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Structure-Activity Relationships of Pentamidine Analogs against *Giardia lamblia* and Correlation of Antigiardial Activity with DNA-Binding Affinity

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1,5-Di(4-amidinophenoxy)pentane (pentamidine) and 38 analogs of pentamidine were screened for in vitro activity against the enteric protozoan *Giardia lamblia* WB (ATCC 30957). All compounds were active against *G. lamblia* as measured by a [*methyl*-^{3}H]thymidine incorporation assay. Antigiardial activity varied widely, with 50% inhibitory concentrations (IC_{50}s) ranging from 0.51 ± 0.13 μM (mean ± standard deviation) for the most active compound to over 100.0 μM for the least active compounds. The IC_{50} of the most potent antigiardial agent, 1,3-di(4-amidino-2-methoxyphenoxy)propane compared favorably with the IC_{50}s of the compounds currently used to treat giardiasis, i.e., furazolidone (1.0 ± 0.03 μM), metronidazole (2.1 ± 0.80 μM), quinacrine HCl (0.03 ± 0.02 μM), and tinidazole (0.78 ± 0.48 μM). A mode of antigiardial activity for these compounds was suggested by the correlation observed between antigiardial activity and the binding of the compounds to calf thymus DNA and poly(dA) · poly(dT).

Giardia lamblia (also known as *G. intestinalis*) is an important cause of acute and chronic gastrointestinal disease throughout the world and has been identified as the etiologic agent in numerous waterborne outbreaks of diarrheal disease. Although *G. lamblia* is among the most prevalent enteric protozoal infections in humans, it is relatively recently that improvements in the in vitro cultivation of this organism have allowed reliable, reproducible tests to assess the in vitro activity of therapeutic agents against *G. lamblia* (3, 19, 21). The agents currently used to treat giardiasis, furazolidone, metronidazole, quinacrine HCl, and tinidazole, were developed for treatment of other infections and were empirically discovered to be effective against *G. lamblia* (3, 21). Treatment failures and undesirable side effects have been reported following the use of all four of these compounds (6, 29, 45). Thus, there is a need for more effective and less toxic antigiardial agents. In this report, we show that *G. lamblia* is susceptible to dicationic molecules and that the antigiardial activities of some of the more potent pentamidine analogs compare favorably with those of agents presently used to treat this infection.

Aromatic diamidines have been used clinically since their antiprotozoal activity was first noted in the 1930s by investigators searching for agents with activity against African trypanosomiasis (24). Several parasitic organisms have been reported to be susceptible to aromatic diamidines, including *Acanthamoeba* sp. (1, 26), *Babesia* sp. (13, 31), *Crithidia fasciculata* (16, 44), *Leishmania* sp. (2, 25, 36, 39), *Plasmodium* sp. (2, 8, 14), *Pneumocystis carinii* (20, 22, 39, 41, 42), and *Trypanosoma* sp. (17, 18, 30, 39). In addition to antiparasitic activity, these compounds have also been reported to exhibit antibacterial (11), antiviral (43), antifungal (11), and antitumor (27) activities. Traditionally, aromatic diamidines, particularly pentamidine, have been used to treat African trypanosomiasis (17, 18, 30, 39), antimony-resistant leishmaniasis (4, 39), and more recently, *P. carinii* pneumonia (10, 20, 35, 39). The recent increase in life-threatening *P. carinii* pneumonia in patients with the acquired immune deficiency syndrome has resulted in a revived interest in pentamidine and related derivatives. Although pentamidine has been used for decades, little is known about its mechanism(s) of action.

The broad spectrum of activity exhibited by aromatic diamidines and the strongly basic nature of the molecules have hindered efforts to determine the mechanism(s) by which they exert their activity. Many different mechanisms have been proposed to explain the antimicrobial activities of pentamidine and related compounds. A single mechanism of action may not be responsible for the action of aromatic diamidines against microorganisms. Additionally, the mode(s) of action of this class of compounds may differ from one organism to another. It has been suggested that these compounds interfere with nucleic acid synthesis and/or metabolism (16, 40, 44). Pentamidine and other diamidines, such as 4′,6-diamidino-2-phenylindole and berenil, have been shown to bind to DNA and exhibit a preference for binding to A+T-rich regions in the minor groove of B-DNA (12, 28, 32, 34, 38). To assess their range of activity further and better understand the mechanism(s) of antimicrobial action, we examined the structure-activity relationships of pentamidine and 38 analogs of pentamidine in an in vitro assay against *G. lamblia* WB (ATCC 30957) and correlated the antigiardial activities of these dicationic molecules with their DNA-binding affinities.

MATERIALS AND METHODS

Chemotherapeutic agents. Pentamidine and the 38 analogs of pentamidine used in this study were synthesized as mono- and dihydrochloride salts in the laboratories of Richard R. Tidwell, Department of Pathology, School of Medicine,

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University of North Carolina at Chapel Hill. Syntheses were carried out by previously described methods (41, 42). High-performance liquid chromatography, elemental analyses and nuclear magnetic resonance spectroscopy were used to determine the purity of the compounds. Quinacrine HCl, furazolidone, metronidazole, and tinidazole were purchased from Sigma (St. Louis, Mo.).

Axenic culture of *G. lamblia* trophozoites. *G. lamblia* WB (ATCC 30957) was grown in filter-sterilized TYI-S-33 medium supplemented with bile (23) and containing 10% heat-inactivated fetal bovine serum, 10 μg of ampicillin (Sigma) per ml, and 10 μg of gentamicin sulfate (Sigma) per ml. Stock cultures of trophozoites were grown in screw-cap borosilicate glass tubes (13 by 100 mm) at 37°C. Organisms were subcultured every 72 h by chilling the culture tube in an ice water bath for 5 min. The trophozoites were dislodged from the glass by inverting the chilled tube vigorously. The number of organisms per milliliter was determined, and 5×10^4 organisms from a logarithmically growing culture were transferred into fresh medium.

Microculture and parasite growth. The relationship between uptake of [*methyl*-^{3}H]thymidine and parasite growth was examined to validate the radiometric assay (3, 19). *G. lamblia* trophozoites were grown to early log phase in filter-sterilized TYI-S-33 medium supplemented with bile and containing 10% fetal bovine serum. The used medium was aspirated from the tube and replaced with assay medium consisting of TYI-S-33 medium with bile, which was further modified by reducing the amounts of fetal bovine serum, Trypticase peptone (Becton Dickinson Microbiology Systems, Cockeysville, Md.), and yeast extract by 50% to reduce nonspecific binding of strongly polar compounds to medium components. The tubes were chilled, and the numbers of organisms per milliliter were determined. Logarithmically growing trophozoites (2.5×10^4) in assay medium were placed into the wells of 96-well microtiter plates. To one-half of the wells was added 1.5 μCi of [*methyl*-^{3}H]thymidine per well (1 to 10 Ci/mmol). The plates were gassed with nitrogen and incubated at 37°C for 4 to 72 h. The growth of the trophozoites in the microtiter plates was determined by microscopic counts by using a hemacytometer to determine the number of trophozoites per milliliter. Organisms incubated in the presence of [*methyl*-^{3}H]thymidine were harvested with a multimash-type cell harvester onto glass microfiber paper by vigorous washing with ice-cold deionized water. Washed and dried filters were placed into scintillation vials and counted to determine [*methyl*-^{3}H]thymidine uptake.

Drug susceptibility testing. Serial dilutions of dicationic compounds, furazolidone, metronidazole, quinacrine HCl, and tinidazole in assay medium were prepared in duplicate rows of 96-well microtiter plates. The plates were gassed with nitrogen and incubated at 37°C. After 24 h, [*methyl*-^{3}H] thymidine was added to yield 1.5 μCi per well. At 42 h, the cells were harvested and [*methyl*-^{3}H]thymidine uptake was determined. The antigiardial activities of metronidazole, pentamidine (compound 4), and 1,3-di(4-amidino-2-methoxyphenoxy)propane (DAMP) (compound 16) were also examined by visual microscopic counts to confirm the validity of the use of a radiometric assay to screen compounds for antigiardial activity. Four sets of serial dilutions were made for each compound in experiments comparing cell numbers with [*methyl*-^{3}H]thymidine uptake.

DNA-binding assay. Binding of pentamidine analogs to calf thymus DNA (Worthington Biochemicals, Freehold, N.J.), poly(dA) · poly(dT) (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and poly(dG-dC) · poly(dG-dC) (Pharmacia-LKB Biotechnology, Inc., Piscataway, N.J.) was evaluated by using a thermal denaturation assay which measures the temperature at which drug-DNA complexes denature. This assay allows calculation of the change in the midpoint (ΔT_m) of the thermal denaturation curve of calf thymus DNA, poly(dA) · poly(dT), and poly(dG-dC) · poly (dG-dC). The binding constant of the compounds under the same conditions is approximately proportional to the magnitude of ΔT_m (°C). This procedure has been described previously in detail (5).

Analysis of data. Data on [*methyl*-^{3}H]thymidine uptake were fitted to a logistic-logarithmic concentration-response function by a nonlinear regression method by using software developed for use with an IBM-AT computer, and drug concentrations required to inhibit 50% incorporation of [*methyl*-^{3}H]thymidine (IC_{50}s) were determined (9, 37). Statistical analysis of data on in vitro antigiardial activity was performed on data from two separate determinations, each of which consisted of duplicate serial dilutions of each compound, to obtain the mean ± the standard deviation. Because of the large number of compounds presented, it was impossible to screen all of the compounds in duplicate at a single time. The results, measured in counts per minute, are therefore interpreted as percent control. A coefficient of determination (r^2) was generated for each concentration-response curve used to determine the IC_{50}. Data were acceptable if r^2 was ≥0.90. Pentamidine was used as a control in each of the six experiments to confirm the reproducibility of the assay.

The data concerning the DNA binding of the compounds also represent the mean ± the standard deviation from two to four separate determinations for each compound. The multiple regression analyses to determine the correlations between DNA-binding affinity and antigiardial activity used these mean values. Simple linear regression analysis was used to examine the relationship between trophozoite growth and [*methyl*-^{3}H]thymidine uptake in the absence and presence of test compounds. Statistical analyses were performed by using the StatView 512+ software package (Brainpower, Inc., Calabasas, Calif.) on a Macintosh II microcomputer. The ClogP values were obtained by using MedChem Software 3.5 (Daylight Chemical Information System, New Orleans, La.).

RESULTS

Parasite growth. [*methyl*-^{3}H]thymidine uptake by *G. lamblia* trophozoites grown in microtiter plates correlated directly with trophozoite growth determined microscopically (Fig. 1). A highly significant positive correlation ($r^2 = 0.98$, $P = 0.0001$) was confirmed by linear regression analysis.

Drug susceptibility. The validity of the use of a radiometric assay to screen compounds for antigiardial activity was confirmed by comparing the growth of *G. lamblia* trophozoites as determined by microscopic counts and [*methyl*-^{3}H] thymidine uptake in the presence of test agents (Fig. 2). Linear regression analyses reveal significant correlations between trophozoite growth and [*methyl*-^{3}H]thymidine uptake in the presence of metronidazole (Fig. 2A) ($r^2 = 0.94$, $P = 0.0001$), pentamidine (Fig. 2B) ($r^2 = 0.91$, $P = 0.0001$), and 1,3-di(4-amidino-2-methoxyphenoxy)propane (DAMP) (Fig. 2C) ($r^2 = 0.82$, $P = 0.0003$).

The in vitro antigiardial activities of compounds currently used to treat giardiasis, i.e., furazolidone, metronidazole, quinacrine HCl, and tinidazole, were compared with those of

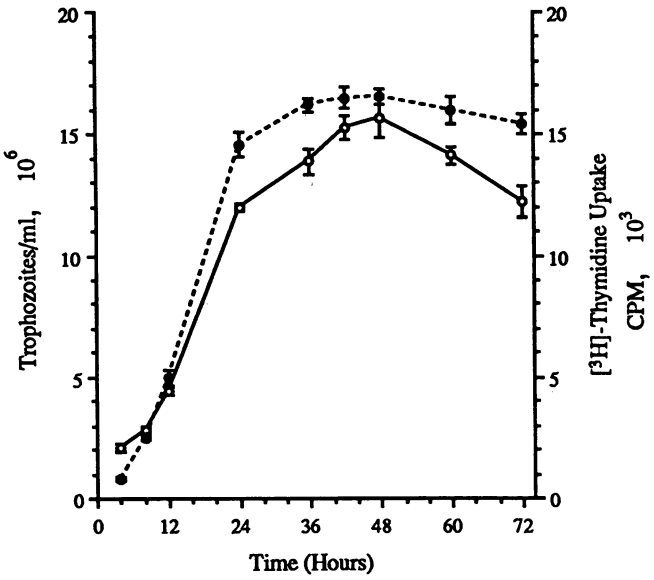


FIG. 1. Graphic representation of the relationship between *G. lamblia* growth as determined by visual counting with a hemacytometer (○) and [^{3}H]thymidine uptake (●). Each point represents the mean of six replicates ± the standard deviation. A highly significant positive correlation ($r^2 = 0.98$, $P = 0.0001$) was confirmed by linear regression.

the most potent dicationic agents identified in this study (Table 1). The IC_{50}s of the most potent dicationic agents compared favorably with those of furazolidone (1.0 ± 0.03 μM) (mean ± standard deviation), metronidazole (2.1 ± 0.80 μM), quinacrine HCl (0.03 ± 0.02 μM), and tinidazole (0.78 ± 0.48 μM) (Table 1).

Pentamidine and 38 analogs of pentamidine were tested for activity against *G. lamblia* and affinity for calf thymus DNA, poly(dA) · poly(dT), and poly(dG-dC) · poly(dG-dC). These data are arranged in tabular form (Tables 2 to 6) on the basis of homogeneous structural modifications. The structure of pentamidine was altered by (i) variation of the length of the alkyl chain connecting the amidinophenoxy moieties from two to six carbons (Table 2), (ii) alteration of the position of the amidino group on the aromatic rings from a position *para* to the ether bridge to the *meta* position (Table 3), (iii) placement of substituent groups on the aromatic rings in positions *ortho* to the ether linkage (Table 4), (iv) isosteric replacement of the ether oxygens with nitrogens (Table 5), and (v) replacement of the amidino groups with imidazolino moieties (Table 6).

Pentamidine and each of the 38 analogs of pentamidine tested exhibited in vitro activity against *G. lamblia* WB. The antigiardial activities of these dicationic molecules varied widely, with IC_{50}s ranging from 0.51 ± 0.13 to over 100 μM for some of the less potent compounds. The parent compound, pentamidine (compound 4), was used as a control in each experiment to ensure reproducibility and was found to be very active against *G. lamblia*, having an IC_{50} of 8.5 ± 2.7 μM (n = 6). Propamidine (compound 2), the three-carbon analog of pentamidine, had an IC_{50} of 1.7 ± 0.37 μM and was one of the three most potent compounds tested in this study. The remaining two compounds which exhibited the greatest antigiardial activity also had alkyl chains of three carbons. The most potent dicationic molecule identified in this study was DAMP (compound 16), in which methoxy groups have been placed on the aromatic rings in positions *ortho* to the ether linkage. Likewise, the three-carbon methoxy-substituted compound in which the amidino groups were replaced with imidazolino moieties, 1,3-di(4-imidazolino-2-methoxyphenoxy)propane (DIMP) (compound 35), exhibited excellent activity against *G. lamblia*. The methoxy-substituted compounds DAMP and DIMP, had IC_{50}s of 0.51 ± 0.13 and 1.9 ± 0.03 μM, respectively.

Within each homogeneous structural group, compounds with an alkyl chain length of three carbons were generally the most potent, followed by those with chain lengths of five and six carbons. Each of the three most potent antigiardial compounds had three methylenes in their alkyl chains. The two- and four-carbon compounds generally exhibit decreased potency relative to the corresponding three-, five-, and six-carbon compounds. The association between antigiardial activity and alkyl chain length is illustrated in Table 2, in which the length of the alkyl chain separating the amidinophenoxy moieties varies from two to six methylenes. Propamidine, the three-carbon compound (compound 2), was approximately threefold more active than pentamidine

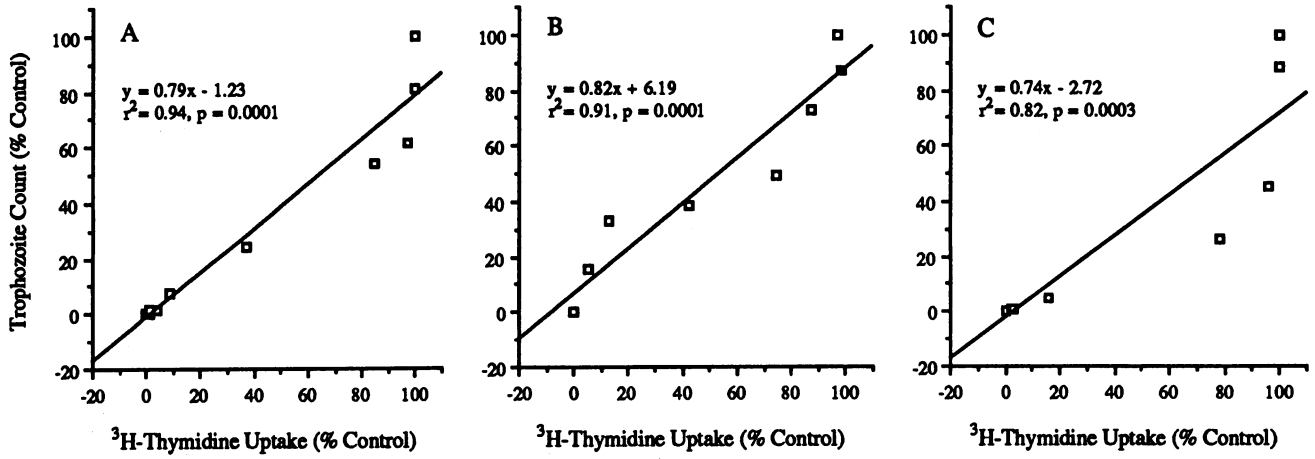
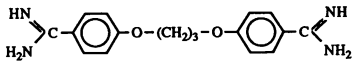
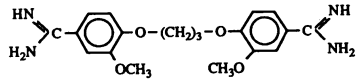
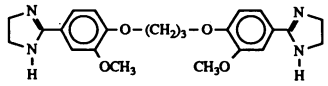
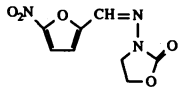
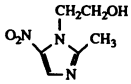
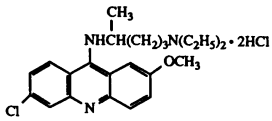
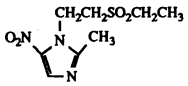


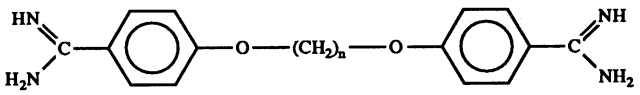
FIG. 2. Graphic representation of the relationship between *G. lamblia* growth and [^{3}H]thymidine uptake in the presence of test agents metronidazole (A), pentamidine (B), and DAMP (C).

TABLE 1. Antigiardial activities of compounds in clinical use versus those of the most potent dicationic compounds

Compound	Structure	Mean IC_{50} (μM) ± SD[a] for *G. lamblia*
Propamidine		1.7 ± 0.37
DAMP		0.51 ± 0.13
DIMP		1.9 ± 0.03
Furazolidone		1.0 ± 0.03
Metronidazole		2.1 ± 0.80
Quinacrine HCl		0.03 ± 0.02
Tinidazole		0.78 ± 0.48

[a] Values are the means for two separate determinations for each compound (two serial dilutions per determination) ± the standard deviations.

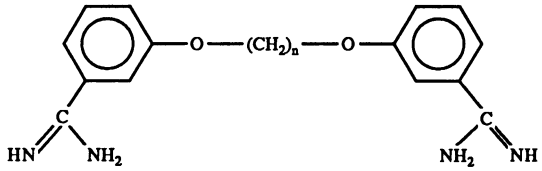
TABLE 2. Biological activities of α,ω-di(4-amidinophenoxy)alkanes



Compound no.	n	Mean IC_{50} (μM) ± SD[a] for *G. lamblia*	Mean DNA binding (ΔT_m [°C]) ± SD[b]			ClogP
			Calf thymus DNA	Poly(dA) · poly(dT)	Poly(dG-dC) · poly(dG-dC)	
1	2	280 ± 3.3	5.4 ± 0.2	7.5 ± 2.6	1.3 ± 0.03	3.3
2	3	1.7 ± 0.37	14.7 ± 1.7	32.0 ± 0.7	4.5 ± 0.2	3.5
3	4	16 ± 0.45	8.3 ± 2.5	17.9 ± 1.6	3.1 ± 0.4	4.0
4	5	8.5 ± 2.7[c]	10.7 ± 0.6	22.9 ± 0.7	3.2 ± 0.5	4.6
5	6	5.5 ± 1.4	9.1 ± 1.8	19.0 ± 0.6	2.9 ± 0.4	5.1

[a] Values are the means for two separate determinations for each compound (two serial dilutions per determination) ± the standard deviations.
[b] Values are the means for two to four separate determinations ± the standard deviations (5a, 41).
[c] Value is the mean for six separate determinations for pentamidine (two serial dilutions per determination) ± the standard deviation.

TABLE 3. Biological activities of α,ω-di(3-amidinophenoxy)alkanes



Compound no.	n	Mean IC_{50} (μM) ± SE[a] for *G. lamblia*	Mean DNA binding (ΔT_m [°C]) ± SD[b]			ClogP
			Calf thymus DNA	Poly(dA) · poly(dT)	Poly(dG-dC) · poly(dG-dC)	
6	3	99 ± 0.66	7.6 ± 1.1	8.5 ± 0.5	3.0 ± 0.4	3.5
7	4	21 ± 5.2	8.1 ± 0.6	15.2 ± 1.4	2.6 ± 0.2	4.0
8	5	11 ± 2.6	7.6 ± 0.6	7.6 ± 1.5	2.2 ± 0.2	4.6
9	6	6.7 ± 0.95	7.3 ± 0.5	8.1 ± 0.6	2.5 ± 0.5	5.1

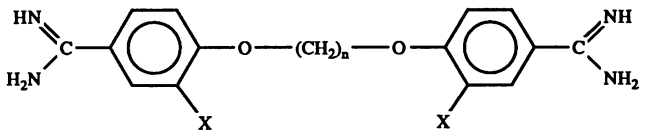
[a] Values are the means for two separate determinations for each compound (two serial dilutions per determination) ± the standard deviations.
[b] Values are the means for two to four separate determinations ± the standard deviations (5a, 41).

(compound 4). Hexamidine (compound 5), the six-carbon analog, was similar in activity to pentamidine (compound 4), while the two (compound 1)- and four (compound 3)-carbon compounds were much less potent. The *meta*-amidinophenoxy compounds (Table 3) were notable exceptions to this generalization.

The effect of shifting the amidino group on the aromatic ring from the position *para* to the ether bridge to a *meta* position is shown in Table 3. In this group of compounds, improved potency was observed with addition of methylenes to the alkyl bridges separating the amidino-phenoxy moieties. The three-carbon alkyl chain analog (compound 6) exhibited the least antigiardial activity, with an IC_{50} of 99 ± 0.66 μM, over 50-fold less active than propamidine (compound 2), the corresponding *para*-amidinophenoxy compound. The six-carbon *meta*-amidinophenoxy compound (compound 9) exhibited the greatest antigiardial activity of the *meta*-amidinophenoxy compounds, with an IC_{50} of 6.7 ± 0.95 μM.

The antigiardial activities of analogs in which five different substituents were placed *ortho* to the ether bridge on both aromatic rings are shown in Table 4. As with the unsubstituted compounds (Table 2), compounds with alkyl chains of three and five carbons appeared to be more active than the corresponding two- and four-carbon derivatives. The most interesting compounds of this group are those in which methoxy groups were placed upon the aromatic rings (compounds 16 to 18). This substitution produced compounds with increased antigiardial activities relative to the unsubstituted compounds (compounds 2 to 4). The three-carbon alkyl-chain methoxy-substituted analog DAMP (compound 16), had an IC_{50} of 0.51 ± 0.13 μM and was the most active dicationic compound screened in this study. Addition of nitro (compounds 10 to 12) and amino (compounds 13 to 15)

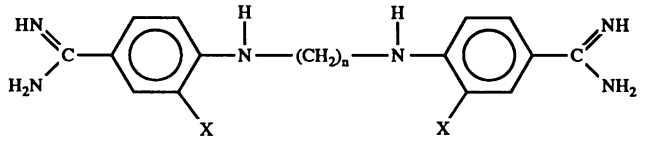
TABLE 4. Biological activities of α,ω-di(4-amidino-2-substituted-phenoxy)alkanes



Compound no.	n	X	Mean IC_{50} (μM) ± SD[a] for *G. lamblia*	Mean DNA binding (ΔT_m [°C]) ± SD[b]			ClogP
				Calf thymus DNA	Poly(dA) · poly(dT)	Poly(dG-dC) · poly(dG-dC)	
10	2	NO_2	85 ± 11	5.6 ± 0.3	10.3 ± 0.2	1.9 ± 0.2	2.8
11	4	NO_2	16 ± 0.30	9.6 ± 1.3	14.7 ± 0.5	3.5 ± 0.6	3.6
12	5	NO_2	8.2 ± 5.5	9.7 ± 1.2	21.6 ± 1.7	3.2 ± 0.2	4.1
13	2	NH_2	126 ± 0.30	8.8 ± 0.2	16.1 ± 0.9	3.0 ± 0.3	1.2
14	3	NH_2	2.3 ± 1.4	13.6 ± 0.1	32.2 ± 0.7	5.2 ± 0.4	1.4
15	4	NH_2	33 ± 1.6	9.6 ± 0.3	19.4 ± 2.3	3.7 ± 1.3	1.9
16	3	OCH_3	0.51 ± 0.13	16.3 ± 0.1	36.3 ± 3.0	3.4 ± 0.4	2.6
17	4	OCH_3	7.7 ± 1.6	10.2 ± 0.2	22.9 ± 0.9	1.1 ± 0.5	3.1
18	5	OCH_3	2.9 ± 0.62	12.0 ± 0.7	27.2 ± 0.1	2.7 ± 0.1	3.6
19	4	Cl	10 ± 3.9	8.9 ± 0.6	20.3 ± 0.5	4.2 ± 0.6	5.2
20	5	Cl	2.9 ± 0.01	10.5 ± 0.4	24.3 ± 0.3	4.6 ± 0.51	5.7
21	5	Br	2.6 ± 0.38	11.6 ± 0.2	25.2 ± 0.2	5.2 ± 0.4	6.0

[a] Values are the means for two separate determinations for each compound (two serial dilutions per determination) ± the standard deviations.
[b] Values are the means for two to four separate determinations ± the standard deviations (5a, 41).

TABLE 5. Biological activities of α,ω-di(4-amidinoanilino)alkanes



Compound no.	n	X	Mean IC_{50} (μM) ± SD[a] for *G. lamblia*	Mean DNA-binding (ΔT_m [°C]) ± SD[b]			ClogP
				Calf thymus DNA	Poly(dA) · poly(dT)	Poly(dG-dC) · poly(dG-dC)	
22	3	H	3.1 ± 0.33	11.6 ± 0.9	25.7 ± 4.5	3.0 ± 0.2	2.7
23	4	H	12 ± 2.7	10.3 ± 0.8	18.7 ± 2.9	2.8 ± 0.2	3.2
24	5	H	5.4 ± 0.38	11.3 ± 0.2	20.7 ± 0.3	4.4 ± 0.2	3.7
25	6	H	2.2 ± 0.51	9.1 ± 1.2	12.9 ± 1.1	2.9 ± 1.1	4.3
26	3	NO_2	5.9 ± 3.9	12.4 ± 0.8	26.6 ± 1.6	5.0 ± 0.6	2.2
27	5	NO_2	5.0 ± 1.6	12.7 ± 0.2	22.1 ± 0.6	6.2 ± 0.6	3.3
28	2	NH_2	440 ± 2.8	9.8 ± 0.5	16.8 ± 1.9	3.0 ± 0.4	0.2
29	4	NH_2	36 ± 2.4	10.5 ± 0.7	13.2 ± 0.6	4.6 ± 0.4	0.7
30	5	NH_2	16 ± 6.9	11.0 ± 0.1	20.9 ± 0.7	3.3 ± 0.4	1.3
31	6	NH_2	7.2 ± 2.9	8.3 ± 0.6	15.0 ± 0.5	3.6 ± 1.9	1.8

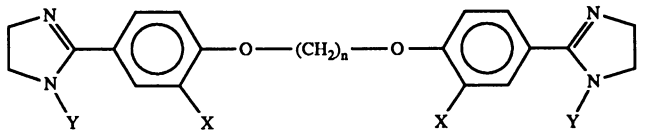
[a] Values are the means for two separate determinations for each compound (two serial dilutions per determination) ± the standard deviations.
[b] Values are the means for two to four separate determinations ± the standard deviations (5a, 41).

groups on the aromatic rings did not significantly alter antigiardial activity. Chloro (compounds 19 and 20)- and bromo (compound 21)-substituted compounds exhibited increased antigiardial activity relative to the corresponding unsubstituted compounds (compounds 3 and 4).

The effect of isosteric replacement of the ether oxygens with nitrogens upon antigiardial activity is reported in Table 5. Except for the six-carbon amidinoanilino compound (compound 25), the anilino compounds (compounds 22 to 25) exhibited antigiardial activity approximately equal to that of the corresponding phenoxy compounds (Table 2). The six-carbon amidinoanilino compound (compound 25) was over twofold more active than hexamidine (compound 5), the corresponding amidinophenoxy analog. Amino substitution of anilino compounds (compounds 28 to 31) resulted in a slight decrease in antigiardial activity compared with the unsubstituted anilino compounds, while nitro substitution (compounds 26 and 27) did not greatly alter activity in relation to the unsubstituted anilino compounds.

Replacement of the amidino groups with imidazolino moieties (Table 6) produced compounds with similar or only slightly altered activity against *G. lamblia* compared with the corresponding *para*-amidinophenoxy compounds (Table 2). Methoxy substitution of imidazolino compounds (compounds 35 to 37) had little or no effect on antigiardial activity relative to the corresponding imidazolino compounds or the corresponding methoxy-substituted amidinophenoxy compounds. Methylation of the imidazolino nitrogens produced compounds (compounds 38 and 39) with greatly reduced antigiardial activities. The antiprotozoal activities of these imidazolino compounds are especially important, because it has been suggested that diamidino compounds have antipro-

TABLE 6. Biological activities of α,ω-di(4-imidazolinophenoxy)alkanes



Compound no.	n	X	Y	Mean IC_{50} (μM) ± SD[a] for *G. lamblia*	Mean DNA binding (ΔT_m [°C]) ± SD[b]			ClogP
					Calf thymus DNA	Poly(dA) · poly(dT)	Poly(dG-dC) · poly(dG-dC)	
32	3	H	H	3.1 ± 1.2	12.7 ± 0.6	26.9 ± 0.4	4.8 ± 0.4	5.6
33	4	H	H	8.2 ± 1.8	12.0 ± 0.1	15.6 ± 0.6	4.8 ± 0.04	6.1
34	5	H	H	5.7 ± 1.7	10.5 ± 0.7	22.1 ± 0.6	4.8 ± 0.6	6.6
35	3	OCH_3	H	1.9 ± 0.03	13.1 ± 1.9	30.4 ± 3.7	3.7 ± 1.5	4.6
36	4	OCH_3	H	9.8 ± 1.1	10.1 ± 0.15	22.7 ± 0.2	3.9 ± 0.5	5.1
37	5	OCH_3	H	5.4 ± 1.3	10.4 ± 1.5	22.4 ± 0.3	4.6 ± 0.2	5.7
38	4	H	CH_3	110 ± 28	7.2 ± 0.1	4.1 ± 0.1	1.5 ± 0.4	7.9
39	5	H	CH_3	78 ± 0.19	5.6 ± 1.9	10.0 ± 0.2	3.4 ± 0.1	8.4

[a] Values are the means for two separate determinations for each compound (two serial dilutions per determination) ± the standard deviations.
[b] Values are the mean for two to four separate determinations ± the standard deviations (5a, 41).

tease activity (15). While amidinophenoxy compounds are effective inhibitors of trypsin (15, 41), imidazolino compounds are devoid of antitrypsin activity and yet are active against *G. lamblia*, *Leishmania mexicana amazonensis* (2), and *Plasmodium falciparum* (2) in vitro and *Pneumocystis carinii* (22) in vivo.

DNA binding affinity and its correlation to antigiardial activity. By using the mean IC_{50}s of these compounds against *G. lamblia* and the mean affinity of these compounds to calf thymus DNA, poly(dA) · poly(dT), and poly(dG-dC) · poly (dG-dC), it was possible to determine correlations between antigiardial activities and the affinities of these compounds for DNA. The structure-activity relationships are represented by the following equations:

$$-\log IC_{50} = -4.21 + 0.20\ (\pm 0.02)CT + 0.19\ (\pm 0.05)n + 0.11\ (\pm 0.03)ClogP - 0.78\ (\pm 0.27)I_{(CH_3)}$$
$$(n = 39,\ r^2 = 0.81,\ F = 36.7,\ s = 0.30)$$

$$-\log IC_{50} = -3.46 + 0.06\ (\pm 0.01)AT + 0.22\ (\pm 0.05)n + 0.08\ (\pm 0.04)ClogP - 0.62\ (\pm 0.29)I_{(CH_3)}$$
$$(n = 39,\ r^2 = 0.80,\ F = 34.9,\ s = 0.31)$$

$$-\log IC_{50} = -2.57 + 0.18\ (\pm 0.08)GC + 0.15\ (\pm 0.08)n + 0.09\ (\pm 0.06)ClogP - 1.26\ (\pm 0.47)I_{CH_3)}$$
$$(n = 39,\ r^2 = 0.43,\ F = 6.5,\ s = 0.52)$$

where n is the sample number; r^2 is variance; F is the F test; s is the standard error of the estimate; IC_{50} is in μM; calf thymus DNA, poly(dA) · poly(dT), and poly(dG-dC) · poly (dG-dC) are represented by CT, AT, and GC, respectively; n is the length of the alkyl bridge connecting the amidinophenoxy moieties; ClogP is a calculated value for the transport of the molecules across an octanol-water partition; and $I_{(CH_3)}$ is an indicator variable to note the presence of methyl groups upon the imidazolino moieties (compounds 37 and 38). These variables allowed calculation of the r^2 values for the correlation of antigiardial activity with calf thymus DNA, poly (dA) · poly(dT), and poly(dG-dC) · poly(dG-dC). There was a strong correlation between antigiardial activities and the affinities of these compounds for calf thymus DNA (r^2 = 0.81) and poly(dA) · poly(dT) (r^2 = 0.80) and a much lower correlation between antigiardial activities and the affinities of the compounds for poly(dG-dC) · poly(dG-dC) (r^2 = 0.43).

All of these compounds exhibited affinity for calf thymus DNA, poly(dA) · poly(dT), and poly(dG-dC) · poly(dG-dC) in the thermal denaturation assay. In all but two cases, compounds 8 and 38, the data suggest that these compounds showed higher affinities for poly(dA) · poly(dT) than for calf thymus DNA. These compounds also appeared to exhibit higher affinities for calf thymus DNA and poly(dA) · poly (dT) than for poly(dG-dC) · poly(dG-dC). The ΔT_ms ranged from 5.4 ± 0.2 to 16.3 ± 0.1°C and from 7.5 ± 2.6 to 36.3 ± 3.0°C for calf thymus DNA and poly(dA) · poly(dT), respectively. The affinity for poly(dG-dC) · poly(dG-dC) did not seem to alter as greatly within each homogeneous structural group, and ΔT_ms for poly(dG-dC) · poly(dG-dC) ranged from 1.1 ± 0.5 to 6.2 ± 0.6°C. For these reasons and because there was no strong correlation between antigiardial activities and the affinities of the compounds for poly(dG-dC) · poly(dG-dC), these data will not be considered in detail.

Compounds with alkyl chain lengths of three or five carbons showed greater affinities for DNA than did those with alkyl chain lengths of two, four, or six carbons. Within homogeneous structural groups, the three-carbon compounds showed the greatest affinities for calf thymus DNA and poly(dA) · poly(dT). This is illustrated in Table 2, in which the three-carbon compound propamidine (compound 2) exhibited ΔT_ms of 14.7 ± 1.7 and 32.0 ± 0.7°C for calf thymus DNA and poly(dA) · poly(dT), respectively. An exception to this generalization are the *meta*-amidinophenoxy compounds (Table 3), which showed reduced affinity for calf thymus DNA and poly(dA) · poly(dT) relative to the corresponding *para*-amidinophenoxy compounds, and the two compounds in which methylene groups have been placed upon the imidazolino moieties (compounds 38 and 39).

In general, placing substituent groups upon the aromatic rings *ortho* to the ether bridge (Table 4) slightly improved or did not greatly alter the affinities of the compounds for calf thymus DNA and poly(dA) · poly(dT). DAMP, the compound with the most pronounced antigiardial activity (compound 16), also exhibited the strongest affinities for calf thymus DNA and poly(dA) · poly(dT), with ΔT_ms of 16.3 ± 0.1 and 36.3 ± 3.0°C, respectively. Isosteric replacement of the ether oxygens with nitrogens (compounds 22 to 25) did not greatly change the affinities of the compounds for calf thymus DNA and poly(dA) · poly(dT) relative to those of the corresponding amidinophenoxy compounds (compounds 2 to 5). Nor did placement of nitro and amino groups upon the aromatic rings of the amidinoanilino compounds greatly alter their affinities for calf thymus DNA or poly(dA) · poly(dT) (compounds 26 to 31). Likewise, replacement of the amidino groups with imidazolino moieties (Table 6) had little effect on the affinity of the compounds for calf thymus DNA and/or poly(dA) · poly(dT) relative to the corresponding amidinophenoxy compounds (compounds 2 to 4 and 16 to 18). Methylation of the nitrogens on the imidazolino moiety produced compounds (compounds 38 and 39) with greatly reduced affinities for both calf thymus DNA and poly(dA) · poly(dT) relative to those of the corresponding amidinophenoxy compounds (compounds 3 and 4).

DISCUSSION

Current interest in the development of new pentamidine analogs has been spurred by the increase in *P. carinii* pneumonia associated with the acquired immune deficiency syndrome. This epidemic has led to extensive investigations to identify more potent, less toxic analogs of pentamidine for treatment of this and other parasitic infections. The compounds described in this study and similar analogs have been tested in the rat model of *P. carinii* pneumonia (22, 41, 42). Preliminary data suggest that many of these compounds are more potent against *P. carinii* pneumonia and less toxic than the parent compound (22, 41, 42). These compounds have also been found to have activity in vitro against *P. falciparum* and *L. mexicana amazonensis* (2). The data described in the current study reveal that *G. lamblia* trophozoites are susceptible to strongly dicationic diamidines and di-imidazolines. Further, these data confirm previously published reports showing the radiometric assay to be a valid, reproducible, and sensitive method for assessment of the antigiardial activities of therapeutic agents in a microculture system (3, 19). The strong correlation between antigiardial activities and the affinities of these compounds for calf thymus DNA and poly(dA) · poly(dT) suggests that DNA binding is a mechanism by which these compounds exert antigiardial activity. In addition, the antigiardial activities of some of the compounds tested in this study compared favorably with those of the drugs currently used to treat giardiasis.

Aromatic diamidines have not been previously reported to be active against *G. lamblia* trophozoites. Given the side-effects and treatment failures associated with the compounds currently used to treat giardiasis, it is obvious that more

efficacious and less toxic drugs could have clinical applications in the treatment of this infection. This study has identified three compounds with promising activity against *G. lamblia* in vitro, each of which has a three-carbon alkyl chain. DAMP (compound 16) was the most potent pentamidine analog identified in this study and is also significantly more potent than pentamidine against *P. carinii* pneumonia in the rat model of disease at one-half of the dosage of the parent compound (41). Propamidine (compound 2) and DIMP (compound 35) were also very active against *G. lamblia*. DIMP (compound 35) has demonstrated 10 times the activity of pentamidine against *P. carinii* pneumonia in the rat model of disease and is active upon oral administration (22).

The potency of the three-carbon pentamidine analogs against *G. lamblia* illustrates an important factor in the structure-activity relationships of these compounds. That is, the number of methylenes in the alkyl chain separating the amidinophenoxy moieties is an important factor in both their antiprotozoal activity and their affinity for calf thymus DNA and poly(dA) · poly(dT). Compounds with three, five, or six methylenes in their alkyl chains are generally more potent against *G. lamblia* than those with two- or four-carbon alkyl chains. The relationship between chain length and antiprotozoal activity was also observed when these compounds were tested in vitro against *L. mexicana amazonensis* and *P. falciparum* (2). Except for the *meta*-amidinophenoxy compounds, analogs with alkyl chains consisting of odd numbers of carbons also exhibited higher affinities for calf thymus DNA and poly(dA) · poly(dT) than did compounds with even numbers of carbons in their alkyl chains. The three most potent compounds (compounds 2, 16, and 35) each have alkyl chains consisting of three methylenes and are among the best DNA-binding agents. Additionally, the great reduction in antigiardial activity produced when methylene groups are placed upon the imidazolino moieties corresponds to a greatly reduced affinity of these compounds for calf thymus DNA and poly(dA) · poly(dT).

The strong correlation between antigiardial activity and drug affinity for poly(dA) · poly(dT) and the poor correlation between antigiardial activity and drug affinity for poly(dG-dC) · poly(dG-dC) may be explained by the sequence specificity exhibited by diamidines in their binding to DNA. Diamidines such as pentamidine, 4′,6-diamidino-2-phenylindole, and berenil have been shown to bind preferentially to A+T-rich regions in minor grooves of B-DNA without intercalating between base pairs (12, 28, 32, 34, 38). The preferential binding of diamidino compounds to A+T-rich regions is further confirmed by the disruption of A+T-rich kinetoplastic DNA in leishmania (7) and trypanosomes (33). The DNA-binding abilities of these agents may not, however, be their primary mechanism of action. 4′,6-Diamidino-2-phenylindole has been shown to inhibit the activity of a type II topoisomerase (46), and it has been suggested that pentamidine also inhibits a type II topoisomerase of trypanosomes (40). Thus, DNA binding might be important in the activity of these agents in that binding to DNA brings them into proximity to enzymes, such as topoisomerases, which are involved in DNA synthesis and/or metabolism.

This study has identified a new class of agents with in vitro activity against *G. lamblia* and has contributed to a better understanding of the structure-activity relationships of analogs of pentamidine and the possible mechanism(s) by which these compounds exert their antimicrobial activities. It has been determined that the number of methylenes in the alkyl chain is important in both antigiardial activity and the affinity of the compounds for DNA. The correlation between antigiardial activity and the affinities of these compounds for both natural and synthetic DNAs suggests that DNA binding is important in the antimicrobial action of these agents. The information obtained from this study may aid in the design of more potent and less toxic antiprotozoal agents which are needed because of the continuing emergence of drug-resistant parasites. Further, *G. lamblia* provides a valuable new model to assist in determining the mechanism(s) of action of this important class of compounds.

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