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Laboratory note

Synthesis and anti-*Pneumocystis carinii* activity of conformationally restricted analogues of pentamidine

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Abstract – A series of conformationally restricted analogues of pentamidine in which the flexible central bridge has been replaced by trans-cyclopropyl, phenyl, pyridinyl, piperazinyl or homopiperazinyl groups as conformationally restricted linkers have been synthesized. The anti-*Pneumocystis carinii* activity of these compounds was evaluated in a cell culture model and the DNA binding affinity was determined by thermal denaturation measurements. At 1 μM, compounds **2**, **3**, **5**, **7**, **9** and pentamidine were highly effective and caused total inhibition of *P. carinii* growth in culture. At 0.1 μM, compounds **2**, **5**, **7** and **10** were more active than pentamidine with N, N′-bis(4-amidinophenyl)piperazine **7** being approximately 15-fold more effective than pentamidine. The most active compounds, **7** and **10**, showed strong binding affinities for calf thymus DNA and poly(dA-dT); however, a clear correlation between DNA binding affinity and the in vitro anti-*P. carinii* activity of these compounds was not observed. The results suggest that the nature of the central linker influences the biological actions of these compounds. © Elsevier, Paris

***Pneumocystis carinii* pneumonia / pentamidine analogues / conformationally restricted / DNA / poly(dA-dT)**

1. Introduction

Pentamidine, 1,5-bis(4-amidinophenoxy)pentane, is an effective agent widely used for the treatment and prophylaxis of *Pneumocystis carinii* pneumonia (PCP), an opportunistic infection commonly seen in patients with acquired immunodeficiency syndrome (AIDS). Despite its clinical usefulness against PCP, the drug is plagued with an extraordinary number of serious adverse reactions [1, 2]. We hypothesize that the multiple pharmacological actions of the drug might be due to its conformational flexibility resulting in indiscriminate binding to both target and non-target macromolecules. Therefore, we are interested in studying the effect of restricting the conformational flexibility of pentamidine analogues on their anti-*P. carinii* activity. We [3–5] and others [6–8] have recently reported on the synthesis and promising anti-*P. carinii* activities of a series of cis- and trans-butamidine analogues [3, 4], piperidine-linked aromatic diimidazolines [5], furans [6, 7], and 2,4-diarylpyrimidines [8] as conformationally restricted analogues of pentamidine. Several of these compounds were found to be more active and less toxic than pentamidine when evaluated against PCP in the immunosuppressed rat model [3, 4, 6, 7].

Although the precise mechanism of action of pentamidine and its analogues as anti-PCP agents is unknown, recent evidence suggests that these compounds bind in the minor groove of DNA and interfere with the normal functions of DNA-dependent enzymes (e.g. topoisomerases) that regulate transcription in the pathogen [9]. Using crystallographic [6, 10, 11], foot-printing [12], and other biophysical techniques [13], it has been shown that pentamidine and its analogues interact with DNA in the minor groove and prefer AT to GC rich DNA sequences. The interactions involve hydrogen-bonding between the amidine groups of pentamidine analogues and the bases and sugars of DNA. In addition, Van der Waals contacts also occur between the drug and the walls of the narrow AT-rich minor groove [6, 10, 11]. Despite investigations into the DNA-binding mode of pentamidine analogues, a

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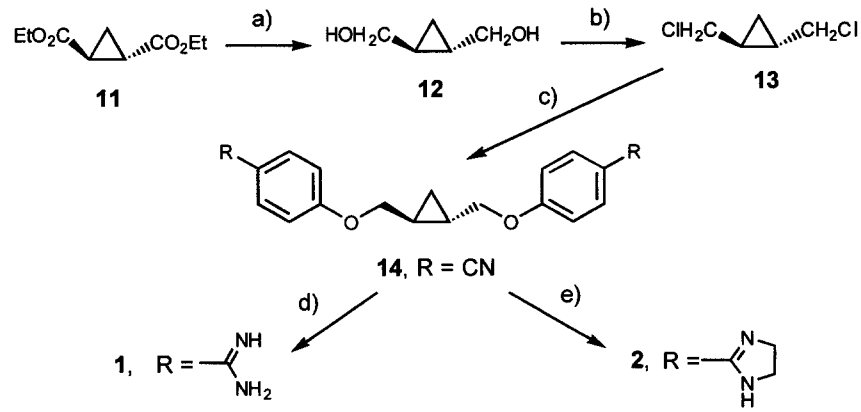


Figure 1. Reagents used for the synthesis of compounds **1** and **2:** a) $LiAlH_4$/ether, reflux; b) $SOCl_2$/pyridine/THF; c) 4-cyanophenol, Na/EtOH; d) HCl (g)/EtOH/THF; NH_3 (g)/MeOH, reflux; e) HCl (g)/EtOH/THF; ethylenediamine/MeOH, reflux.

clear correlation between the DNA binding affinity and their in vivo anti-PCP actions has not been established. More recently, inhibition of an endo/exonuclease in *P. carinii* has been implicated as a mode of anti-*P. carinii* action for several dicationic furans [7].

In this paper we describe the synthesis and in vitro anti-*P. carinii* activity of a series of conformationally restricted analogues of pentamidine *(table I)*. The binding affinity of these compounds to calf thymus DNA and poly(dA-dT) was also determined. Several compounds with strong binding affinity to DNA effectively inhibited the growth of *P. carinii* in culture.

2. Chemistry

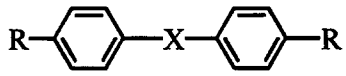
The synthesis of compounds **1–10** was accomplished according to the procedures shown in *figures 1–4*. For the synthesis of **1** and **2** *(figure 1)*, reduction of diethyl trans-1,2-cyclopropanedicarboxylate **11** by $LiAlH_4$ in ether gave the diol **12** which was heated with $SOCl_2$ to give the dichloride **13**, which was then reacted with the anion of 4-cyanophenol to give the dinitrile **14**. Treatment of **14** with dry HCl gas in anhydrous EtOH and anhydrous THF gave the imidate ester, which was refluxed either in methanolic ammonia to give the diamidine **1** or with excess ethylenediamine in anhydrous EtOH to give the diimidazoline **2** [14]. Similar procedures were used to convert the other dinitriles to the corresponding diamidines or diimidazolines **3–10**. In *figure 2*, **11** was hydrolysed to the corresponding dicarboxylic acid **15** which was reacted with $SOCl_2$ to give the acid dichloride **16**. Treatment of **16** with two moles of 4-cyanoaniline in the presence of N,N-diisopropylethylamine gave the dinitrile **17**. Similar reactions of acid dichlorides **18** and **19** with 4-cyanoaniline gave the corresponding dicyano compounds **20** and **21** *(figure 3)*. The dicyano compounds **17**, **20** and **21** were converted to the diamidines and diimidazolines **3** and **4** *(figure 2)*, **5** and **6** *(figure 3)* in a manner similar to **1** and **2**. In *figure 4*, an aromatic nucleophilic displacement reaction between 4-fluorobenzonitrile **24** and piperazine **22** or homopiperazine **23** afforded the corresponding dinitrile **25** or **26**. The dinitriles **25** and **26** were easily converted to the corresponding diamidines **7** and **9**, or the diimidazolines **8** and **10**.

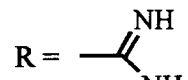
3. Biological results and discussion

The in vitro anti-*P. carinii* activity and DNA binding properties of the synthesized compounds **1–10** are shown in *table II*. The compounds were tested in a short-term culture assay system using human embryonic lung cells with *P. carinii* from infected rat lung as described previously [15, 16]. This culture model has been used effectively as a rapid screen to predict the anti-*P. carinii* activities of experimental compounds [16, 17].

In this study, pentamidine and the untreated control were used as the positive and negative controls, respectively. The compounds were tested in the culture model at 1.0 and 0.1 μM concentrations in two separate experiments (indicated as exp. 1 and exp. 2 in *table II*). Compounds **2**, **3**, **4**, **6**, **7**, **9** and **10** were evaluated in experiment 1 and compounds **1**, **5**, and **8** were evaluated in experiment 2. Of the nine compounds **1–9** tested at 1.0 μM, five compounds (i.e. **2**, **3**, **5**, **7** and **9**) and pentamidine were highly effective and totally inhibited the growth of *P. carinii* in culture. Compound **1** also showed

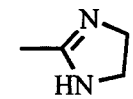
Table I. Structures and physical properties of pentamidine analogues **1–10.**



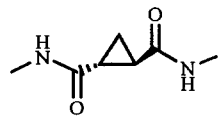
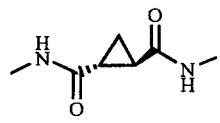
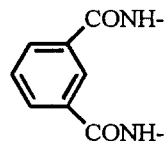
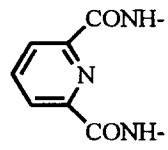
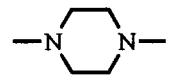
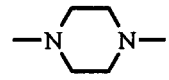
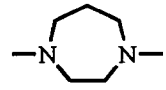
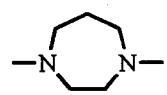


(AM)

or



(IM)

Cpd	R	X	Yield %[a]	M.p. (°C)[b]	^{1}H NMR[c]
1	AM		66	257–259	9.23 (s, 4H), 9.02 (s, 4H), 7.83 (d, 4H), 7.12 (d, 4H), 4.14 (m, 4H), 1.88 (m, 4H).
2	IM		42	201–203	10.64 (s, 4H), 8.03 (d, 4H), 7.14 (d, 4H), 4.14 (m, 4H), 3.91 (s, 8H), 1.86 (m, 4H).
3	AM		64	> 360	11.10 (s, 2H), 9.15 (s, 8H), 7.79 (m, 8H), 2.46 (m, 2H), 1.38 (m, 2H).
4	IM		63	> 360	11.16 (s, 2H), 10.55 (bs, 4H), 8.00 (d, 4H), 7.87 (d, 4H), 3.97 (s, 8H), 2.45 (m, 2H), 1.40 (m, 2H).
5	AM		63	> 360	11.23 (s, 2H), 9.30 (s, 4H), 9.02 (s, 4H), 8.94 (s, 1H), 8.22 (d, 2H), 8.20 (d, 4H), 7.88 (d, 4H), 7.74 (t, 1H).
6	AM		58	318	11.72 (s, 2H), 9.33 (s, 4H), 9.04 (s, 4H), 8.44 (d, 4H), 8.32 (m, 3H), 7.93 (d, 4H).
7	AM		81	356	9.02 (s, 4H), 8.69 (s, 4H), 7.79 (d, 4H), 7.15 (d, 4H), 3.58 (s, 8H).
8	IM		65	> 360	10.17 (s, 4H), 7.87 (d, 4H), 7.09 (d, 4H), 3.91 (s, 8H), 3.61 (s, 8H).
9	AM		56	340	8.91 (s, 4H), 8.61 (s, 4H), 7.73 (d, 4H), 6.92 (d, 4H), 3.76 (s, 4H), 3.53 (m, 4H), 1.96 (m, 2H).
10	IM		71	326	10.14 (s, 4H), 7.85 (d, 4H), 6.94 (d, 4H), 3.89 (s, 8H), 3.78 (s, 4H), 3.55 (m, 4H), 1.93 (m, 2H).

[a]Yield from the corresponding nitrile. [b]Dihydrochloride except for **2**, which is the free base. [c]Dihydrochloride in DMSO-d_6.

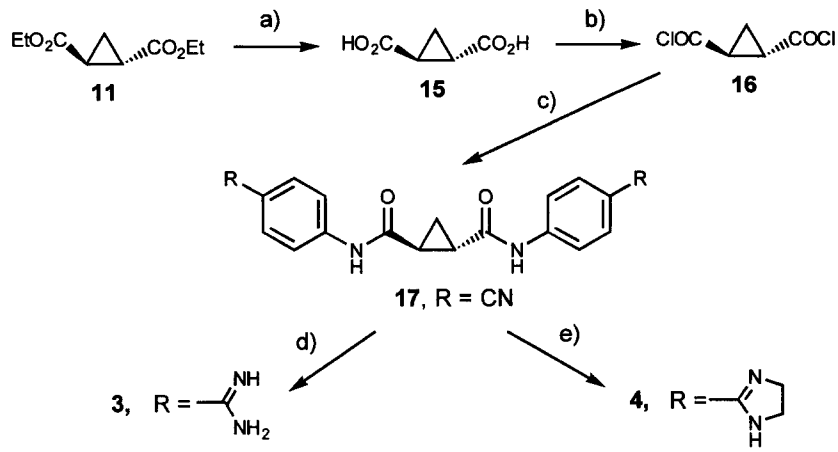


Figure 2. Reagents used for the synthesis of compounds **3** and **4:** a) 10% NaOH, reflux; b) $SOCl_2$; c) 4-cyanoaniline/DIEA/THF; d) HCl (g)/EtOH/THF/$CHCl_3$, NH_3 (g)/EtOH, reflux; e) HCl (g)/EtOH/THF; ethylenediamine/EtOH, reflux.

strong inhibitory activity (93% inhibition) at 1.0 μM. When all ten compounds were tested at 0.1 μM, four compounds (i.e. **2**, **5**, **7** and **10**) were significantly more active than pentamidine. Compound **7** with a piperazine ring as the central linker connecting the benzamidine groups, was the most active and was 15-fold more effective than pentamidine. Among the bisbenzamidines, **1**, **3**, **5**, **6**, **7** and **9**, the rank order of potency against

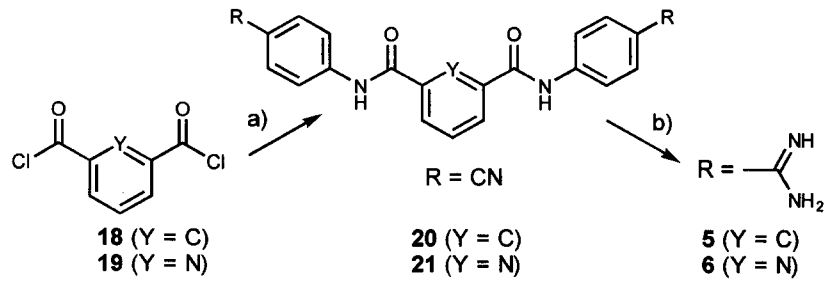


Figure 3. Reagents used for the synthesis of compounds **5** and **6:** a) 4-cyanoaniline/DIEA/THF; b) HCl (g)/EtOH/THF, NH_3 (g)/EtOH, reflux.

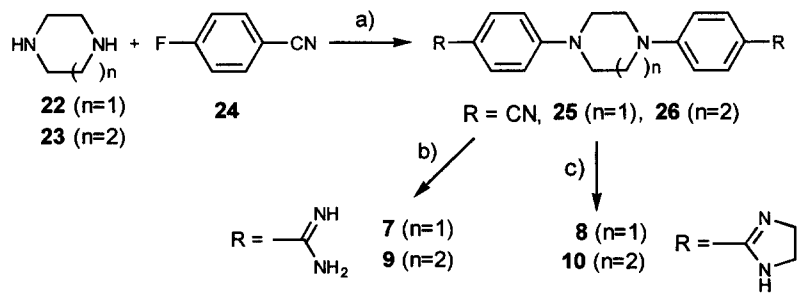


Figure 4. Reagents used for the synthesis of compounds **7–10:** a) K_2CO_3/DMSO, 120 °C; b) HCl(g)/EtOH/$CHCl_3$/THF; NH_3(g)/EtOH, reflux; c) HCl (g)/EtOH/$CHCl_3$, ethylenediamine/EtOH, reflux.

Table II. Anti-*P. carinii* activity and DNA binding affinity of pentamidine analogues.

Exp., compound concentration (μM)	Change in numbers of *P. carinii* trophozoites from day 1 through day 5 in culture ($\times 10^{-5}$)[a]	% of control growth	DNA binding (ΔT_m, °C)	
			Calf thymus DNA[b]	Poly(dA-dT)[c]
Exp. 1, Control	24.10 ± 4.02			
Pentamidine			11.1	20.6
1.0	−3.98 ± 1.64	< 0		
0.1	11.00 ± 5.23	46		
Exp. 2, Control	25.47 ± 3.08			
Pentamidine				
1.0	−2.22 ± 0.70	< 0		
0.1	5.85 ± 2.42	23		
Exp. 2, Compd **1**			11.1	20.3
1.0	1.83 ± 0.70	7		
0.1	7.61 ± 1.17	30		
Exp. 1, Compd **2**			10.0	20.1
1.0	−2.93 ± 1.87	< 0		
0.1	2.15 ± 2.34	9		
Exp. 1, Compd **3**			7.1	11.1
1.0	−0.12 ± 2.03	< 0		
0.1	25.23 ± 3.43	105		
Exp. 1, Compd **4**			6.0	9.6
1.0	12.67 ± 2.85	53		
0.1	17.74 ± 2.46	74		
Exp. 2, Compd **5**			8.0	9.9
1.0	−1.29 ± 1.36	< 0		
0.1	3.43 ± 1.56	13		
Exp. 1, Compd **6**			7.1	15.9
1.0	9.36 ± 2.22	39		
0.1	23.40 ± 3.59	97		
Exp. 1, Compd **7**			17.0	23.8
1.0	−5.97 ± 0.66	< 0		
0.1	0.78 ± 1.72	3		
Exp. 2, Compd **8**			15.0	19.4
1.0	4.99 ± 2.26	20		
0.1	21.20 ± 5.46	83		
Exp. 1, Compd **9**			15.0	23.1
1.0	−3.32 ± 1.60	< 0		
0.1	14.62 ± 3.35	61		
Exp. 1, Compd **10**			15.0	24.0
0.1	2.73 ± 1.79	11		

[a]Values are reported as means ± SEMs. Values are calculated by subtracting the numbers of organisms on day 1 of culture from the numbers detected on day 5; thus, positive values denote growth, numbers near zero denote little change, and negative numbers denote decreasing organisms in the culture. [b]Increase in thermal melting of calf thymus DNA. [c]Increase in thermal melting of Poly(dA-dT).

P. carinii at 0.1 μM was **7** > **5** > **1** > **9** > **6** ≈ **3**. Compound **7** with a piperazine linker was 35-fold more potent than **3** which contains the *trans*-1,2-cyclopropanediamide moiety as the central linker. Among the bisbenzimidazolines, **2**, **4**, **8** and **10**, the rank order of potency against *P. carinii* at 0.1 μM was **2** ≈ **10** > **4** ≈ **8**. In this series, compound **2** was about 9-fold more potent than **8**. Substitution of the amidino groups in **1**, **3**, **7** and **9**, with the cyclic imidazolino groups resulted in either, a slight to modest increase (1.4 to 5.5-fold) in anti-*P. carinii* activity at 0.1 μM for **2**, **4**, and **10**, or a dramatic 28-fold reduction in anti-*P. carinii* activity for **8**. These results indicate that the piperazine linker connecting the benzamidine groups in **7** appears to present the optimal conformation for potent anti-*P. carinii* activity in this series of compounds. Compound **7** is also significantly more active than a series of piperidine-linked aromatic diimidazolines reported earlier [5].

The most active compounds, **7** and **10**, were also the strongest DNA binders (ΔT_m: 17.0 °C and 15.0 °C for calf thymus DNA, 23.8 °C and 24.0 °C for poly(dA-dT), respectively) in this series. All ten compounds were found to bind to both calf thymus DNA and poly(dA-dT). Binding to poly(dA-dT) was consistently stronger, in agreement with the observation that pentamidine analogues prefer AT-rich DNA sequences [7, 10, 11]. Compounds containing the more basic amidino groups (e.g. **1**, **3**, and **7**) generally showed stronger binding affinity for calf thymus DNA and poly(dA-dT) than their corresponding counterparts bearing the imidazolino groups (e.g. **2**, **4**, and **8**). The rank orders of binding affinity to poly(dA-dT) by the bisamidines and bisimidazolines were **7** > **9** > **1** > **6** > **3** > **5**, and **10** > **2** > **8** > **4**, respectively. Although the most active compounds, **7** and **10**, have strong binding affinity for DNA, a clear correlation between the in vitro anti-*P. carinii* activity and DNA binding affinity for the compounds in this series was not observed.

4. Conclusions

Ten conformationally restricted analogues of pentamidine with different central linkers have been synthesized and evaluated for anti-*P. carinii* activity. The binding affinity of these compounds to calf thymus DNA and poly(dA-dT) was also measured. Of the ten compounds, the diamidine **7** with a piperazine linker and diimidazoline **10** with a homopiperazine linker were found to be the most potent (15- and 4-fold more active than pentamidine at 0.1 μM, respectively) and had the strongest affinity for DNA. The results indicate that the central linker influences the biological actions of these compounds.

5. Experimental protocols

5.1. Chemistry

Melting points were determined on a Haake-Buchler melting point apparatus and are uncorrected. The ^{1}H NMR spectra were recorded on a Varian Gemini-300 or a GE Omega-500 spectrometer and d_6-dimethylsulfoxide was used as the solvent unless otherwise noted. The chemical shifts (δ) are reported in ppm relative to TMS, 0.00 ppm. Elemental analyses (C, H, N) were performed by M-H-W Laboratories, Phoenix, AZ and are within ± 0.4% of the theoretical values. All chemicals and solvents were purchased from Aldrich Chemical Company. THF was distilled from Na and benzophenone before use and other chemicals were used as received. Anhydrous ethanol and methanol were used throughout this study unless stated otherwise.

5.1.1. General procedure for the synthesis of diamidines and diimidazolines **1–10** *from the corresponding dinitriles*

To a cooled, stirred solution of a dinitrile compound in EtOH, anhydrous THF and/or $CHCl_3$, anhydrous HCl gas was bubbled through for 1 h. The solution was then allowed to reach room temperature and stirred for 3 d. The solvent was concentrated to near dryness below 40 °C under reduced pressure and ether was added to precipitate the imidate salt. The solid, after filtration, was immediately dissolved in MeOH or EtOH. For the synthesis of amidines, anhydrous ammonia gas was bubbled through the solution for 1 h, during which time the solution was heated and then refluxed for 4 h. The solvent was then removed under reduced pressure and ether was added to precipitate the crude product after cooling. Recrystallization from the appropriate solvent(s) gave the pure amidine as the dihydrochloride salt. For the synthesis of imidazolines, excess ethylenediamine was added to the solution of the imidate salt in MeOH or EtOH. The mixture was refluxed for 2–4 h before the solvent was evaporated. Ether was added to the residue to afford the imidazoline free base, which was refluxed in saturated HCl-EtOH solution for 2–4 h. The solvent was then removed under reduced pressure and the residue was purified by recrystallization in the appropriate solvent(s) to give the desired imidazoline as the dihydrochloride salt, with the exception of **2** which was obtained as a free base without refluxing in HCl-EtOH.

5.1.2.1. Trans-1,2-bis(4-cyanophenoxymethylene) cyclopropane **14**

Anhydrous ether (60 mL) was added to $LiAlH_4$ powder (1.20 g, 30.0 mmol) and the mixture was stirred under a N_2 atmosphere. After 10 min, a solution of diethyl trans-1,2-cyclopropanedicarboxylate **11** (4.66 g, 25.0 mmol) in ether (20 mL) was added at such a rate as to keep the ether refluxing gently. The suspension was stirred for an additional 30 min, during which time an extra portion of ether (60 mL) was added to dilute the suspension. Water (1 mL), 15% NaOH (1 mL) and water (3 mL) were added dropwise in sequence to the stirred reaction system under cooling. The resulting mixture was then filtered and the filtrate was dried over Na_2SO_4. Evaporation of the solvent gave **12** (1.40 g, 55%) as a white liquid. It was used without further purification. ^{1}H NMR ($CDCl_3$): δ 4.94 (s, 2H), 3.46–3.37 (m, 4H), 0.99–0.92 (m, 2H), 0.45–0.40 (m, 2H).

To a stirred solution of diol **12** (1.22 g, 12.0 mmol) and pyridine (0.25 mL) in anhydrous THF (40 mL), cooled in an ice-bath, was slowly added a solution of $SOCl_2$ (4.5 mL, 20.6 mmol) in anhydrous THF (20 mL). The

mixture was stirred overnight and then brought to reflux for 4 h. After cooling, cold water was added and the mixture was extracted with ether (2 × 50 mL). The extracts were combined and washed with 10% $NaHCO_3$ until neutral pH and then with water (2 × 20 mL) and dried over $MgSO_4$. Distillation gave **13** (1.23 g, 67%) as a pale yellow oil. It was used without further purification. ^1H NMR ($CDCl_3$): δ 4.04 (dd, 4H, J = 14.5 and 7.0 Hz), 1.98 (t, 2H, J = 7.0 Hz), 1.30 (t, 2H, J = 7.0 Hz).

A solution of 4-cyanophenol (1.58 g, 13.3 mmol) in EtOH (10 mL) was slowly added to a solution of sodium (0.33 g, 14.4 mmol) and EtOH (10 mL). To this mixture was slowly added a solution of **13** (0.92 g, 6.62 mmol) in EtOH (5 mL). The mixture was then heated and refluxed for 5 d and filtered after cooling. The solid was washed with copious amounts of water and dried at 40 °C for 4 h to give **14** (1.52 g, 75%) as a white solid. ^1H NMR ($CDCl_3$): δ 7.58–7.55 (m, 4H), 6.92–6.90 (m, 4H), 4.07–4.05 (m, 4H), 2.00–1.98 (m, 4H).

5.1.2.2. Trans-1,2-bis(4-amidinophenoxymethylene) cyclopropane **1**

Following the general procedure (*5.1.1.*), a reaction of **14** (0.76 g, 2.50 mmol) in EtOH (10 mL) and anhydrous THF (60 mL) with HCl (g) gave the imidate salt, which was treated with anhydrous ammonia in MeOH (60 mL) to give **1** (0.68 g, 66%) as a white solid after recrystallization (MeOH/EtOAc: 1/5). Anal. $C_{19}H_{22}N_4O_2 \cdot 2HCl \cdot 2H_2O$ (C, H, N).

5.1.2.3. Trans-1,2-bis(4-imidazolinophenoxymethylene)cyclopropane **2**

Following the general procedure (*5.1.1.*), a reaction of **14** (0.186 g, 0.61 mmol) in EtOH (5 mL) and anhydrous THF (30 mL) with HCl (g) gave the imidate salt, which was treated with ethylenediamine (1 mL) in MeOH (30 mL) to give **2** (0.10 g, 42%) as a white solid after recrystallization from methanol, m.p. 201–203 °C. For the determination of ^1H NMR, the free base was converted to its dihydrochloride form by refluxing in HCl-EtOH. The elemental analysis was determined for the free base form. Anal. $C_{23}H_{26}N_4O_2$ (C, H, N).

Compounds **3–10** were prepared according to the above procedures, and the physical data of compounds **1–10** are listed in *table I*.

5.1.3. General procedure for the synthesis of dinitriles **17**, **20** *and* **21**

Following a known procedure [18], a solution of the appropriate acid dichloride in THF was added to a stirred solution of 4-aminobenzonitrile (2 eq) and N, N-diisopropylethylamine (DIEA, 2 eq) in THF at 0 °C under a N_2 atmosphere. After 30 min, another portion of DIEA (2 eq) in THF was added and the mixture stirred overnight. The solvent was removed under reduced pressure. Recrystallization of the residue in the appropriate solvent(s) gave the corresponding dinitrile.

5.1.3.1. N,N'-Bis(4-cyanophenyl)trans-1,2-cyclopropanediamide **17**

A solution of diethyl trans-1,2-cyclopropanedicarboxylate **11** (10.0 g, 11.4 mmol) in 100 mL of 2 M NaOH was refluxed for 1 h and then stirred overnight at room temperature. The mixture was acidified with 2 M HCl to pH 3 and extracted with EtOAc (2 × 60 mL) after saturation with NaCl. The combined organic layers were washed with brine and dried over Na_2SO_4. Evaporation of the solvent afforded **15** (5.15 g, 81%) as a white solid. ^1H NMR: δ 12.60 (s, 2H), 1.86–1.83 (m, 2H), 1.24–1.20 (m, 2H).

Under a N_2 atmosphere, a mixture of **15** (3.54 g, 27.2 mmol) and $SOCl_2$ (10 mL) was heated at 50–60 °C for 30 min. Removal of the excess of $SOCl_2$ gave 3.2 g (80%) of **16** which was used without further purification. This compound **16** was immediately used in the next reaction because of its instability.

Following the general procedure (*5.1.3.*), **17** was prepared from **16** in 62% yield after recrystallization (DMSO/H_2O: 1/1). ^1H NMR: δ 10.80 (s, 2H), 7.75–7.71 (m, 8H), 2.31 (t, 2H, J = 7.0 Hz), 1.34 (t, 2H, J = 7.0 Hz).

5.1.3.2. N,N'-Bis(4-cyanophenyl)1,3-benzodiamide **20**

Yield 77%, after recrystallization (DMSO/MeOH: 1/1). ^1H NMR: δ 10.80 (s, 2H), 8.51(s, 1H), 8.16 (dd, 2H, J = 10.0 and 8.0 Hz), 7.98 (d, 4H, J = 8.5 Hz), 7.82 (d, 4H, J = 8.5 Hz), 7.72 (t, 1H, J = 8.0 Hz).

5.1.3.3. N,N'-Bis(4-cyanophenyl)2,6-pyridinediamide **21**

Yield 72%, after recrystallization (DMSO/MeOH: 1/1). ^1H NMR: δ 11.30 (s, 2H), 8.41 (d, 2H, J = 7.5 Hz), 8.32 (t, 1H, J = 6.0 Hz), 8.16 (d, 4H, J = 8.5 Hz), 7.91 (d, 4H, J = 8.0 Hz).

5.1.3.4. N,N'-Bis(4-cyanophenyl)piperazine **25**

Under a N_2 atmosphere, a mixture of piperazine **22** (3.4 g, 40.0 mmol), 4-fluorobenzonitrile **24** (9.7 g, 80.0 mmol) and anhydrous K_2CO_3 (16.5 g, 120.0 mmol) in anhydrous DMSO (100 mL) was heated at 120 °C for 6 h. After it was cooled to room temperature, an excess of water was added to the reaction mixture. The precipitate was filtered, washed with water and dried to give **25** (9.6 g, 83%) as a pale yellow solid. ^1H NMR ($CDCl_3$): δ 7.53 (d, 4H, J = 8.0 Hz), 6.87 (d, 4H, J = 9.5 Hz), 3.52 (s, 8H).

5.1.3.5. N,N'-Bis(4-cyanophenyl)homopiperazine **26**

Following the procedure described for the synthesis of **25**, compound **26** was obtained in 80% yield. ^{1}H NMR ($CDCl_3$): δ 7.48 (d, 4H, J = 7.0 Hz), 6.72 (d, 4H, J = 7.0 Hz), 3.71 (s, 4H), 3.49 (t, 4H, J = 7.0 Hz), 2.13-2.10 (m, 2H).

5.2. Pharmacology

5.2.1. Screening of compounds for anti-P. carinii activity in culture

The method used to evaluate the anti-*P. carinii* activity of the prepared compounds has been described previously [15, 16]. All the compounds were dissolved in DMSO for testing. The data for day 7 are not included for analysis because the controls began to lose viability at that time point.

5.2.2. Thermal denaturation studies

Pentamidine, EDTA, Tris-HCl, calf thymus DNA and poly(dA-dT) used in this study were purchased from Sigma Chemical Company. The method used for the determination of the binding affinity (ΔT_m) of the compounds **1–10** to calf thymus DNA and the nucleic acid homopolymer poly(dA-dT) has been described previously [19, 20]. The binding affinity was measured by determining the change in midpoint (T_m) of the thermal denaturation curves of calf thymus DNA as well as poly(dA-dT) at a 1:5 compound to base pair ratio. The thermal denaturation temperatures of calf thymus DNA or poly(dA-dT) were determined in 5.0 mM, pH 7.55, Tris-HCl buffer containing 50 μM of pure EDTA, 5% DMSO, and the appropriate concentration of the test compounds dissolved in DMSO. Calf thymus DNA and poly(dA-dT) were used at an initial absorbance of about 0.3 at 260 nm. The melting curves were recorded on a Beckman DU Series 640 spectrophotometer, with a jacketed six cuvette *T*m cell holder. The cell holder was heated by the Peltier Temperature Controller that was programmed at a constant rate of 0.5 °C/min. The cell temperature was simultaneously recorded by a temperature probe embedded into the cell block. Data were collected by using the T_m analysis accessory software installed on the spectrophotometer and the program recorded absorbance, temperature, and cuvette number. Each sample was read for 0.10 s, and 5.0 mM, pH 7.55, Tris-HCl buffer containing 50 μM of pure EDTA and 5% DMSO was used as the blank. At the end of each experiment, T_m results were calculated from either the first derivative or the 2-points fit calculation. Under these conditions, the typical T_m values for calf thymus DNA and poly(dA-dT) were 60.1 and 36.1 °C, respectively. Each ΔT_m value reported in *table II* represents the mean of at least two experimental determinations.

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References

[1] Wharton J.M., Coleman D.L., Wofsy C.B., Ann. Intern. Med. 105 (1986) 37–44.

[2] Amsden G.W., Kowalsky S.F., Ann. Pharmacother. 26 (1992) 218–226.

[3] Donkor I.O., Jones S.K., Tidwell R.R., Bioorg. Med. Chem. Lett. 3 (1993) 1137–1140.

[4] Donker I.O., Tidwell R.R., Jones S.K., J. Med. Chem. 37 (1994) 4554–4557.

[5] Huang T.L., Zhang Q., White A.T., Queener S.F., Bartlett M.S., Smith J.W., Donkor I.O., Bioorg. Med. Chem. Lett. 6 (1996) 2087–2090.

[6] Boykin D.W., Kumar A., Spychala J., Zhou M., Lombardy R.J., Wilson W.D. et al., J. Med. Chem. 38 (1995) 912–916.

[7] Boykin D.W., Kumar A., Xiao G., Wilson W.D., Bender B.C., McCardy D.R., Hall J.E., Tidwell R.R., J. Med. Chem. 41 (1998) 124–129.

[8] Kumar A., Boykin D.W., Wilson W.D., Jones S.K., Bender B.K., Dykstra C.C., Hall J.E., Tidwell R.R., Eur. J. Med. Chem. 31 (1996) 767–773.

[9] Dykstra C.C., McClerson D.R., Elwell L.P., Tidwell R.R., Antimicrob. Agents Chemother. 38 (1994) 1890–1898.

[10] Nunn C.M., Neidle S., J. Med. Chem. 38 (1995) 2317–2325.

[11] Trent J.O., Clark G.R., Humar A., Wilson W.D., Boykin D.W., Hall J.E. et al., J. Med. Chem. 39 (1996) 4554–4562.

[12] Bailly C., Perrine D., Lancelot J.C., Saturnino C., Robba M., Waring M.J., Biochemistry 323 (1997) 23–31.

[13] Lombardy R.L., Tanious F.A., Ramachandran K., Tidwell R.R., Wilson W.D., J. Med. Chem. 39 (1996) 1452–1462.

[14] Tidwell R.R., Jones S.K., Geratz J.D., Ohmeng K.A., Cory M., Hall J.E., J. Med. Chem. 33 (1990) 1252–1257.

[15] Queener S.F., Bartlett M.S., Nasr M., Smith J.W., Antimicrob. Agents Chemother. 37 (1993) 2166–2172.

[16] Bartlett M.S., Shaw M.M., Navaran P., Smith J.W., Queener S.F., Antimicrob. Agents Chemother. 39 (1995) 2436–2441

[17] Queener S.F., J. Med. Chem. 38 (1995) 4739–4759.

[18] Wang W., Lown J.W., J. Med. Chem. 35 (1992) 2890–2897.

[19] Cory M., McKee D.D., Kagan J., Henry D.W., Miller J.A., J. Am. Chem. Soc. 107 (1985) 2528–2536.

[20] Fairley T.A., Tidwell R.R., Donkor I.O., Naiman N.A., Ohemeng K.A., Lombardy R.J., Bentley J.A., Cory M., J. Med. Chem. 36 (1993) 1746–1753.