ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1994, p. 1890–1898
0066-4804/94/$04.00+0

Vol. 38, No. 9

Selective Inhibition of Topoisomerases from *Pneumocystis carinii* Compared with That of Topoisomerases from Mammalian Cells

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Received 29 November 1993/Returned for modification 9 March 1994/Accepted 8 June 1994

Type I and II topoisomerase activities were partially purified from *Pneumocystis carinii*. The catalytic (strand-passing) activities of both enzymes were selectively inhibited by members of a series of dicationic-substituted bis-benzimidazoles compared with those of topoisomerases of mammalian (calf thymus) origin. The most active inhibitors of the parasite enzymes were also highly effective in an in vivo animal model of *P. carinii* pneumonia. Selected dicationic-substituted bis-benzimidazoles also strongly inhibited the induction of the topoisomerase I- and II-mediated cleavable complex, suggesting that the biologically active DNA minor groove-binding molecules inhibit the enzyme-DNA binding step of the topoisomerase reaction sequence. The apparent selectivities for the parasite enzymes and the low levels of toxicity to mammalian cells for the biologically active bis-benzimidazoles suggest that these compounds hold promise as effective therapeutic agents in the treatment of a life-threatening AIDS-related disease, *P. carinii* pneumonia.

Dicationic-substituted aromatic molecules related to pentamidine have long been known to be effective antiparasitic agents (35). Recent studies have found that a number of direct analogs of pentamidine have activity against *Pneumocystis carinii* (19), *Giardia lamblia* (3), *Toxoplasma gondii* (25), *Cryptosporidium parvum* (7), *Leishmania amazonensis* subsp. *mexicana* (5), and *Plasmodium falciparum* (5). A strong correlation was observed between compound activity against *G. lamblia* in vitro and DNA-binding ability (3). On the basis of those initial results, studies of antiparasitic compounds were extended to a series of compounds with much stronger DNA-binding affinities, the dicationic bis-benzimidazoles. As observed with the pentamidine analogs, a strong correlation was found between DNA binding strength and antigiardial activity for the bis-benzimidazole series [r^2 = 0.96 versus calf thymus DNA and r^2 = 0.97 versus poly(dA)·(dT)](4). Other studies confirmed that the effective antigiardial compounds were strong DNA minor groove-binding agents with an AT base pair preference (14). Molecular modeling calculations in that study showed that the DNA-binding strength for this class of compound depended on the radius of curvature on the basis of four defined moieties within the molecules, the distance between cationic moieties, the electronic effects from cationic substituents, and hydrogen bonding. There was no evidence of either an intercalative or a covalent interaction of these compounds with nucleic acids. The antigiardiasis study also revealed a convincing correlation between antitopoisomerase II activity and in vitro activity against *G. lamblia* for the dicationic bis-benzimidazoles (r^2 = 0.91) (4). It is unclear whether these compounds manifest their antiparasitic activity primarily by binding to DNA, topoisomerase, or the enzyme-DNA binary complex.

As part of a continuing investigation to determine the mechanism of the anti-*P. carinii* action of these dicationic molecules and to develop new agents to treat *P. carinii* pneumonia (PCP), the compounds were examined for their inhibitory effects on both *P. carinii* and mammalian topoisomerases. An initial study showed that several pentamidine analogs were effective inhibitors of a crude preparation of the *P. carinii* topoisomerase II enzyme (12). The current study expands on that study by examining the inhibitory effects of a new series of dicationic molecules against both type I and II topoisomerases purified from *P. carinii* and type I and II mammalian (calf thymus) topoisomerases. The results strongly suggest that the dicationic bis-benzimidazoles act by preventing the first step of topoisomerase action, site recognition and cleavage, with a specificity for the parasite enzyme over that for the mammalian enzyme. The original inhibitor studies dealt only with direct pentamidine analogs against the enzyme, but the present study was directed at the dicationic-substituted bis-benzimidazoles. These compounds were selected for the current study on the basis of the previously observed correlation between their DNA-binding strengths, abilities to inhibit topoisomerase, and activities against *G. lamblia*. The primary goal of the present study was to determine if similar correlations could be established for these compounds with *P. carinii* as was seen with *G. lamblia*. In addition, a better understanding of the relationship between DNA binding and topoisomerase inhibition should provide important clues to aid in the design of effective and selective antiparasitic drugs.

MATERIALS AND METHODS

Nucleic acid substrates. Catenated *Crithidia fasciculata* kinetoplast DNA (kDNA) was purchased from TopoGEN Inc. (Columbus, Ohio). The isolation of P_4 phage knotted, supercoiled pBR322 and pBluescriptII KS$^+$ plasmid DNAs has been described previously (6, 12). The procedure for 3′ end labeling of *Eco*RI-digested pBR322 DNA was described previously by Liu et al. (27).

Chemicals. Dithiothreitol, β-mercaptoethanol (β-ME), phe-

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nylmethylsulfonyl fluoride (PMSF), double-stranded DNA cellulose, agarose, and ATP (grade 1) were obtained from Sigma Chemical Co. (St. Louis, Mo.). The ATP was initially dissolved in a small volume of 2 M Tris-HCl (pH 7.5), and the concentration was determined spectrophotometrically by using an extinction coefficient of 15.4×10^3 cm^{-1} M^{-1}. Hydroxyapatite and heparin Sepharose were purchased from Bio-Rad Co. (Hercules, Calif.). Q-Sepharose was obtained from Pharmacia Co. (Piscataway, N.J.). All other reagents were of analytical grade.

Test compounds. The bis-benzimidazole series were synthesized in the laboratory of one of us (R.R.T.) and have been described previously (14, 40). The following DNA minor groove-binding compounds were used as controls: Berenil, 4′,6′-diamidino-2-phenylindole (DAPI), Hoechst 33258, netropsin, and distamycin. All were obtained from Sigma Chemical Co.

Sources of *P. carinii* organisms. In all experiments performed in the present study, we used enzyme obtained from *P. carinii* organisms isolated from the lungs of 100 rats. The organisms were harvested after the rats had been immunosuppressed for 10 to 12 weeks by using a standard protocol that included the administration of daily doses of dexamethasone and tetracycline and a low-protein diet (19). The lungs were excised from euthenized rats after their weights dropped from 200 g to at least 120 g.

Purification of *P. carinii* topoisomerases I and II. The topoisomerase were partially purified from *P. carinii* organisms isolated from immunosuppressed rat lungs as follows. A crude extract from 12 g of purified *P. carinii* (12) in buffer A (50 mM Tris [pH 7.5], 10% glycerol, 1 mM EDTA, 10 mM β-ME, 0.1 mM PMSF) was applied to a Q-Sepharose column (2.5 by 35 cm). All column fractionations were controlled with a fast-performance liquid chromatography system (Pharmacia). The column was washed with two column volumes of buffer A with 0.1 M NaCl, and protein was eluted from the column with a gradient of from 100 mM to 2 M KCl. ATP-dependent and -independent relaxing activities were obtained from fractions that eluted after 0.8 M KCl. The pooled fractions (14.1 mg of protein) were immediately applied to a hydroxyapatite column (2.5 by 10 cm) and were washed with three volumes of buffer P (20 mM KPO_4 [pH 7.0], 10% glycerol, 10 mM β-ME, 0.1 mM PMSF). The column was developed with a gradient of from 20 to 700 mM KPO_4, and ATP-dependent and -independent relaxing activities coeluted at approximately 250 mM KPO_4 (pH 7.0). The pooled activity (900 μg of protein) was applied to a heparin-Sepharose column (1.5 by 12 cm), and after washing with buffer A, the column was developed with a gradient of from 0 to 1.0 M NaCl in buffer A. Active fractions eluted at approximately 0.25 M NaCl. A nuclease activity copurified with the topoisomerase activities through this column. After dialysis against buffer A to reduce the salt, the activity was applied to a monoQ (5/5) column. After washing with buffer A and eluting with a gradient of from 0 to 2.0 M NaCl, the ATP-independent activity eluted at approximately 0.5 M and the ATP-dependent topoisomerase activity eluted at approximately 0.7 M NaCl, with the nuclease activity appearing slightly later. The ATP-independent activity was pooled and dialyzed against 50% glycerol–buffer A and was stored at −20°C. There was a rapid loss of 50% of this activity, but the remaining activity was stable for more than 1 year. One unit of *P. carinii* topoisomerase I was defined as the amount of enzyme required to relax 50% of the form I supercoiled plasmid DNA into form II relaxed molecules at a concentration of 250 nM at 37°C in 30 min. The early-eluting ATP-dependent topoisomerase fractions were pooled separately from the fractions containing the nuclease, dialyzed into 50% glycerol, and stored at −20°C. This activity was stable for more than 1 year and did not contain appreciable nuclease or ATP-independent topoisomerase activity. One unit of *P. carinii* topoisomerase II enzyme was defined as the amount of enzyme with the activity required to completely decatenate 125 ng of kDNA in 30 min at 37°C under the conditions described below. While the efficiency of purification could not be determined, the final total activities achieved were approximately 3,000 U for the topoisomerase I (1,000 U/ml) and 800 U for the topoisomerase II (1,500 U/ml) enzymes.

TABLE 1. Optimization of *P. carinii* catalytic topoisomerase reaction conditions[a]

Reaction condition	Topoisomerase I		Topoisomerase II	
	Concn (mM)	% of maximum	Concn (mM)	% of maximum
Cations				
$MgCl_2$	7.5–10	100	10	100
$MgSO_4$	10	90	5–10	100
$MnSO_4$	5–40	75	NA[b]	
$ZnCl_2$	NA		ND[c]	
$CoCl_2$	NA		ND	
$CaCl_2$	ND		5–10	90
Salt				
KCl	150	100	50	90
NaCl	200	90	100	90
$(NH_4)_2SO_4$	100	80	200	100

[a] The optimum pH for topoisomerase I is 7 to 7.5. The optimum pH for topoisomerase II is 7 to 8.5, and the optimum ATP concentration for topoisomerase II is 1.0 mM. Both enzymes have an absolute requirement for a divalent cation.
[b] NA, no activity.
[c] ND, not determined.

***P. carinii* topoisomerase assays.** The optimum pH and the optimum cation, salt, and ATP (for topoisomerase II) concentrations were determined for the *P. carinii* topoisomerases before performing the drug inhibition studies (Table 1). For the characterization of nuclease activities and cleavable complex formation we used KS^+ DNA restricted with the *Hin*dIII and *Pst*I enzymes and filled in at the *Hin*dIII site with Klenow DNA polymerase and radiolabeled deoxynucleoside triphosphates (3,000 Ci/mmol). The optimized assays are described below.

(i) Supercoiled relaxation assay. The topoisomerase I reaction buffer contained 50 mM Tris (pH 7.5), 50 mM ammonium acetate, 5 mM $MgSO_4$, 35 μg of bovine serum albumin (BSA) per ml, and 25 nM supercoiled KS^+ DNA in a final volume of 15 μl. For topoisomerase II, ATP (Li salt [pH 8.0]) was added at a final concentration of 1 mM. Topoisomerase enzyme was added and the mixture was incubated at 37°C for 30 min and was then stopped by the addition of sodium dodecyl sulfate (SDS) to 1%. After electrophoresis through a 0.8% agarose gel, the samples were stained with ethidium bromide and photographed under UV light.

(ii) Decatenation assay. The topoisomerase II-specific decatenation reaction buffer contained 50 mM Tris (pH 7.5), 50 mM KCl, 50 mM NaCl, 10 mM $MgCl_2$, 0.5 mM sodium EDTA, 30 μg of BSA per ml, 20 mM dithiothreitol, 1 mM ATP, and 130 ng of kDNA in a final volume of 15 μl (13, 28). ATP-dependent topoisomerase from *P. carinii* was added, and the reaction mixture was incubated at 37°C for 30 min and was

then stopped by the addition of SDS to 1%. After electrophoresis through a 0.8% agarose gel, the samples were stained with ethidium bromide and photographed under UV light. The catenated kDNA remained in the wells of the gel, and the decatenated DNA was resolved into relaxed, supercoiled, and multiple moieties. The *P. carinii* enzyme primarily resolves the kDNA into relaxed monomers, with approximately 20% multimers and 5% partially supercoiled moieties also observed. No linearized kDNA was observed under these conditions, and no activity was observed in the absence of ATP.

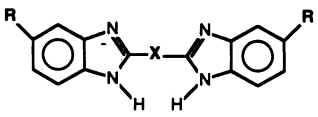
(iii) *P. carinii* topoisomerase I and II catalytic inhibition assays. The results of the *P. carinii* topoisomerase I drug inhibition assays were reported as the concentration of compound required to inhibit 50% of the relaxation of the supercoiled pBluescriptII KS^+ DNA. The test compounds were mixed with the DNA under the reaction conditions described above; this was followed by the addition of 0.5 U of *P. carinii* topoisomerase I and incubation at 37°C for 30 min. The reactions were stopped by the addition of SDS to 1%. After electrophoresis of the samples and photography of the gels, the supercoiled and relaxed DNA substrate bands on the photographic negatives were quantitated by scanning with an LKB laser densitometer.

The *P. carinii* topoisomerase II drug inhibition assays were based on the concentration of compound that inhibited 50% of the decatenation activity. This reaction is specific for type II topoisomerases because kDNA can be decatenated only by a double-strand breakage and resealing. As with the topoisomerase I enzyme, the drugs were mixed with the DNA before the addition of 1.0 U of *P. carinii* ATP-dependent topoisomerase. The reaction mixture was incubated at 37°C for 30 min, and the reaction was then stopped by the addition of SDS to 1%. After electrophoresis through a 0.8% agarose gel, the samples were stained with ethidium bromide and photographed under UV light. Each compound was assayed at least two times, and the concentration of drug required to inhibit 50% of the decatenation activity was determined by densitometric scanning of photographic negatives in the same manner as described above for the type I topoisomerase assay.

Calf thymus DNA topoisomerases I and II. Purified calf thymus DNA topoisomerases I and II were purchased from TopoGEN Inc. and were used as described by the manufacturer.

(i) Calf thymus topoisomerase I and II catalytic inhibition assays. The topoisomerase I relaxation inhibition assay, with supercoiled plasmid pBR322 used as the substrate, was performed as described by Hertzberg et al. (17). The topoisomerase I enzyme was used at a concentration of 1,000 U/ml. The topoisomerase II unknotting inhibition assay, with P_4 phage knotted DNA used as the substrate, was performed as described by Besterman et al. (6). This assay is equivalent to the kDNA decatenation reaction. The topoisomerase II enzyme was used at a concentration of 800 U/ml.

(ii) Calf thymus topoisomerase I and II cleavable complex inhibition assays. The topoisomerase I-mediated cleavable complex assay was performed as described by Kjeldsen et al. (21), with the following modification; in addition to using camptothecin at 10 μM, various concentrations of potential cleavable complex inhibitors were added to the reaction mixtures at time zero. The topoisomerase II-mediated cleavable complex assays were carried out as described by Nelson et al. (30) and Tewey et al. (39), with the following modification; in addition to *m*-AMSA at 20 μM, various concentrations of potential cleavable complex inhibitors were added to the reaction mixtures before the addition of topoisomerase. Since the enzymes purchased from TopoGen did not have sufficient activities for us to perform cleavable complex assays, calf thymus topoisomerases with much higher specific activities were obtained from Peter Leitner of Glaxo Inc. (Research Triangle Park, N.C.). The topoisomerase I from this source had a catalytic activity of 6.5×10^6 U/ml. In the topoisomerase I cleavable complex assay we used 1.3×10^2 U per reaction tube. The topoisomerase II had a catalytic activity of 2.5×10^5 U/ml, and in the cleavable complex assay we used 2.5×10^2 U per reaction tube.



Cmpd. no.		R	X
1	BABIM	Am	CH_2
2	004NAN081	Im	CH_2
3	004SL031	Am	$(CH_2)_2$
4	004NAN089	Im	$(CH_2)_2$
5	004SL037	Am	$(CH_2)_3$
6	40-067	Im	$(CH_2)_3$
7	004KG017	Am	$(CH_2)_4$
8	005NAN021	Im	$(CH_2)_4$
9	BBE	Am	-HC=CH-
10	SB58	IsopropylAm	-HC=CH-

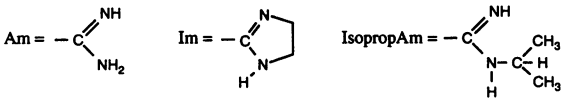


FIG. 1. Structures of dicationic bis-benzimidazoles.

RESULTS

While the yields of the ATP-dependent and -independent topoisomerase activities from *P. carinii* could not be calculated accurately, the preparations were devoid of any detectable endo- or exonuclease activity, as determined on radiolabeled linear DNA substrates (data not shown). The *P. carinii* topoisomerase II enzyme is active on both supercoiled and catenated DNA substrates with an absolute ATP requirement, but it has not been isolated in sufficient concentrations or in the amounts required to perform the drug-dependent DNA cleavage assays. The topoisomerase I activity isolated from *P. carinii* was not stimulated by ATP, and this enzyme preparation was also insufficiently potent for use in DNA cleavage experiments. However, there was enough enzyme to perform catalytic inhibition assays.

Inhibition of *P. carinii* topoisomerase I and II catalytic activities. The primary goal of the study was to determine whether *P. carinii* topoisomerases are potential targets for the antimicrobial action of the bis-benzimidazole compounds. The abilities of these compounds to inhibit the catalytic (strand-passing) activity of partially purified *Pneumocystis* topoisomerases I and II were compared with their observed activities in the rat model of PCP. The structures of the compounds analyzed in the study are shown in Fig. 1. An example of a typical gel used to determine drug inhibition of the topoisomerase II kDNA-decatenating activity is shown in Fig. 2. In this example, both compound 3 and berenil inhibited the

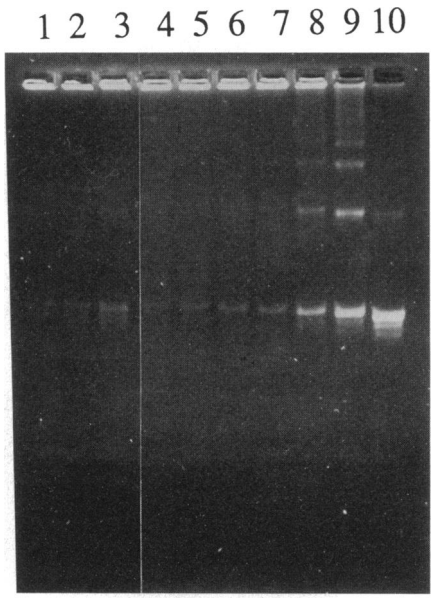


FIG. 2. Inhibition of *P. carinii* topoisomerase II kDNA decatenation by compound 3 and berenil. The assay is described in Materials and Methods. The results presented here are representative of those of the experiments performed with kDNA as the substrate for the *P. carinii* topoisomerase II enzyme. Lanes 1 to 3, twofold dilutions of berenil (25, 12.5, and 6.25 μM) mixed with the substrate before the addition of the *P. carinii* topoisomerase II; lanes 4 to 9, twofold dilutions of compound 3 (20, 10, 5, 2.5, 1.25, and 0.625 μM) mixed with the kDNA substrate before the addition of the *P. carinii* topoisomerase II enzyme; lane 10, no-drug, enzyme-only control.

decatenating activity of the *P. carinii* topoisomerase II enzyme at all concentrations tested. Only in lane 10 of Fig. 2 (the no-drug control) was the kDNA substrate completely relaxed by the enzyme. The catalytic inhibitory activities of all of the compounds tested plus those of four control DNA minor groove-binding drugs are summarized in Table 2. For comparison purposes the values for the DNA-binding strengths and activities against PCP for these compounds are also given in Table 2. Compounds 4, 8, 9, and 10 were the most potent *P. carinii* topoisomerase II inhibitors, with 50% inhibitory concentrations (IC_{50}s) ranging from 0.3 to 1.25 μM. Compounds 3, 4, 8, and 9 were the most active against *P. carinii* topoisomerase I, with IC_{50}s of between 1.25 and 3 μM. Compounds 7 and 10 also showed good activity (IC_{50} = 6 to 10 μM) against the parasite topoisomerase I. There was no quantitative correlation between efficacy in the rat model of disease and the antitopoisomerase activities of the compounds (Table 2). All of the compounds were active against PCP, yet some were significantly less effective as topoisomerase inhibitors (compounds 1, 2, 5, and 6). The compounds that were less effective against the topoisomerases were also relatively weaker DNA-binding compounds. The inability to determine an IC_{50} for the animal model of PCP also made it impossible to assess whether antiparasitic activity and enzyme inhibition were correlated.

The most active topoisomerase inhibitors in this series also exhibited potency against the enzymes in the same range as other potent DNA minor groove-binding compounds such as netropsin, DAPI, berenil, and Hoechst 33258. A correlation could be made between DNA binding strength of the bis-benzimidazoles on poly(dA) · (dT) and inhibition of the topoisomerases from *P. carinii* (r^2 = 0.787 for topoisomerase I and r^2 = 0.691 for topoisomerase II).

It would be very useful to determine whether the most biologically active compounds were able to fragment DNA in combination with topoisomerase II. Many anticancer drugs (e.g., *m*-AMSA and the epipodophyllotoxins) induce the so-called cleavable complex in association with topoisomerase II in addition to inhibiting the enzyme's strand-passing effect. As to which activity is the most predictive of biological usefulness, Rowe et al. (33) concluded that the formation of the cleavable complex correlates much better with cytotoxic potency than

TABLE 2. Effects of dicationic bis-benzimidazoles on *P. carinii* topoisomerase I and II catalytic activity, DNA binding, and PCP

Compound	Topoisomerase I IC_{50} (μM)		Ratio[c]	Topoisomerase II IC_{50} (μM)		Ratio[d]	DNA binding (ΔT_m [°C])[a]			% Control *P. carinii* cysts/g of lung[b]
	P. carinii DNA	Calf thymus DNA		*P. carinii*	Calf thymus DNA		Calf thymus DNA	Poly (dA) · poly (dT)	Poly (dGC) · poly (dGC)	
1	1,500	>50	>0.033	400	>100	<0.25	7.6	11.5	3.5	—[e]
2	300	50	0.17	62	100	1.6	6.9	10.7	4.1	1.57
3	3.1	50	16.1	0.625	>100	>160	18.5	34.6	3.0	0.99
4	3–6	50	16.6	10	>100	<10	18.5	37.2	4.0	1.71
5	>500	50	<0.1	30	>100	>3.3	10.6	20.1	3.3	31.82
6	>160	50	<0.31	50	>100	>2.0	9.9	20.1	3.4	4.43
7	10–12	>50	>5.0	12.5	>100	>8	15.9	31.3	3.3	0.30
8	1.25	>50	>40	1.25	>100	>80	15.3	28.9	4.6	0.08
9	3.1	50	16.1	1.25	100	80	21.8	42.4	5.8	2.68
10	6.25	50	8.0	0.312	50	160	22.8	32.6	5.5	—
DAPI	25–50	12.5	0.5	1–3	50	50	21.5	38.8	6.9	ND[f]
Berenil	250–500	ND	ND	10	ND	ND	ND	ND	ND	75.02
Camptothecin	>100	ND	ND	ND	ND	ND	ND	ND	ND	ND
Hoechst 33258	12.5	12.5–25	1.0	25	12.5	0.5	14.1	32.4	2.5	108.03

[a] The data are described by Fairley et al. (14). ΔT_m, change in melting temperature.
[b] All compounds were tested at 10 mg/kg. These data are described by Tidwell et al. (41).
[c] Ratio of topoisomerase I IC_{50} for calf thymus to that for *P. carinii*.
[d] Ratio of topoisomerase II IC_{50} for calf thymus to that for *P. carinii*.
[e] —, the effectiveness of this compound was quantitated by a histological scoring system (19). It was as effective as compounds 2, 3, and 4.
[f] ND, not done.

does inhibition of catalytic activity. Unfortunately, the *Pneumocystis* topoisomerase II preparation was insufficiently active for us to be able to perform the cleavable complex assay.

Inhibition of calf thymus topoisomerase I and II catalytic activities. In order for a topoisomerase-targeting drug to be selective, it must interact with the enzyme(s) or the enzyme-DNA binary complex of the parasite at lower concentrations than it interacts with the host enzymes. Therefore, it was necessary to determine how active these dicationic compounds were against enzymes of mammalian (calf thymus) origin. The results of this analysis are also outlined in Table 1. The highest drug concentration tested in these in vitro assays was 100 μM. DAPI and Hoechst 33258 were the most effective inhibitors of topoisomerase I, with IC_{50}s of 12.5 μM. None of the bis-benzimidazoles at less than 50 μM showed any activity against the catalytic activity of calf thymus topoisomerase I. DAPI, Hoechst 33258, and distamycin at levels of 5.0, 2.8, and 2.7 μM, respectively, have been reported to inhibit 50% of the catalytic activity of topoisomerase I isolated from L1210 cells (29, 42). Our results showed that these binders to the minor groove of duplex DNA were slightly less active. For mammalian topoisomerase II, only Hoechst 33258 (IC_{50}, 12.5 μM) had a significant inhibitory effect.

None of the dicationic bis-benzimidazoles (tested up to 100 μM) alone generated in vitro cleavable complex formation with either calf thymus topoisomerase I or II, although positive controls with known cleavable complex-generating compounds were fully active when tested at the same time (data not shown). These effects are comparable to those reported for berenil (11), which inhibits calf thymus topoisomerase II decatenation at >150 μM, and netropsin, which inhibits calf thymus topoisomerase II decatenation activity at >25 μM in vivo (2); these two compounds also do not generate cleavable complex formation.

Effect of dicationic bis-benzimidazoles on cleavable complex formation by mammalian topoisomerases. Agents that bind to the minor groove of duplex DNA have been shown, however, to effectively inhibit the drug-induced cleavable complex formation mediated by both topoisomerases I and II (1, 2, 29, 42). Therefore, the minor groove-binding bis-benzimidazoles were also tested for this property. Figures 3, 4, and 5 and Table 3 show the results of those experiments. *m*-AMSA (20 μM) was used to generate topoisomerase II-dependent strand breakage (Fig. 3), and camptothecin (10 μM) was used in the topoisomerase I cleavage experiments (Fig. 4). As shown in Table 3, only the compounds with the ethene bridge (compounds 9 and 10) significantly inhibited cleavable complex formation by both topoisomerases I and II from calf thymus. In addition, compounds 9 and 10 showed cleavage inhibitory activities equivalent to those of the classical minor groove binders DAPI, distamycin, Hoechst 33258, and netropsin (Table 3). It is interesting that compounds 3, 4, and 8, which were the most potent inhibitors of the catalytic activity of *P. carinii* topoisomerases I and II (Table 1), had little or no effect in the calf thymus topoisomerase cleavable complex inhibition assay.

The DNA cleavage profiles shown in Fig. 3 and 4 revealed a very interesting phenomenon. In addition to completely inhibiting DNA cleavage at high concentrations, the active bis-benzimidazole compounds stimulated specific drug-dependent cleavage sites at low concentrations. Figure 3 shows the topoisomerase II-mediated cleavable complex inhibition profile for compound 9. Compound 1, a noninhibitor (Table 3), was included as a negative control. The dominant sites for *m*-AMSA-induced DNA cleavage by calf thymus topoisomerase II are located at approximately nucleotides 3380, 2790, 1630, and 560 relative to the *Eco*RI site. At a concentration of

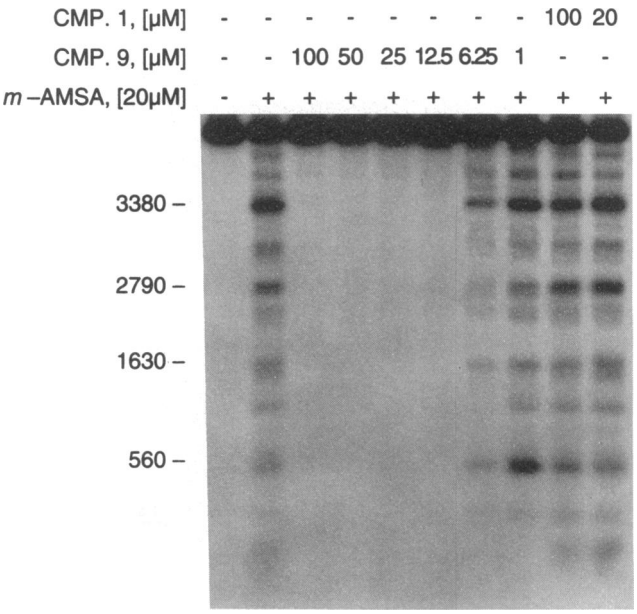


FIG. 3. Dicationic compounds inhibit *m*-AMSA-dependent cleavage of pBR322 by calf thymus topoisomerase II. The experiments were performed to determine the data in Table 3 on the effects of the bis-benzimidazoles on the *m*-AMSA-dependent DNA cleavage reaction. Compounds 9 and 1 (CMP. 1 and 9) at various dilutions were mixed with the end-labeled linear DNA substrate after the addition of *m*-AMSA but before the addition of the calf thymus topoisomerase II enzyme. Compound 9 but not compound 1 inhibited the formation of the cleavable complex by the topoisomerase. After electrophoresis the gels were quantitated by measuring the radioactivity in each band in an AMBIS beta-imager.

12.5 μM (and greater), all of the cleavage sites were completely inhibited by compound 9. At 6.25 μM, the fragment at nucleotide 2790 was essentially absent, whereas the other three fragments were beginning to reappear. Interestingly, the cleavage at the site at nucleotide 560 was dramatically stimulated with 1 μM compound 9 in comparison with the stimulation of the control treated with *m*-AMSA only. Compound 10, which was identical to compound 9 except for an isopropyl group on the amidino groups (Fig. 1), did not induce hypercleavage at the site at nucleotide 560 at a concentration of 0.1 or 1.0 μM (data not shown). However, compound 10 at even lower concentrations appeared to significantly increase DNA cleavage at the site at nucleotide 3380 in comparison with the cleavage observed for the control treated with *m*-AMSA only. Compound 3 gave a DNA fragment profile identical to that of compound 4 in the in vitro cleavable complex inhibition assay (data not shown). Notably, none of the control compounds (DAPI, distamycin, Hoechst 33258, and netropsin) showed similar concentration-dependent biphasic fluctuations in cleavage site intensities. These fluctuations in fragment intensities also tended to complicate the determination of IC_{50}s in this assay. The IC_{50}s were computed by measuring the total counts in each lane relative to the counts in the lanes containing topoisomerase, radiolabeled DNA, and the cleavable complex-inducing standard compound (*m*-AMSA for topoisomerase II and camptothecin for topoisomerase I).

Figure 4 shows the topoisomerase I-mediated cleavable complex inhibition profile for compound 9. The dominant cleavage sites for camptothecin-induced fragmentation of pBR322 DNA span specific stretches of nucleotides designated

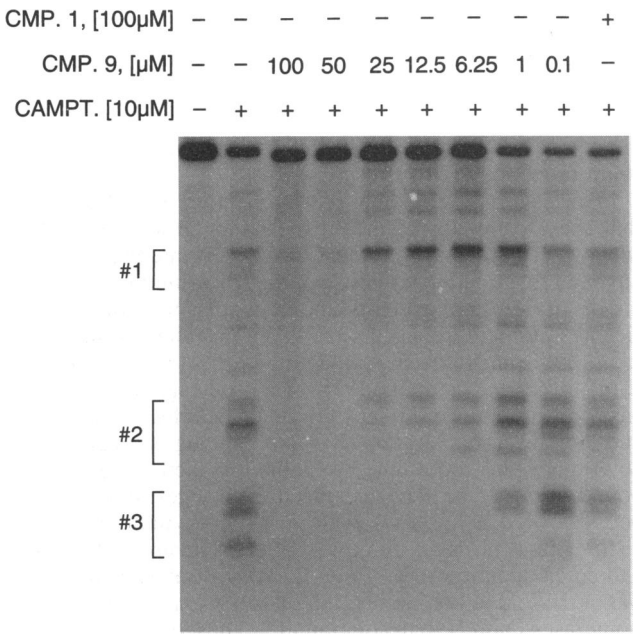


FIG. 4. Dicationic bis-benzimidazoles inhibit camptothecin (CAMPT)-dependent cleavage of pBR322 by calf thymus topoisomerase I. The results presented here exemplify those in studies similar to those for which the results are presented in Fig. 3 performed on the camptothecin-dependent cleavable complex formation catalyzed by calf thymus topoisomerase I. The experiment was performed in the same manner as that for topoisomerase II cleavable complex formation, except that camptothecin and topoisomerase I were used. Note that there is a stimulation of the production of some of the bands relative to the control lane with just camptothecin. These results are quantitated in Fig. 5.

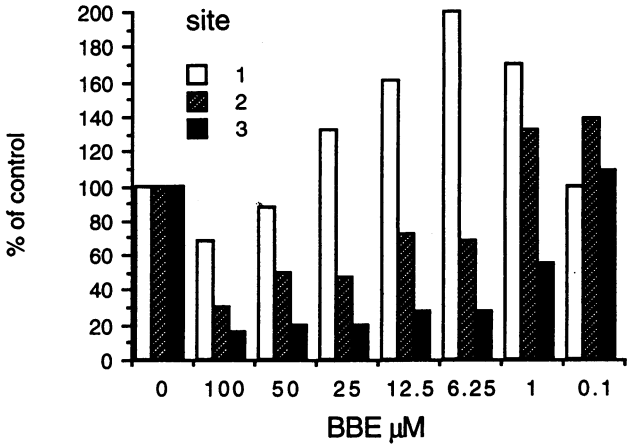


FIG. 5. Compound 9 [*trans*-1,2-bis(5-amidino-2-benzimidazolyl) ethene (BBE)] selectively stimulates the production of the topoisomerase I-camptothecin-dependent cleavage products. During quantitation of the autoradiograph presented in Fig. 4, the three major areas of cleavage products (from top to bottom, sites 1, 2, and 3) were separately determined and compared with the no-drug control lane. The values in the graph are expressed as a percentage of the value in the control lane for the three areas of the gel. This bar graph shows that compound 9 actually stimulates the production of the largest band at 1 to 25 μM and that of the band in the second region at 0.1 to 1 μM. We found that this observation is specific for the topoisomerase I enzyme and compound 9.

at sites 1, 2, and 3. At a concentration of 50 μM (and greater), compound 9 totally inhibited cleavage of the DNA at sites 2 and 3 and inhibited site 1 by at least 95%. Similar to what was observed with the cleavable complex inhibition profile for topoisomerase II, certain cleavage sites were significantly stimulated by low concentrations of compound 9. Two examples of this phenomenon for compound 9 are cleavage site 1 at concentrations of 6.25 and 1.0 μM and cleavage site 3 at 0.1 μM (Fig. 4). The cleavage profile around site 1 was even more intriguing because stimulation disappeared for compound 9 at 0.1 μM. This peak of cleavage was at 6.25 μM, with a twofold increase in the production of this band. These data are graphically represented in Fig. 5 for the three areas shown in Fig. 4, illustrating the dramatic effect on region 1. Significantly, this phenomenon was observed for compound 9 only.

TABLE 3. Effects of dicationic bis-benzimidazoles on calf thymus topoisomerase I and II inhibition in drug-dependent cleavage reactions

Compound	Drug-dependent cleavage inhibition (IC_{50} [μM])	
	Camptothecin topoisomerase I	*m*-AMSA topoisomerase II
1	>100	>100
2	>100	>100
3	>100	>100
4	67	>100
5	>100	>100
6	>100	>100
7	>100	>100
8	>100	>100
9	19.9	7.02
10	14.5	4.7
Netropsin	24.6	9.56
Distamycin	10.5	3.22
DAPI	11.2	3.27
Hoechst 33258	15.6	6.76

Unlike compound 9, compounds 2, 3, 4, and 10 did not stimulate cleavage at nucleotide region 3 at low concentrations (data not shown). However, similar to compound 9, they all significantly stimulated cleavage at site 1 over a concentration range of 1 to 10 μM (data not shown). Interestingly, the four minor groove-binding drugs, DAPI, distamycin, netropsin, and Hoechst 33258, acted in the same manner. Each compound failed to stimulate cleavage at nucleotide region 3, but they significantly stimulated cleavage at site 1 at concentrations of between 1 and 10 μM. These two regions of the linear DNA template differed markedly with respect to the effects that minor groove-binding agents have on the subsequent recognition and binding of mammalian DNA topoisomerase(s).

Selectivity of the dicationic bis-benzimidazoles: mammalian versus *Pneumocystis* DNA topoisomerases I and II. Table 2 shows the ratios of IC_{50}s (when the endpoints could be determined) of the bis-benzimidazole series against the catalytic activities of the *P. carinii* topoisomerases versus those of the calf thymus topoisomerases. The larger the value, the more sensitive the *P. carinii* enzyme is to the compound compared with the sensitivity of the mammalian enzyme (i.e., the more selective the compound). Compounds 3, 4, 9, and 10 were the most selective in terms of their activities against DNA topoisomerase I, exhibiting 1 order of magnitude preference for the *Pneumocystis* enzyme. Compounds 4 and 10 were by far the most selective for the *Pneumocystis* topoisomerase II, with a

differential of 2 orders of magnitude compared with their preference for the mammalian topoisomerase II. While compounds 3, 4, 8, and 9 are quite active in the rat model of PCP, the limited availability of compound 10 prevented the testing of compound 10 for its anti-PCP activity. Although the ratios for compound 8 could not be precisely determined, it selectively inhibited both types of topoisomerases. The ratio was at least 40 for topoisomerase I and 80 for topoisomerase II. It is worth noting that compound 8 was among the most effective compounds in the treatment of PCP in the rat model (41).

DISCUSSION

The current work originated with studies on pentamidine analogs that clearly showed a correlation between DNA minor groove-binding strength and antiparasitic activity (3). On the basis of the results of those studies, nine dicationic-substituted bis-benzimidazoles with potent minor groove-binding properties were examined for their in vitro activities against *G. lamblia*. The results of that analysis showed a strong correlation between in vitro antigiardial activity, DNA-binding affinity, and the ability to inhibit the catalytic activity of giardial DNA topoisomerase II (4). This correlation suggested that the antiparasitic activity of the bis-benzimidazoles is linked to DNA binding and further suggested that inhibition of giardial DNA topoisomerase II is a factor in drug activity. On the basis of the results of the studies with *G. lamblia*, these compounds were tested against *P. carinii* in a rat PCP model (41). Several of the dicationic bis-benzimidazoles were found to be highly effective anti-*P. carinii* agents. The primary goal of the current study was to determine whether *Pneumocystis* DNA topoisomerases serve as primary targets for any of these compounds and whether a correlation exists between the strength of DNA binding, enzyme inhibition, and biological activity, similar to the relationship found with *G. lamblia*.

The anticipated correlation between topoisomerase inhibition and antiparasitic activity was not observed with *P. carinii*. However, the biologically active bis-benzimidazoles did show significant selectivity for the parasite enzymes over that for their mammalian (calf thymus) counterparts. It is not feasible to generate 50% protective dose data in the animal model of pneumocystosis, and this may account for the failure to establish a convincing correlation between enzyme inhibition and antiparasitic activity. There are other possible explanations. (i) DNA topoisomerases are not the primary targets; perhaps other DNA-dependent enzymes are key targets for biologically active bis-benzimidazoles. (ii) The complexities of drug metabolism and tissue distribution in animal studies make meaningful correlations between in vitro and in vivo systems difficult, if not impossible. Studies are planned to determine the effect of the compounds on other parasite-derived enzymes in vitro and in vivo.

The results of the present study suggest that selected dicationic bis-benzimidazoles can affect topoisomerase function presumably by their DNA minor groove-binding properties. While intercalators cause major structural distortions, ligands which interact with DNA by groove binding effect only minor changes such as widening of the minor groove of DNA and localized bending of the DNA helix (29). Despite the minimal conformational changes caused by these agents, both distamycin and netropsin have been shown to alter DNA-directed enzyme reactions such as DNase I cleavage (15, 24) and RNA polymerase transcription (8, 37) in vitro. In addition, McHugh et al. (29) have reported that the DNA minor groove-binding drugs distamycin A, Hoechst 33258, and DAPI significantly modulate the enzymatic activities of both topoisomerases I and II purified from L1210 cells. Since topoisomerase activity is essential for viability, even in nonreplicating cells, DNA-binding ligands that significantly alter the binding affinity, the binding site(s), and/or the subsequent enzymatic activity of these important enzymes may be agents of cell death, either directly or by initiating a cascade of events culminating in cell death (e.g., an SOS-like response).

In terms of DNA binding, previous studies have determined that these compounds bind in the minor groove of B-DNA with an AT base pair preference and do not intercalate (14). It has been suggested that the size of the DNA-binding site by the minor groove-binding compound is important to their potencies as antimicrobial agents (42). We postulate from DNase footprinting and molecular modeling studies (14) (unpublished data) that these compounds cover a 4-bp region with an AT base pair preference similar to those of Hoechst 33258, DAPI, and berenil (16, 32, 43). If DNA-binding strength alone determines the topoisomerase inhibitory activity of a compound, it would be expected that the strongest-binding compounds would be the strongest topoisomerase inhibitors. Table 2 shows that this is not the case. Whereas compounds 7 and 8 had equal affinities for all three DNA substrates, compound 8 was a 10-fold more potent inhibitor of *Pneumocystis* topoisomerases I and II than compound 7. Thus, binding affinity to DNA is only part of the story with respect to the ability of these compounds to inhibit the catalytic activities of *P. carinii* topoisomerases. Perhaps this situation is similar to the postulated mechanism of quinolone inhibition of DNA gyrase. For example, Shen et al. (34) suggest that the inhibition of DNA gyrase by norfloxacin occurs as a result of drug binding to an enzyme site which appears after the formation of the binary (gyrase-DNA) complex.

Perhaps the most convincing evidence that bis-benzimidazoles are topoisomerase-interacting agents came as a result of the cleavable complex formation suppression assay. Beerman et al. (2) reported that the minor groove-binding agent netropsin suppresses the catalytic activity of purified topoisomerase II and topoisomerase I- and II-mediated DNA-protein cross-links (cleavable complex formation) in the isolated nuclei of 935.1 cells. The observed inhibitory effects of netropsin against both enzymes have led to investigators conclude that minor groove binders interfere with the action of both topoisomerases I and II in a qualitatively similar manner (29, 42). The nature of this interaction is presumed to be that DNA topoisomerases are unable to interact with DNA sites where the minor groove is already occupied by netropsin (2). As Table 3 shows, compounds 9 and 10 are both more potent inhibitors of calf thymus topoisomerase I- and II-induced cleavable complexes than netropsin.

Another feature that these two bis-benzimidazoles share with some classical minor groove-binding agents is the enhancement of topoisomerase activity at low compound concentrations (Fig. 3 and 4). This concentration-dependent variation in cleavage patterns has been reported for netropsin (2) and distamycin A (29). Stimulation of topoisomerase activity may be related to the ability of certain minor groove-binding compounds to induce DNA bending. It has been postulated that the observation of enzyme stimulation or inhibition depends on the extent to which drug-binding sites are saturated. Thus, drugs at low levels interact with only a fraction of the available sites, resulting in localized distortions of the helix that favor interaction with topoisomerase and a resultant enhancement of enzyme activity (cleavage). At closer to saturating drug levels, these localized distortions that previously favored an enzyme-DNA interaction are reduced, resulting in decreased catalysis (29). The most direct study that addressed this issue

was reported by Chen et al. (10), who showed that Hoechst 33258, Hoechst 33342, distamycin A, berenil, and netropsin induce limited but highly specific topoisomerase I-dependent single-strand breaks at extremely low drug concentrations (<1 μM). The cleavage-stimulating drug concentrations, however, are much less than the cytotoxic concentrations of these compounds, bringing into question the physiological relevance of their observations.

The DNA cleavage profile induced by compound 9 and topoisomerase I at site 1 of linear pBR322 DNA (Fig. 4) is more difficult to interpret. Compound 9, over a concentration range of 1 to 25 μM, significantly stimulated cleavage at site 1 relative to that stimulated by the control with *m*-AMSA only, whereas at 0.1 μM, cleavage at this site was slightly less than that by the control. The biphasic nature of this concentration-dependent response might be explained in terms of a stable distortion in the DNA template that at some critical threshold of drug binding would begin to reverse its configuration. Therefore, the enzyme activity around this site would progress from hypercleavage to hypocleavage within a rather narrow range of drug concentrations. It is perhaps easier to visualize this possibility in the case of a constrained (e.g., supercoiled) DNA template than it is with linear DNA with a free end of rotation. However, there is precedence for localized, stable DNA distortions within a linear DNA fragment. As observed with other topoisomerase inhibitors, it is likely that a ternary complex of drug-DNA-topoisomerase is formed and stabilized, but not in a covalent manner. Experiments to determine on-off rates for the drugs and enzymes on the DNA substrates as well as measurements of direct drug-enzyme interactions will give a clearer picture of this situation.

There is a considerable amount of interest focused on novel topoisomerase-targeting drugs that do not induce cleavable complex formation but instead inhibit the enzyme via an alternative mechanism (18, 38). For example, Sorensen et al. (36) have shown that the anthracycline aclarubicin inhibits the DNA cleavage activity of topoisomerase II by preventing the initial noncovalent DNA-binding reaction of the enzyme. The experimental evidence presented in the current study strongly suggests that the bis-benzimidazoles also act at an early stage in the processive reactions of topoisomerases I and II, before the initial strand cleavage can occur. There appear to be profound differences in the modes of action of different agents targeting topoisomerase(s). These enzymes can be adversely affected by inhibitors at either the DNA-binding, cleavage, or religation step of the total topoisomerase reaction.

The present study unequivocally shows that the biologically active bis-benzimidazoles selectively inhibit *Pneumocysitis* topoisomerases rather than their mammalian counterparts. It is well established that topoisomerases have preferred recognition sequences within which they cleave with a much higher frequency than they do other nucleotide sequences (20, 31). Since *P. carinii* has an AT-rich genome, perhaps its DNA topoisomerases have evolved to preferentially bind AT sequences, the same sequences preferred by the bis-benzimidazoles. This coincidence may explain the selectivities that these compounds have for the topoisomerases of the parasite. In addition, Pommier et al. (31) have shown that the distributions of the cleavage sites are changed by cleavable complex-inducing drugs. Another consideration is the AT base pair preference for the drug binding. Modeling studies of these compounds on DNA have used the sequence GGCAATTGCC (41a). Certain runs of oligomeric adenines with regular spacing can cause bending of the DNA (22), and these bent sites are preferred interaction sites for topoisomerases (9, 26). Furthermore, it was demonstrated that the topoisomerase I-DNA interaction is stabilized by a helix curvature (23). These studies also indicated that topoisomerase I senses the writhe rather than the twist of the substrate. If a minor groove-binding drug (with an AT preference) straightens the intrinsically curved DNA and reduces the writhe, rather than blocks a topoisomerase-binding site, a second mechanism of inhibition besides interference with binding could be contemplated. Finally, any specific interactions of these compounds, through their charged moieties, directly with the enzymes cannot be ruled out at this time. It is entirely possible that the mechanism of inhibition for this series of compounds is through some sort of ternary complex of drug-DNA-enzyme.

In summary, the bis-benzimidazoles analyzed in the present study were significantly more effective inhibitors of *P. carinii* topoisomerases I and II than they were of enzymes of mammalian origin. The ability to selectively inhibit topoisomerases from different species makes these enzymes attractive targets for future antiparasitic drugs.

ACKNOWLEDGMENTS

We acknowledge the support of Susan Jones, Wynella Brake, and Brendan Bender for assistance with the production of purified *P. carinii* organisms and Mike Cory and Richard Lombardy for helpful discussions.

This work was supported by National Institutes of Health grant 1-PO-1-AI-3363-02.

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