Structure-Activity Studies of Dicationically Substituted Bis-Benzimidazoles against *Giardia lambia*: Correlation of Antigiardial Activity with DNA Binding Affinity and Giardial Topoisomerase II Inhibition

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Nine dicationically substituted bis-benzimidazoles were examined for their in vitro activities against *Giardia lambia* WB (ATCC 30957). The potential mechanisms of action of these compounds were evaluated by investigating the relationship among in vitro antigiardial activity and the affinity of the molecules for DNA and their ability to inhibit the activity of giardial topoisomerase II. Each compound demonstrated antigiardial activity, as measured by assessing the incorporation of [methyl-3H]thymidine by giardial trophozoites exposed to the test agents. Three compounds exhibited excellent in vitro antigiardial activities, with 50% inhibitory concentrations which compared very favorably with those of two currently used drugs, quinacrine HCl and metronidazole. Putative mechanisms of action for these compounds were suggested by the strong correlation observed among in vitro antigiardial activity and the affinity of the molecules for natural and synthetic DNA and their ability to inhibit the relaxation activity of giardial topoisomerase II. A strong correlation between the DNA binding affinity of these compounds and their inhibition of giardial topoisomerase II activity was also observed.

*Giardia lambia* is a common cause of endemic and epidemic diarrheal disease throughout the world. Some individuals harbor asymptomatic infections, while others may exhibit acute or chronic gastrointestinal disease. Four agents are presently used to treat giardiasis: the nitroimidazoles metronidazole and tinidazole, the nitrofuran furazolidone, and quinacrine HCl, an acridine. Many problems are associated with the currently used chemotherapeutic agents, including treatment failures, unpleasant side effects, activity against normal intestinal flora, and possible carcinogenicity. While treatment of symptomatic individuals is recommended, there is controversy as to whether asymptomatic cyst passers should be treated, especially in light of the problems associated with the antigiardial agents presently available. More-effective and less-toxic agents are therefore needed for the treatment of giardiasis.

The search for new antigiardial agents has been aided by improvements in axenic culturing of the organism (13) and drug susceptibility testing (3, 12). Among the classes of compounds recently examined for antigiardial activity are anthelmintic benzimidazoles (1, 8, 14, 15) and derivatives of the antiprotozoal compound pentamidine (2). Pentamidine and 38 related dicationically substituted molecules were examined for in vitro activities against *G. lamblia* (2). The 50% inhibitory concentration values (IC50s) of the pentamidine analogs demonstrating the most potency against *G. lamblia* compared favorably with those of the compounds which are used at present to treat the infection (2). In addition, there was a strong correlation between the antigiardial activities of the pentamidine analogs and their affinity for calf thymus DNA and poly(dA) · poly(dT). The ability of pentamidine to bind to DNA has been proposed as a mechanism of action for this and related compounds. Pentamidine and related molecules have also been identified as potential inhibitors of the activity of type II topoisomerases (7, 17).

A number of dicationically substituted bis-benzimidazoles, originally developed as protease inhibitors and DNA binding agents, were available in our laboratory for in vitro antimicrobial susceptibility testing. These compounds have shown antiarthritic activity in an animal model (11) and have also demonstrated in vivo activity against *Pneumocystis carinii* pneumonia in the rat model of disease (20). Several of the bis-benzimidazoles also exhibit affinities for DNA stronger than those of the pentamidine derivatives (9). This fact, coupled with the correlation between the antigiardial activity and DNA binding affinity of the pentamidine derivatives, prompted us to expand our studies. The present report describes the examination of the structure-activity relationships of nine dicationically substituted bis-benzimidazoles against *G. lamblia* in vitro, the correlation between their antigiardial activities and their affinities for natural and synthetic DNA, and their ability to inhibit the activity of giardial topoisomerase II.

**MATERIALS AND METHODS**

**Chemotherapeutic agents.** The bis-benzimidazoles used in the present study were synthesized according to previously described methods (6, 9, 18, 19). The purity of the compounds was determined by high-performance liquid chroma-
tography, elemental analyses, and nuclear magnetic resonance spectroscopy. Quinacrine-HCl and metronidazole were purchased from Sigma (St. Louis, Mo.).

Cultivation and isolation of parasites. G. lamblia (WB) trophozoites (ATCC 30957) were grown axenically in TYI-S-33 medium supplemented with bile (Sigma) and 10% fetal bovine serum (13). The organisms were grown in borosilicate glass tubes (12 by 100 mm) at 37°C, inclined approximately 30° from the horizontal. Trophozoites were subcultured every 60 to 96 h by chilling the tubes in an ice-water bath for 5 min and then vigorously inverting the tubes to dislodge the trophozoites. The number of trophozoites per milliliter was determined, and 5.0 x 10⁴ trophozoites were inoculated into fresh medium.

Large-scale cultures were cultivated in outside in roller bottles (Bellco Glass, Inc., Vineland, N.J.), which provide a high surface area-to-volume ratio (10). Logarithmically growing trophozoites (2 x 10⁶) were inoculated into each bottle containing approximately 625 ml of medium. The roller bottles were incubated at 37°C and rotated at 6 revolutions per hr for 70 to 80 h, until an examination of the surfaces of the bottles by inverse microscopy revealed a dense monolayer of trophozoites. The bottles were chilled in an ice-water bath for 15 min, and the contents were placed in 250-ml polypropylene bottles and centrifuged in a Sorvall GSA rotor at 6,000 rpm for 20 min. The pellets were resuspended in twice their volume of buffer A (50 mM Tris-HCl [pH 7.5; analytical grade], 100 mM KCl [analytical grade], 10% glycerol [analytical grade], 10 mM β-mercaptoethanol [Sigma], 1 mM phenylmethylsulfonyl fluoride [PMSF; Sigma], 0.5 mM dithiothreitol [Sigma], 0.5 mM Na₄EDTA [Sigma], 2 mg of aprotinin per ml [Sigma]). The cell suspension was dropped into liquid nitrogen and stored at −70°C.

Susceptibility to bis-benzimidazoles. A microculture system for assessing the antigiardial activity of chemotherapeutic agents has been previously reported (2, 3). The addition of Trypticase peptone in the media caused some of the bis-benzimidazole compounds to fall out of solution, a problem previously observed in the analysis of pentamidine analogs (2). Therefore, Trypticase peptone was omitted from the assay medium for all bis-benzimidazoles and control compounds. This alteration did not greatly interfere with the growth of the trophozoites during the assay period. G. lamblia trophozoites were grown to early log phase in filter-sterilized TYI-S-33 medium supplemented with bile and 10% fetal bovine serum. Serial dilutions of drugs suspended in assay medium were prepared in duplicate rows of a 96-well microtiter plate. Spent medium was aspirated from the culture tubes and replaced with assay medium before the tubes were chilled, and 2.5 x 10⁴ logarithmically growing trophozoites were added to wells containing the test agents. The plates were placed in an anaerobic chamber, gassed with nitrogen, and incubated at 37°C. After 24 h, [methyl-³H]thymidine (1 to 10 Ci/mmol; ICN, Irvine, Calif.) was added to yield 1.5 to 2 μCi per well. At 42 h, the cells were harvested with a PHD cell harvester (Cambridge Technology, Inc., Watertown, Mass.) onto glass microfiber paper. Washed and dried filters were counted in a Packard Tri-Carb 46-40 scintillation counter.

DNA-binding assay. The binding of this series of bis-benzimidazoles to calf thymus DNA, poly(dA·dA)·poly(dT·dT), poly(dA·dT)·poly(dA·dT), and poly(dG·dC)·poly(dG·dC) was evaluated by a thermal denaturation assay and has been previously reported (9).

Topoisomerase assays. The activity of topoisomerase II was monitored throughout the purification steps by utilizing a relaxation assay. The relaxation of supercoiled plasmid DNA upon enzyme treatment is among the methods most commonly used for assessing topoisomerase activity. This assay takes advantage of the decreased mobility of DNA in an agarose gel following enzymatic treatment. For the relaxation assay, 0.5 μg of supercoiled plasmid DNA (pBluescript II KS⁺; Stratagene, La Jolla, Calif.) under the conditions of the assay obtained according to standard methods (16) was used in a 15-μl reaction mixture containing Topoisomerase II Reaction Buffer (10 mM Tris-HCl [pH 7.9], 50 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 15 μg of bovine serum albumin per ml, 1 mM ATP; U.S. Biochemicals, Cleveland, Ohio). A reaction mixture lacking ATP was also prepared to differentiate between ATP-dependent (topoisomerase II) and ATP-independent (topoisomerase I) relaxing activities. The reaction mixtures were incubated at 37°C for 15 min and stopped by the addition of 5 μl of stop buffer containing 5% sodium dodecyl sulfate, 25% glycerol, and 0.5 μg of bromophenol blue per ml. The samples were applied to 0.8% horizontal agarose gels and electrophoresed in Tris-borate-EDTA buffer for 4 h at 4 V/cm. The gels were stained with ethidium bromide and photographed under UV light. The extent of relaxation was determined based on the migration of the DNA in the gels. Negative photographs of the gels were scanned with an LKB laser densitometer, and the peak areas were evaluated. One unit of topoisomerase II was defined as the amount of enzyme that relaxes 50% of the supercoiled plasmid DNA in an ATP-dependent manner. Figure 1 illustrates the ATP dependence of the reaction.

Preparation of cell extract. The trophozoite suspension containing approximately 3.8 x 10⁹ organisms (1.6 g [wet weight]) was thawed at room temperature and lysed by the addition of an equal amount of buffer A containing 2 M KCl. The mixture was maintained on ice for 30 min, with periodic mixing, and then centrifuged in a Sorvall SS34 rotor at 12,000 rpm for 20 min to remove cell debris. The supernatant was dialyzed against 1 liter of buffer A for 2 h to reduce the
KCl concentration, and the precipitate was removed by centrifugation.

**Enzyme purification.** All purification steps were performed at 4°C. Chromatographic separation was performed with a Pharmacia LKB fast-performance liquid chromatography system consisting of an LKB Controller LCC-500 Plus, two LKB P-500 pumps, an LKB FRAC-100 fraction collector, an LKB UV-M Monitor, and an LKB 2210 Chart Recorder (Pharmacia LKB, Piscataway, N.J.). Protein concentrations were measured according to the method described by Bradford (4).

**CDR.** Cell Debris Remover (CDR) (Whatman Biosystems, Inc., Clifton, N.J.) is a modified cellulose product specifically designed for the clarification of crude lysates. CDR absorbs polar lipids, nucleic acids, fats, and cell debris. The centrifuged dialysate (fraction I) was applied to a CDR column (1.5 by 5 cm) equilibrated with buffer A and was washed with 25 ml of buffer A. Protein-containing fractions were combined for subsequent chromatography (fraction II).

**Q-Sepharose fast flow (Pharmacia).** Fraction II obtained from the CDR column was applied to a Q-Sepharose column (1.5 by 10 cm) equilibrated with buffer A. The column was washed with 40 ml of buffer A, and the proteins were eluted with a 50-ml linear gradient of 100 mM to 2 M KCl in buffer A. The fractions were examined for their abilities to cause relaxation of supercoiled DNA (form I) to the fully relaxed state (form II) in the presence (topoisomerase II) and absence (topoisomerase I) of ATP. Fractions eluting from the Q-Sepharose column between 450 and 750 mM KCl containing ATP-dependent relaxation activity were dialyzed against 1 liter of buffer B (100 mM potassium phosphate [pH 7.0], 10% glycerol, 10 mM β-mercaptoethanol, 1 mM PMSF). The dialysate (fraction III) was clarified by centrifugation for subsequent chromatography.

**Hydroxylapatite.** Fraction III obtained from the Q-Sepharose chromatography was applied to a Bio-Gel HT hydroxylapatite column (1.5 by 5 cm; Bio-Rad Laboratories, Richmond, Calif.). The column was washed with 40 ml of buffer B, and the protein was eluted with a 40-ml linear gradient of potassium phosphate (100 mM to 1 M) in buffer B. The fractions were examined for ATP-dependent relaxation activity. The ATP-dependent relaxation activity eluted from the hydroxylapatite column between 550 and 700 mM potassium phosphate. The active fractions were pooled (fraction IV) and dialyzed for 2 h against 500 ml of buffer C (50 mM Tris-HCl [pH 7.5], 100 mM KCl, 20% glycerol, 10 mM β-mercaptoethanol, 5 mM dithiothreitol, 0.1 mM Na₂EDTA). Fraction IV was dialyzed for an additional 2 h against buffer C containing 50% glycerol. The dialysate was aliquoted into 0.65-ml microcentrifuge tubes and stored at −70°C (stable for over 6 months at −70°C).

**Inhibition of topoisomerase II relaxation activity by bis-benzimidazoles.** The possible inhibitory effects of bis-benzimidazoles upon ATP-dependent relaxation were determined. Serial dilutions of the test compounds were made in 96-well microtiter plates. Various concentrations of the compounds were added to the reaction mixture in 0.65-ml microcentrifuge tubes. Two units of the enzyme was then added to each tube, and the reaction mixtures were incubated and electrophoresed as described above. The amounts of drug (micromolar) required to inhibit 50% of the supercoiled DNA relaxation were determined.

**Data analysis.** IC₅₀,₉₅ for in vitro antigiardial activity and for DNA binding affinity were determined as previously described (2). The IC₅₀,₉₅ for the inhibition of the relaxing activity of giardial topoisomerase II were determined by using scanning densitometry to compare peak areas on photographic negatives of reactions with and without dicationic agents. The points obtained by scanning densitometry were graphed, and the IC₅₀,₉₅ were determined. Statistical analyses were performed by using the StatView 512+ software package (Brainpower, Inc., Calabasas, Calif.) on a Macintosh LC II microcomputer.

### RESULTS

**Enzyme purification.** Results from a representative purification of *G. lamblia* topoisomerase II are shown in Table 1. The enzyme purification was performed with a crude cell extract of approximately $5.8 \times 10^9$ trophozoites (1.6 g [wet weight]). This crude extract had a total protein concentration of 67.4 mg and a total enzyme activity of about $1.4 \times 10^7$ U. The crude lysate was passed over a CDR column. The CDR column removed approximately 28% of the total protein and resulted in a loss of approximately 39% of the starting ATP-dependent relaxing activity. Protein-containing fractions from the CDR column were applied to a Q-Sepharose column. ATP-dependent relaxation activity was eluted from this column between 450 and 750 mM KCl. Passage over the Q-Sepharose column resulted in a 97% reduction of the total protein content relative to that of the crude lysate. The specific activity of the pooled fractions from the Q-Sepharose column represents nearly a 10-fold increase over that of the crude lysate. The pooled active fractions from the Q-Sepharose column were applied to a hydroxyapatite column. Enzyme activity eluted from the hydroxyapatite column between 550 and 700 mM of potassium phosphate. The hydroxyapatite further reduced the total protein content to less than 0.5% of the starting protein concentration and raised the specific activity to approximately $1.2 \times 10^5$ U/mg of protein. The total topoisomerase II activity recovered after all of the chromatographic steps was approximately 6.4%. There was no ATP-independent relaxing activity or apparent nuclease in the final fraction. Figure 1 shows that the relaxation reaction is ATP dependent after the addition of either 3 or 30 U of topoisomerase.

**Drug susceptibility.** Nine bis-benzimidazoles were tested for in vitro activities against *G. lamblia* and for their abilities to inhibit the relaxation of supercoiled DNA by giardial topoisomerase II. These data, along with the affinities of the compounds for calf thymus DNA, poly(dA·dT)·poly(dA·dT), poly(dA·dT)·poly(dA·dT), and poly(dG·dC)·poly(dG·dC) (9), are presented in Table 2. The compounds are structurally similar, differing only in the presence of either amidino or imidazolino moieties upon the aromatic rings, in the number of methylene groups (one to four carbon atoms) in the linking group joining the benzimidazole groups, or in the presence of a double bond in the alkyh chain.

| Table 1. Purification of type II topoisomerase from *G. lamblia* |
|---------------------|---------------------|---------------------|---------------------|
| Fraction            | Total protein content (mg) | Total activity (U) | Sp act (U/mg of protein) | Activity recovered (%) |
| I (crude lysate)    | 67.4                | 1.4 × 10⁵          | 2.1 × 10⁵             | 100                  |
| II (CDR)            | 48.3                | 8.5 × 10⁵          | 1.8 × 10⁵             | 60.7                 |
| III (Q-Sepharose)   | 1.9                 | 4.5 × 10⁴          | 2.3 × 10⁴             | 32.1                 |
| IV (hydroxyapatite) | 0.08                | 9.0 × 10³          | 1.2 × 10³             | 6.4                  |

* The enzyme purification was performed with approximately $5.8 \times 10^9$ *G. lamblia* trophozoites (1.6 g [wet weight]).

* One unit relaxes half of the supercoiled DNA under the assay conditions.
While each compound demonstrated in vitro activity against G. lamblia, the antigiardial activities of these compounds varied widely. The IC\textsubscript{50} ranged from 0.04 ± 0.01 for the most-potent molecule to more than 300 μM. The parent molecule bis(5-amidino-2-benzimidazolyl)ethene (BABIM; compound 8), a very potent inhibitor of trypsin-like proteases and a promising antiarthritic agent (11, 19), demonstrated very poor in vitro activity against G. lamblia, with an IC\textsubscript{50} of 307 ± 28.3 μM. Compound 9, differing from BABIM only in the substitution of amidino moieties for the amidino groups, also demonstrated poor in vitro antigiardial activity. The addition of a second methylene in the link between the benzimidazole groups (compounds 2 and 4) resulted in greatly increased in vitro activity against G. lamblia. The IC\textsubscript{50} for the amidino-substituted (compound 4) and the amidino-substituted (compound 2) methylene derivatives were 0.33 ± 0.15 and 0.21 ± 0.03 μM, respectively. Addition of a third methylene (compounds 6 and 7) to the alkyl bridge resulted in decreased activity relative to those of the compounds with two methylenes (compounds 2 and 4) but resulted in activity better than that of the compounds with one methylene joining the benzimidazole groups (compounds 8 and 9). Compounds with four methylenes (compounds 3 and 5) were more-potent antigiardial inhibitors than compounds with either one (compounds 8 and 9) or three (compounds 6 and 7) methylenes, but they demonstrated less in vitro antigiardial activity than the compounds with two methylenes (compounds 2 and 4). A bis-benzimidazole in which the benzimidazole groups are joined by an ethylene group (compound 1) was the most-potent antigiardial compound tested in this study. The antigiardial activity of trans-1,2-bis(5-amidino-2-benzimidazolyl)ethene (BBE) (IC\textsubscript{50} = 0.04 ± 0.01 μM) compares favorably with those of two compounds currently used to treat this infection, metronidazole (IC\textsubscript{50} = 1.1 ± 0.56 μM) and quinacrine-HCl (IC\textsubscript{50} = 0.05 ± 0.01 μM).

**DNA binding affinity and its correlation with antigiardial activity.** Each of the nine bis-benzimidazoles exhibited an affinity for calf thymus DNA, poly(dA)·poly(dT), poly(dA-dT)·poly(dA-dT), and poly(dG-dC)·poly(dG-dC) in a thermal denaturation assay (9). These compounds showed a higher affinity for poly(dA)·poly(dT) and poly(dA-dT)·poly(dA-dT) than for calf thymus DNA or for poly(dG-dC)·poly(dG-dC). BBE (compound 1), the compound which demonstrated the strongest antigiardial activity, also showed the greatest affinity for DNA. Compounds 2 and 4, which demonstrated pronounced in vitro antigiardial activities, also bound to calf thymus DNA, poly(dA)·poly(dT), and poly(dA-dT)·poly(dA-dT) with high affinity. Likewise, compounds 3 and 5 with four methylenes in the alkyl bridge demonstrated both potent in vitro antigiardial activity and affinity for calf thymus DNA and the poly(dA)·poly(dT) and poly(dA-dT)·poly(dA-dT) homopolymers. The molecules with one (compounds 8 and 9) and three (compounds 6 and 7) methylenes joining the benzimidazole moieties showed lower affinity for calf thymus DNA, poly(dA)·poly(dT), and poly(dA-dT)·poly(dA-dT) relative to the other compounds.

The mean IC\textsubscript{50} for in vitro activity and for DNA binding affinity were used to determine the correlations between the antigiardial activities of these molecules and their affinities for DNA. The structure-activity relationships may be represented by the following equations:

\[ -\log IC_{50} = 0.26 (±0.02) CT - 0.29 (±0.1) n - 3.67 \]

\[ n = 9 \quad r^2 = 0.96 \quad F = 72.9 \quad s = 0.31 \quad (1) \]
−log IC₅₀ IV = 0.11 (±0.01) AT − 0.49 (±0.09) n − 3.11
n = 9 \quad r² = 0.97 \quad F = 91.1 \quad s = 0.28 \quad (2)

−log IC₅₀ IV = 0.12 (±0.01) AltAT − 0.37 (±0.09) n − 3.17
n = 9 \quad r² = 0.97 \quad F = 98.0 \quad s = 0.27 \quad (3)

−log IC₅₀ IV = 0.68 (±0.57) GC − 0.08 (±0.44) n − 3.65 (NS)
n = 9 \quad r² = 0.19 \quad F = 0.73 \quad s = 1.39 \quad (4)

where n is the sample number; r² is the variance; F is the F test; s is the standard error of the estimate; −log IC₅₀ IV is the −log of the in vitro IC₅₀ (micromolar); CT, AT, Alt AT, and Alt GC, respectively, are calf thymus DNA, poly(dA) · poly(dT), poly(dA-dT), poly(dG-dC), and poly(dG-dC); NS is not significant; and n is the length of the alkyl bridge connecting the benzimidazole moieties. These variables allowed the calculation of the r² values for the correlation of antigiardial activity with DNA binding affinity. There was a very strong correlation between in vitro antigiardial activity and the affinities of the compounds for calf thymus DNA (r² = 0.96), poly(dA) · poly(dT) (r² = 0.97), and poly(dA-dT) · poly(dA-dT) (r² = 0.97). There was no significant correlation between antigiardial activity and the affinity of molecules for poly(dG-dC) · poly(dG-dC) (r² = 0.26).

**Topoisomerase II inhibition by bis-benzimidazoles and its correlation with in vitro antigiardial activity and DNA binding affinity.** Each compound inhibited the relaxation of supercoiled DNA to some degree. These compounds showed a wide range of topoisomerase II inhibitory concentrations, with IC₅₀ values ranging from 0.1 to more than 600 μM. BBE (compound 1), which demonstrated the most potency against *G. lamblia* in vitro and which showed the strongest affinity for DNA, was also the best inhibitor of the relaxation assay for topoisomerase II activity. The compounds with a single methylene connecting the benzimidazole moieties (compounds 8 and 9) were the least active in the antigiardial compounds, showed relatively poor affinity for DNA, and also showed the least inhibitory activity in the topoisomerase II relaxation assay. Compounds 2 and 4 with two methylenes showed excellent antigiardial activity and strong affinity for DNA and were also good inhibitors of the relaxation of supercoiled DNA by giardial topoisomerase II. Compounds 3 and 5 with four methylenes connecting the benzimidazole moieties showed more-moderate antigiardial activity, intermediate levels of affinity for DNA, and moderate to good ability to inhibit the activity of topoisomerase II.

The −log IC₅₀ IV for the inhibition of giardial topoisomerase II and in vitro activity and the values for DNA binding affinity were used to determine the correlations among topoisomerase II inhibition and the antigiardial activities and DNA binding affinities of the molecules. There was a strong correlation between the ability of the compounds to inhibit the relaxation activity of giardial topoisomerase II and their in vitro activity against *G. lamblia* trophozoites (r² = 0.91), as is indicated by the following equation, where the −log of the concentration of drug required to inhibit 50% of the giardial topoisomerase II relaxation activity is indicated by

− log IC₅₀ IV = 1.102 (±0.14) − log TopoII IC₅₀ − 0.45
(±0.16) n + 1.37

n = 9 \quad r² = 0.91 \quad F = 30.98 \quad s = 0.46 \quad (5)

Strong correlations were also displayed between the topoisomerase II inhibitory activities of bisbenzimidazoles and the affinities of the molecules for calf thymus DNA (r² = 0.88), poly(dA) · poly(dT) (r² = 0.84), and poly(dA-dT) · poly(dA-dT) (r² = 0.90). However, there was no significant correlation between topoisomerase II inhibition by these compounds and their affinities for poly(dG-dC) · poly(dG-dC) (r² = 0.14). These relationships are illustrated by the following equations:

−log IC₅₀ TII = 0.22 (±0.03) CT − 4.09
n = 9 \quad r² = 0.88 \quad F = 53.36 \quad s = 0.46 \quad P = 0.0002

−log IC₅₀ TII = 0.09 (±0.02) AT − 3.78
n = 9 \quad r² = 0.84 \quad F = 35.92 \quad s = 0.55 \quad P = 0.0005

−log IC₅₀ TII = 0.11 (±0.01) AltAT − 3.78
n = 9 \quad r² = 0.90 \quad F = 64.60 \quad s = 0.42 \quad P = 0.0001

−log IC₅₀ TII = 0.55 (±0.51) GC − 3.11 (NS)
n = 9 \quad r² = 0.14 \quad F = 1.16 \quad s = 1.25 \quad P = 0.32

**DISCUSSION**

The prevalence of giardial infections in the United States and in other parts of the world, coupled with the treatment failures and side effects associated with current antigiardial therapies, has led to increased interest in the development of new antigiardial agents. Benzimidazoles have been shown to be effective in the treatment of this infection (1). This fact, along with the demonstrated antigiardial activity of dicarboxylic pentamide-like molecules, led to the in vitro testing of a number of dicarboxylic substituted bis-benzimidazoles against *G. lamblia*. The data described in the present study demonstrate that *G. lamblia* trophozoites are susceptible to these molecules. Three compounds (compounds 1, 2, and 4) demonstrated antigiardial activities comparable to those of drugs currently used to treat giardiasis. The bis-benzimidazoles exhibited a strong affinity for calf thymus DNA and the homopolymers poly(dA) · poly(dT) and poly(dA-dT) · poly(dA-dT), and they were also capable of inhibiting the activity of giardial topoisomerase II. Antigiardial activity correlated to the affinity of the compounds for calf thymus DNA, poly(dA) · poly(dT), and poly(dA-dT) · poly(dA-dT). The ability of the bis-benzimidazoles to interfere with the relaxation activity of giardial topoisomerase II correlated with both their in vitro activities against *G. lamblia* and the affinity of the molecules for calf thymus DNA, poly(dA) · poly(dT), and poly(dA-dT) · poly(dA-dT).

The DNA binding affinity of dicarboxylic substituted molecules was previously shown to be related to alkyl chain length (2, 5). The present study revealed that compounds containing linkers with an even number of carbon atoms and a higher affinity for calf thymus DNA, poly(dA) · poly(dT) and poly(dA-dT) · poly(dA-dT) demonstrated better in vitro antigiardial activity than compounds with an odd number of carbon atoms in the alkyl chains. These compounds, like the pentamide derivatives, bind to A+T-rich regions in the minor groove of DNA without intercalating between base pairs (9). The preferential binding of these molecules to A+T-rich regions may explain the strong correlations observed between the antigiardial activities of the compounds and their affinities for calf thymus DNA, poly(dA) · poly(dT), and poly(dA-dT) · poly(dA-dT) and the lack of correlation between antigiardial activity and the affinity of the molecules for poly(dG-dC) · poly(dG-dC). The conformation of the molecules also appears to be important for their in vitro activities and DNA binding affinities. Fairley et al.
reported an association between the radii of curvature of these molecules and their affinities for DNA (9). Molecules with radius of curvatures most similar to that of the minor groove of DNA, i.e., compounds 1, 2, and 4, were more-potent inhibitors of the in vitro growth of *G. lamblia*, had a stronger affinity for DNA, and were also better inhibitors of giardial topoisomerase II activity.

In conclusion, a new class of agents with promising antiangiardial activity has been identified. This study has also permitted an evaluation of potential mechanisms of action of deazacentrifugally substituted bis-benzimidazoles. A possible mechanism(s) of action of these molecules is suggested by the correlations among their in vitro antiangiardial activities, DNA binding abilities, and topoisomerase II inhibitory activities. The strong correlation observed between the DNA binding affinities of these compounds and their inhibition of giardial topoisomerase II activity suggests a link between the topoisomerase II inhibitory activities of bis-benzimidazoles and their affinities for DNA. The abilities of these molecules to bind to DNA and to inhibit topoisomerase II should be exploited to aid in the design of more-potent antiangiardial molecules.

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