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Preliminary communication

Piperazine-linked bisbenzamidines: a novel class of antileishmanial agents

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Abstract

A series of 13 1,4-diarylpiperazines has been prepared, evaluated for antileishmanial activity and their binding affinity to DNA was measured. Among these compounds, 1,4-bis[4-(1*H*-benzimidazol-2-yl)phenyl]piperazine (**14**) emerged as the most active compound with an IC_{50} value of 0.41 μM which is about sevenfold more potent than pentamidine.

Keywords: Amphotericin B; *Leishmania donovani*; 1,4-Diarylpiperazines; DNA binding; Diamidines; Pentamidine

1. Introduction

Leishmaniases are parasitic diseases transmitted by the bite of the infected female phlebotomine sandfly and manifest with visceral, cutaneous, and mucocutaneous forms. They are a significant cause of morbidity and mortality in the developing countries of the world, and they affect about 2 million people per annum mostly in tropical and subtropical regions, the geographical distribution being limited by the biotope of the insect vector [1].

Chemotherapy for these parasitic diseases is generally ineffective mainly due to the emergence of drug-resistant strains and toxicity of the therapeutic agents [2]. The pentavalent antimonials are widely used as primary therapy whereas alternative drugs include amphotericin B, pentamidine, paromomycin, and azoles [3,4]. However, resistance to antimonials is common [5] and treatments with amphotericin B and pentamidine are plagued by severe toxic side effects [3].

Recently, we reported on the synthesis of a series of novel 1,4-diarylpiperazines as potential antipneumocystis [6] and trypanocidal agents [7]. As part of our research program on the development of novel antifungal and antiparasitic drug candidates, we decided to synthesize an expanded series of 1,4-diarylpiperazines, and investigated their biological effects against the *Leishmania* parasites. In particular, we focused our attention on the preparation of piperazine-linked bisbenzamidines and related derivatives, and evaluated the in vitro antileishmanial activity and DNA binding properties of these compounds.

2. Chemistry

The key step for the preparation of 1,4-diarylpiperazines is a double nucleophilic displacement of fluorine in 4-fluoro derivatives by the nitrogen atoms of piperazine. That reaction, performed in DMF at 120 °C, affords the expected tricyclic molecules in good yields provided the aromatic precursor bears a strong electron-withdrawing group in the para position.

Conversion of the dinitrile **1** into the amidines **3–7** (Scheme 1) was effected by the Pinner reaction [8,9]. Treatment of a solution of **1** in dichloromethane with methanol and gaseous hydrochloric acid afforded the intermediate bisbenzenecarboximidic ester (**2**) which was subsequently reacted with ammonia or the appropriate primary amine (methylamine, propylamine, cyclohexylamine, benzylamine). Action of a diamine (ethylenediamine or propylenediamine)

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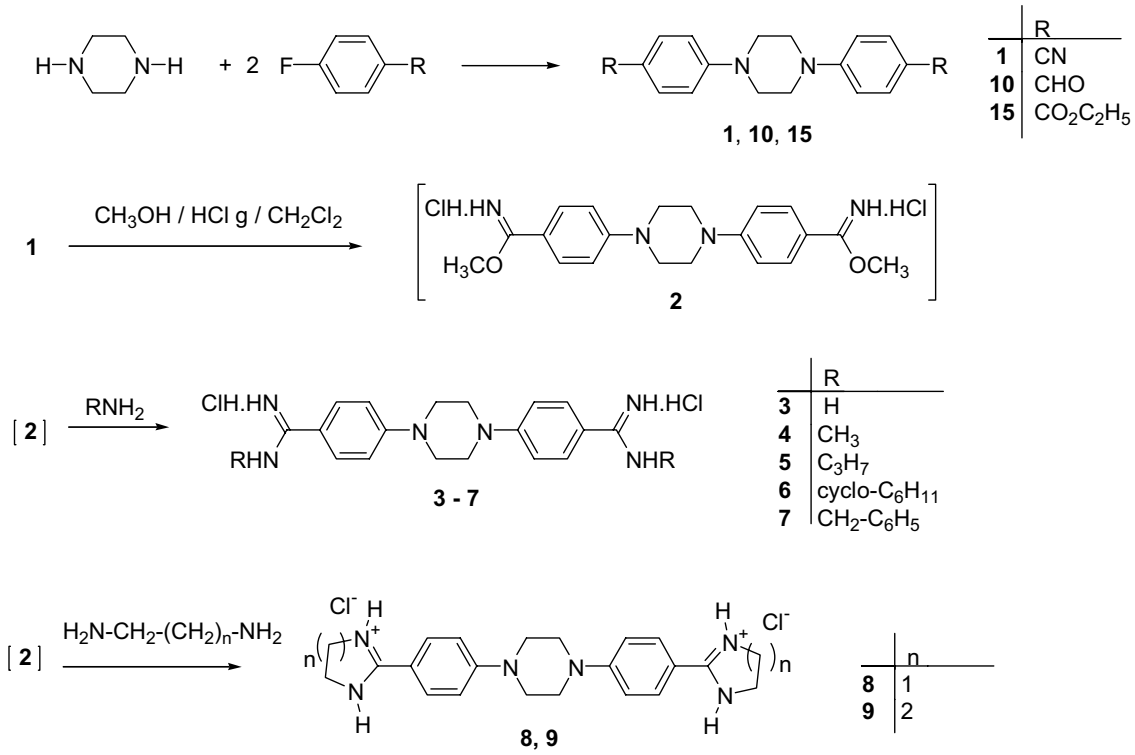


Scheme 1. Synthesis of compounds **1–10** and **15**.

on **2** enabled to prepare **8** and **9** bearing an imidazoline or a tetrahydropyrimidine system as the end groups (Scheme 1).

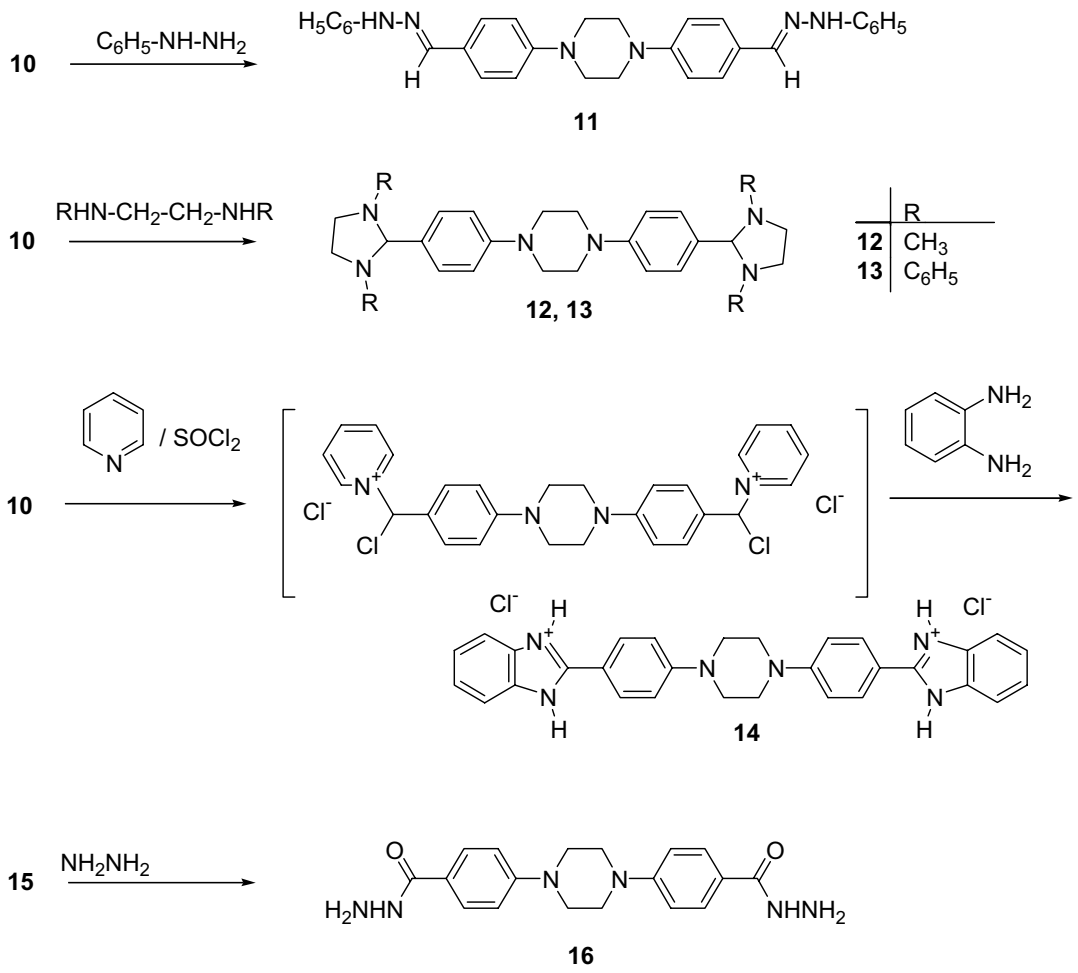
From the dialdehyde **10** (Scheme 2) and phenylhydrazine, we obtained the hydrazone **11**, by azeotropic distillation of water in boiling toluene. Compound **12**, bearing dimethylimidazolidinyl rings was prepared similarly. However, **13**, containing diphenylimidazolidinyl rings, could not be obtained in a pure state by that procedure. Compound **13** was synthesized by refluxing **10** and dianilinoethane in a mixture of ethanol and acetic acid [10]. The bisbenzimidazole compound **14**, on the other hand, could not be synthesized from the bisulfite adduct [11] of **10** and phenylenediamine. It was, however, conveniently isolated after activation of the aldehyde group with pyridine and thionyl chloride and subsequent reaction with the diamine [12,13]. The dicarboxhydrazide compound **16** (Scheme 2) was obtained by hydrazinolysis of the diester **15**.

3. Biological evaluation

In the sandfly vector, *Leishmania* parasites exist as extracellular promastigotes, while in the mammalian hosts they exist primarily as intracellular amastigotes within phagolysosomes of macrophages. There are different methods to evaluate the in vitro leishmaniacidal activity of potential drugs. Most of them are based on testing the action of the compounds on viability, metabolic activity, or proliferation of the promastigotes. Those methods involve microscopic counting of live promastigotes in control and treated culture [14], quantification of intracellular ATP levels [15], the spectrophotometric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2*H*-tetrazolium (MTS)-based assay [16], a radiorespirometric microtest [17], and the fluorometric/colorimetric Alamar blue test [18] which is used in this study. The Alamar blue test measures the effect of the compounds on the growth of the promastigotes in culture. Assessment of effects of potential drugs on the intracellular survival of amastigotes requires amastigote-macrophage cultures [19] or axenic amastigote cultures [14] and typically enables investigation of drug sensitivity at various stages of the life of the parasite.

4. Results and discussion

The data (Table 1) suggests that several 1,4-diarylpiperazines represent interesting leads as antileishmanial agents, and their activity are dramatically dependent on the nature of the aryl substituents. Among this series of compounds, diester **15** and dicarboxhydrazide **16** have no inhibitory effect on the *L. donovani* promastigotes, whereas weak inhibition was observed with the dihydrazone **11**. Clearly, piperazines 1,4-disubstituted by 4-benzamidine groups (e.g. **3, 4, 6** and **7**) or related moieties (e.g. **8** and **14**) emerged as the most promising compounds. The parent diamidine **3**, however, exhibits moderate inhibition and it appears that the inhibitory activity can be modulated by the introduction of an alkyl group on a nitrogen atom of the amidine function (**4–7**). An alkyl group such as propyl (**5**) is detrimental to activity, whereas a smaller alkyl group such as methyl (**4**) or large cyclic moieties such as cyclohexyl (**6**) or benzyl (**7**) increased



Scheme 2. Synthesis of compounds **11–14** and **16**.

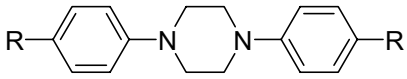
potency by 1.8–5.0-fold based on the IC_{50} values. The benzyl derivative (**7**) has an inhibitory profile comparable to that of pentamidine, a drug clinically used in the treatment of Leishmaniasis. More interestingly, the most potent compound (**14**) is a derivative in which both nitrogen atoms are included in a five-membered cyclic ring. Compound **14** was about sevenfold more potent than pentamidine and almost as potent as amphotericin B in the in vitro study. The size or the rigidity of the cyclic ring seems to play an important role in the mode of action of the agent, since going from a five-membered ring (**8, 14**) to a six-membered ring (**9**) leads to a complete disappearance of the antileishmanial effect. It should be noted that compounds substituted by a saturated five-membered ring, namely the imidazolidinyl-containing compounds **12** and **13** were devoid of activity. It is likely that steric hindrance of the methyl or phenyl groups around the nitrogen atoms may prevent binding of the compounds to the macromolecular target in the parasite.

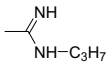
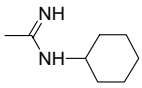
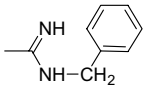
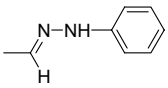
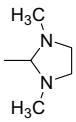
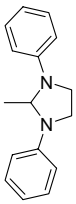
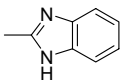
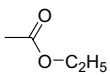
The binding of these compounds to calf thymus DNA and poly(dA-dT) was determined (see Table 1) in order to investigate the relationship between DNA binding and antileishmanial activity. A direct correlation was not apparent for this series of compounds. It is interesting to note that the most active compound, **14**, was one of the weakest DNA binder. However, compounds that showed moderate inhibition of the parasite, namely **3, 4, 6, 7** were good binders of DNA. This suggests that binding to DNA may not be the major mode of antileishmanial action of this class of compounds and that other mechanisms of action such as the disruption of polyamine metabolism [20,21], inhibition of topoisomerase II [22], and inhibition of serine proteases [23] may be involved. In fact, diamidines have been found to be pleotropic (multitarget) compounds, which effect different cellular functions. Uptake of diamidines by *Leishmania* and trypanosome cells occurs through arginine/nucleoside/polyamine transporters [24,25]. Therefore, relative uptake of different diamidine molecules and their effect on other targets functions may also be important for their leishmanicidal action. Based on this observation, it is likely that the compounds reported here may inhibit the parasite by binding to several macromolecular targets in the parasite.

5. Conclusions

In summary, a series of 1,4-diarylpiperazines were synthesized and evaluated for their in vitro inhibitory activity against the *Leishmania* parasite. The DNA binding affinity of

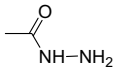
Table 1
Antileishmanial activity and DNA binding affinity of 1,4-diarylpiperazines



Compound Number	*R*	Antileishmanial activity [a] IC_{50} (μM)	IC_{90} (μM)	DNA binding (ΔT_m, °C) Calf thymus DNA	Poly (dA-dT)
3		21.5 ± 4.3	>100.0	16.8	25.3
4		8.5 ± 2.1	>100.0	15.3	22.2
5		NA [b]	–	17.0	26.0
6		11.6 ± 2.6	21.4 ± 5.7	15.5	23.6
7		4.3 ± 1.0	15.7 ± 3.6	18.0	25.1
8		6.7 ± 2.5	10.0 ± 1.3	15.8	18.3
9		NA [b]	NA [b]	12.2	17.9
11		33.7 ± 5.9	>100.0	–0.2	0.0
12		>100.0	–	2.4	1.8
13		NA [b]	–	–1.2	0.0
14		0.41 ± 0.07	0.63 ± 0.20	0.1	2.0
15		NA [b]	–	0.6	0.0

(continued on next page)

Table 1
(continued)

Compound Number	*R*	Antileishmanial activity [a] IC_{50} (μM)	IC_{90} (μM)	DNA binding (ΔT_m, °C) Calf thymus DNA	Poly (dA-dT)
16		NA [b]	–	0.1	0.0
Pentamidine		2.9 ± 0.4	10.1 ± 2.0	14.0	26.0
Amphotericin B		0.14 ± 0.03	0.58 ± 0.12	–	–

[a] Values are given as mean ± S.D. of at least three observations.
[b] NA: no activity (no effect on growth of *Leishmania* cells up to the highest concentration (50 μg ml^{-1}) tested).

these compounds was also measured. A correlation between the in vitro antileishmanial activity and DNA binding affinity of the diamidines reported here was not observed. The antileishmanial effect was dramatically dependent on the nature of the substituents attached on the aryl groups. The bisamidine moiety is essential for activity, although the activity can be readily modulated by introduction of substituents on the nitrogen atoms of the amidine functions. Such modifications led to the identification of 1,4-bis[4-(1*H*-benzimidazol-2-yl)phenyl]piperazine (**14**) as a promising candidate since it was about sevenfold more potent than pentamidine and almost equipotent to amphotericin B. Further studies on compound **14** and optimization of its structure leading to novel analogues with superior biological properties are on going in our laboratories.

6. Experimental protocols

6.1. Chemistry

^{1}H NMR spectra were obtained using a Varian Inova instrument (500 MHz), chemical shifts (δ) are given in ppm using TMS as internal reference. IR spectra were recorded on a Perkin–Elmer Spectrum One instrument operating in the diffuse reflectance mode. Solvents and reagents are commercially available (Aldrich, Acros Organics, Fisher Scientific, Sigma Chemical Co.) and were used without further purification.

Compounds **1** [26], **3** [6,27], **8** [6,27], **10** [28], and **11** [29] have been described in the literature.

Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ.

*6.1.1. General procedures for compounds **4–7** and **9***

A mixture of 4,4′-(1,4-piperazinediyl)bisbenzonitrile (**1**) (2 mmol; 0.6 g) in dichloromethane (250 ml) and methanol (10 ml) was saturated with HCl gas and the reaction medium was left at room temperature for 4 days. The precipitate was filtered and washed with acetone. The crude imidate was used without further purification and treated with the appropriate (di)amine.

*6.1.1.1. 4,4′-(1,4-Piperazinediyl)bis(*N*-methyl benzenecarboximidamide), dihydrochloride salt (**4**).* The compound was obtained by treatment of the crude imidate with methylamine (50 mmol; 3.9 ml of an aqueous solution at 40%) in ethanol (50 ml) at reflux for 30 min. The precipitate was filtered from the hot mixture and washed with ethanol.

Yield: 40%.

M.p.: >300 °C.

NMR (DMSO-d_6): 9.2 (br, 6H); 7.7 (d, 4H, J = 9 Hz); 7.1 (d, 4H, J = 9 Hz); 3.6 (s, 8H); 3.0 (s, 6H) ppm.

IR: 3124; 2847; 1668; 1505; 1452; 1232 cm^{-1}.

$C_{20}H_{26}N_6 \cdot 2HCl$ (423.38). Calc.: C, 56.74; H, 6.67; N, 19.85. Found: C, 56.82; H, 6.53; N, 20.02.

*6.1.1.2. 4,4′-(1,4-Piperazinediyl)bis(*N*-propyl benzenecarboximidamide), dihydrochloride salt (**5**).* The compound was obtained by treatment of the crude imidate with propylamine (20 mmol; 1.7 ml) in ethanol (50 ml) at room temperature for 3 days. The reaction medium was concentrated under reduced pressure and the residue was washed with ether.

Yield: 30%.

M.p.: >300 °C.

NMR (DMSO-d_6): 9.6 – 8.6 (br, 6H); 7.7 (d, 4H, J = 9 Hz); 7.1 (d, 4H, J = 9 Hz); 3.5 (s, 8H); 3.4 (t, 4H); 1.6 (sext, 4H, J = 7 Hz); 0.9 (t, 6H, J = 7 Hz) ppm.

IR: 3048; 1672; 1606; 1515; 1385 cm^{-1}.

$C_{24}H_{34}N_6 \cdot 2HCl$ (479.49). Calc.: C, 60.12; H, 7.57; N, 17.53. Found: C, 59.94; H, 7.32; N, 17.61.

*6.1.1.3. 4,4′-(1,4-Piperazinediyl)bis(*N*-cyclohexyl benzenecarboximidamide), dihydrochloride salt (**6**).* The compound was obtained by treatment of the crude imidate with cyclohexylamine (20 mmol; 2.3 ml) in boiling ethanol (50 ml) for 30 min. The solid was filtered and successively washed with water, ethanol, and ether.

Yield: 25%.

M.p.: >300 °C.

NMR (DMSO-d_6): 9.2 (br, 4H); 9.1 (br, 2H); 7.7 (d, 4H, J = 9 Hz); 7.1 (d, 4H, J = 9 Hz); 3.5 (m, 2H); 3.5 (s, 8H); 1.9 (d, 4H, J = 8 Hz); 1.8 (d, 4H, J = 8 Hz); 1.6 (d, 2H, J = 12 Hz); 1.4 (m, 8H); 1.1 (m, 2H) ppm.

IR: 3053; 1673; 1606; 1516; 1234 cm^{-1}.

$C_{30}H_{42}N_6 \cdot 2HCl$ (559.62). Calc.: C, 64.39; H, 7.92; N, 15.02. Found: C, 64.46; H, 7.72; N, 15.15.

*6.1.1.4. 4,4′-(1,4-Piperazinediyl)bis[*N*-(phenylmethyl) benzenecarboximidamide], dihydrochloride salt (**7**).* The compound was obtained by treatment of the crude imidate with benzylamine (20 mmol; 2.2 ml) in ethanol (50 ml) at reflux for 30 min. The precipitate was filtered from the hot mixture and washed with water, ethanol, and ether.

Yield: 50%.

M.p.: >300 °C.

NMR (DMSO-d_6): 10.0 (s, 2H); 9.3 (s, 2H); 8.9 (s, 2H); 7.8 (d, 4H, J = 8 Hz); 7.4 (m, 8H); 7.3 (t, 2H, J = 7 Hz); 7.1 (d, 4H, J = 8 Hz); 4.7 (s, 4H); 3.6 (s, 8H) ppm.

IR: 3099; 1668; 1607; 1518; 1384; 1235 cm^{-1}.

$C_{32}H_{34}N_6 \cdot 2HCl$ (575.57). Calc.: C, 66.78; H, 6.30; N, 14.60. Found: C, 66.98; H, 6.50; N, 14.41.

*6.1.1.5. 1,4-Bis [4-(1,4,5,6-tetrahydropyrimidin-2-yl) phenyl] piperazine, dihydrochloride salt (**9**).* The compound was obtained by treatment of the crude imidate with 1,3-diaminopropane (30 mmol; 2.5 ml) in ethanol (50 ml) at reflux for 90 min. After cooling, the precipitate was filtered and washed with dimethylformamide (5 ml) and ether.

Yield: 25%.

M.p.: >300 °C.

NMR (DMSO-d_6): 9.5 (br, 4H); 7.6 (d, 4H, J = 9 Hz); 7.1 (d, 4H, J = 9 Hz); 3.5 (s, 8H); 3.4 (t, 8H, J = 5 Hz); 1.9 (mult, 4H, J = 5 Hz) ppm.

IR: 3271, 1638, 1602, 1516, 1231 cm^{-1}.

$C_{24}H_{30}N_6 \cdot 2HCl \cdot 1.8H_2O$ (507.90). Calc.: C, 56.76; H, 7.07; N, 16.55. Found: C, 56.91; H, 7.35; N, 16.67.

*6.1.2. 1,4-Bis(1,3-dimethylimidazolidine-2-yl)piperazine (**12**)*

A mixture of 4,4′-(1,4-piperazinediyl)bisbenzaldehyde (**10**) (2.9 g, 10 mmol), *N,N′*-dimethylethylenediamine (85%, 2.1 g, 20 mmol), and *p*-toluenesulfonic acid (100 mg) in benzene (100 ml) was heated under reflux in a Dean Stark apparatus for 5 h. After cooling the solvent was evaporated under reduced pressure to afford a solid.

The final product was recrystallized from acetonitrile.

Yield: 70%.

M.p.: 204–206 °C.

NMR ($CDCl_3$): 7.4 (d, 4H, J = 9 Hz); 7.0 (d, 4H, J = 9 Hz); 3.4 (m, 4H); 3.3 (s, 8H); 3.2 (s, 2H); 2.5 (m, 4H); 2.2 (s, 6H) ppm.

IR: 2940; 2826; 1611; 1515; 1449; 1227; 1043; 1032 cm^{-1}.

$C_{26}H_{38}N_6$ (434.62). Calc.: C, 71.85; H, 8.81; N, 19.34. Found: C, 72.05; H, 8.71; N, 19.50.

*6.1.3. 1,4-Bis(1,3-diphenylimidazolidine-2-yl)piperazine (**13**)*

A mixture of 4,4′-(1,4-piperazinediyl)bisbenzaldehyde (**10**) (2.9 g, 10 mmol), dianilinoethane (8.5 g, 40 mmol), and acetic acid (10 ml) in ethanol (100 ml) was heated under reflux for 16 h. After cooling, the precipitate was filtered and washed with acetone.

The final product was recrystallized from a mixture (1:1) of ethanol and dioxane.

Yield: 75%.

M.p.: 250–255 °C (decomposition).

NMR (DMSO-d_6): 7.4 (d, 4H, J = 8 Hz); 7.1 (t, 8H, J = 8 Hz); 6.8 (d, 4H, J = 8 Hz); 6.7 (d, 8H, J = 8 Hz); 6.6 (t, 4H, J = 8 Hz); 6.1 (s, 2H); 3.9 (m, 4H); 3.7 (m, 4H); 3.6 (s, protons from the dioxane of recrystallization); 3.1 (s, 8H) ppm.

IR: 3071; 2963; 2826; 1599; 1482; 1236; 750 cm^{-1}.

$C_{46}H_{46}N_6 \cdot 1/2C_4H_8O_2$ (726.40). Calc.: C, 79.31; H, 6.93; N, 11.56. Found: C, 79.67; H, 7.08; N, 11.54.

*6.1.4. 1,4-Bis[4-(1*H*-benzymidazol-2-yl)phenyl]piperazine, dihydrochloride salt (**14**)*

A solution of 4,4′-(1,4-piperazinediyl)bisbenzaldehyde (**10**) (10 mmol; 2.94 g) in dichloromethane (100 ml) was added dropwise to a mixture of thionyl chloride (24 mmol; 1.8 ml) and pyridine (24 mmol; 2 ml) in dichloromethane (100 ml). The reaction medium was stirred for 2 h at room temperature. 1,2-Phenylenediamine (60 mmol; 6.5 g) was slowly added and the mixture was stirred overnight. The precipitate was filtered and washed successively with water, ethanol, and dichloromethane.

Yield: 85%.

M.p.: >300 °C.

NMR (DMSO-d_6): 8.1 (d, 4H, J = 9 Hz); 7.6 (dd, 4H, J = 6 and 3 Hz); 7.3 (dd, 4H, J = 6 and 3 Hz); 7.2 (d, 4H, J = 9 Hz); 3.7 (s, 8H) ppm.

IR: 3600–2200 (br); 1603; 1508; 1229; 1034; 745 cm^{-1}.

$C_{30}H_{26}N_6 \cdot 2HCl$ (543.49). Calc.: C, 66.30; H, 5.19; N, 15.46. Found: C, 66.05; H, 5.32; N, 15.30.

*6.1.5. 4,4′-(1,4-Piperazinediyl)bisbenzoic acid ethyl ester (**15**)*

A mixture of piperazine (0.85 g, 10 mmol), ethyl 4-fluorobenzoate (3.36 g, 3.0 ml, 20 mmol), and potassium carbonate (2.8 g, 20 mmol) in dimethylformamide (10 ml) was heated under reflux for 8 h. After cooling, water (20 ml) was added to the reaction mixture to precipitate the final product that was filtered and washed with water and ethanol.

The final product was recrystallized from acetone.

Yield: 40%.

M.p.: 199–200 °C.

NMR ($CDCl_3$): 7.9 (d, 4H, J = 8 Hz); 6.9 (d, 4H, J = 8 Hz); 4.4 (quad, 4H, J = 9 Hz); 3.5 (s, 8H); 1.4 (t, 6H, J = 9 Hz) ppm.

IR: 2981; 2907; 2846; 1710; 1688; 1601; 1519; 1446; 1402 cm^{-1}.

$C_{22}H_{26}N_2O_4$ (382.45). Calc.: C, 69.09; H, 6.85; N, 7.32. Found: C, 69.18; H, 6.77; N, 7.45.

*6.1.6. 4,4′-(1,4-Piperazinediyl)bisbenzenecarboxhydrazide (**16**)*

A mixture of 4,4′-(1,4-piperazinediyl)bisbenzoic acid ethyl ester (**15**) (0.4 g: 1 mmol), water (0.5 ml), ethanol

(2.5 ml), and hydrazine hydrate (5 ml) was heated under reflux for 8 h. After cooling, the precipitate was filtered and retreated under the same experimental conditions for a further 8 h period. After cooling, the precipitate was filtered and washed with water.

Yield: 80%.

M.p.: >300 °C.

NMR (DMSO-d_6): 9.6 (br, 2H); 7.9 (d, 4H, J = 8 Hz); 7.0 (d, 4H, J = 8 Hz); 4.5 (br, 4H); 3.5 (s, 8H) ppm.

IR: 3307; 2982; 2848; 1690; 1640; 1602; 1278; 940 cm^{-1}.

$C_{18}H_{22}N_6O_2$ (354.41). Calc.: C, 61.00; H, 6.26; N, 23.71. Found: C, 61.26; H, 6.38; N, 23.71.

6.2. Antileishmanial activity

The compounds were dissolved in DMSO at the concentration of 2 mg ml^{-1} and diluted with the culture medium to obtain the desired final concentrations. The solutions were added, in a 96 well microplate assay, to the *L. donovani* promastigotes culture (2×10^6 cell per ml). Controls with corresponding dilutions of DMSO indicated that growth of the parasite was not altered under those experimental conditions. The plates were incubated at 26 °C for 72 h and growth of *Leishmania* promastigotes was determined by Alamar blue assay [18]. Pentamidine and amphotericin B were used as the standard antileishmanial agents. IC_{50} and IC_{90} values for each compound was computed from the growth inhibition curve.

6.3. DNA binding affinity measurements[6,30,31]

The thermal denaturation temperatures 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 spectrometer, with a jacketed six cuvettes 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^{-1}. 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 two-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 the table represents the mean of at least two experimental determinations.

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