a *Leishmania* infected macrophage assay suggesting that these diamidines are not delivered well to the parasites within the macrophage host cell. Further studies focusing on macrophage delivery are necessary to take advantage of the potent intrinsic antileishmanial activity of these azaterphenyl diamidines.

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