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Original Article

Synthesis and In Vitro Leishmanicidal Effects of Conformationally Restricted Analogues of Pentamidine

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Abstract

Four conformationally restricted analogues of pentamidine were prepared. Then, different concentrations (0.039, 0.078, 0.156, 0.312 and 0.625 mg/mL) of each compound and two positive controls (amphotericin B and pentamidine, 0.625 mg/mL), one negative control (culture medium) and one solvent control (DMSO) were prepared and placed in 24-well plates containing 50000 parasite per well. Promastigotes of *Leishmania major* were incubated over a period of 2 days at 25°C; subsequently, percent of viable parasite in each well determined spectrophotometrically using MTT assay. The average EC_{50} for compounds 4a,b and 8a,b in DMSO was 0.098, 0.410, 0.150, 0.720 mg/mL, respectively. The average EC_{50} for positive controls pentamidine and amphotericin B was found to be 0.062 and 0.026 mg/mL. The control solvent had no significant effect on *L. major* promastigotes. All compounds had significant effect compared to DMSO and were less potent than positive controls.

Keywords: Pentamidine; Conformationally restricted; Analogues; Leishmaniasis.

Introduction

Protozoal parasitic diseases continue to pose serious public health in the world. Among them, *Leishmania* spp. are causative agents of mortality and morbidity in the human known as leishmaniasis, and estimated to affect 12 million people, with approximately 350 million individuals at the risk, worldwide. During the last 10 years, there has been the global burden and extensive epidemics form of the disease due to human migration, particularly its coincidence with HIV and the capacity of *Leishmania* to infect specialized immune cells and then to inhibit induction and activation of the immune system (1, 2). Leishmaniases are transmitted by the bite of the infected female phlebotomine sandfly and manifest with visceral, cutaneous, and mucocutaneous forms (3).

They are obligatory intracellular parasites that proliferate and develop to virulence metacyclic stage in an invertebrate vector then can infect diverse vertebrate hosts. The two distinct developmental stages of *Leishmania* are recognized as promastigotes and amastigotes, the first form which is found within midgut of the sandfly has an elongated shape and long flagellum. However, in the mammalian hosts upon the bite of an infected sandfly, it differentiates to amastigote stage, which lack flagella and is an obligatory intracellular pathogen infecting hematopoietic cells of the monocyte/macrophage lineage as professional phagocytic cells (4, 5).

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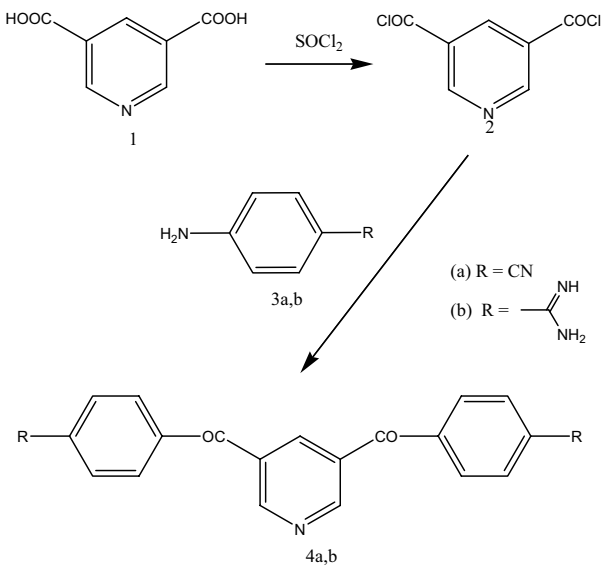


Scheme 1. Synthesis of compounds 4a,b

Chemotherapy for these parasitic diseases is generally ineffective mainly due to the emergence of drug-resistant strains and toxicity of the therapeutic agents (6). The pentavalent antimonials are widely used as primary therapy whereas alternative drugs include amphotericin B, pentamidine, paromomycin, and azoles (7, 8). However, resistance to antimonials is common (9) and treatments with amphotericin B and pentamidine are plagued by severe toxic side effects (10, 11).

It has been hypothesized that multiple pharmacological actions of the pentamidine (12) might be due to its conformational flexibility resulting in indiscriminate binding to both target and non-target macromolecules. Hence, as a part of our research program on the development of novel antiparasitic drug candidates, we decided to synthesize series of conformationally restricted analogues of pentamidine and investigated their biological effects against the *Leishmania* parasites.

Experimental

Synthesis of compounds

The synthesis of compounds **4a, b** and **8a, b** was accomplished according to the procedure shown in scheme 1. Pyridine-3,5-dicarboxylic acid (**1**) was heated with $SOCl_2$ to give the dichloride (**2**), which was then reacted with 4-cyanoaniline (**3a**) and 4-aminobenzamidine (**3b)** to give pyridine -3,5-dinitrile (**4a**) and diamidine (**4b**).

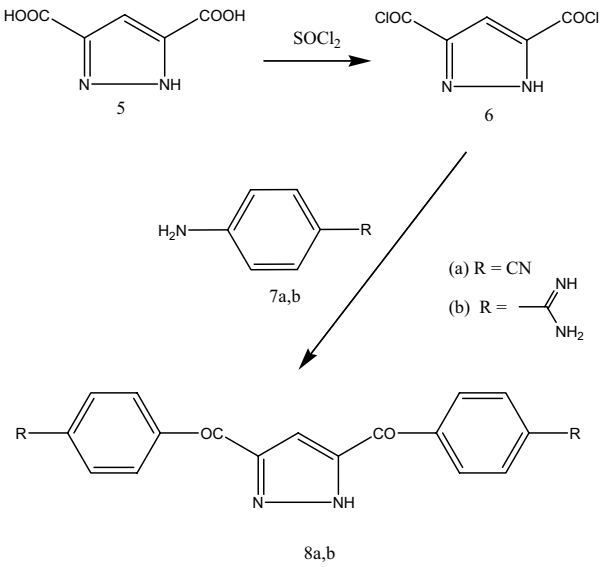
Similar procedures was used to synthesize pyrrazole-3,5-dinitrile (**8a**) and diamidine (**8b**) (scheme 2).

Characterization of compounds

Melting points were determined on Electrothermal Capillary apparatus and are uncorrected. The IR spectra were obtained using a Perkin-Elmer Model 1000. ^{1}H NMR were obtained on Bruker Ac-80 spectrophotometer and chemical shifts (δ) are in ppm relative to internal tetramethylsilane. Errors of elemental analyses were within ±0.4% of theoretical values.

General procedure for the synthesis of dinitriles (4a, 8a)

Following a known procedure (12), a solution of the appropriate acid dichloride in THF was added to a stirred solution of 4-aminobenzonitrile (2 mmol) and triethylamine (2 mmol) in THF at 0°C. After 30 min another portion of triethylamine (2 mmol) was added and the mixture stirred overnight. The



Scheme 2. Synthesis of compounds 8,b

solvent was removed under reduced pressure. Chromatography of the residue with appropriate solvent gave the corresponding dinitriles.

N, N'-bis(4-cyanophenyl)pyridine-3,5-dicarboxamide (4a)

A mixture of 1 (0.5 g, 3 mmol) and $SOCl_2$ (20 mL) was refluxed for 6 h. Removal of the excess $SOCl_2$ gave 0.6 g of 2 which was used without further purification. This compound was immediately used in the next reaction because of its instability.

Following the general procedure described above, 4a was prepared from 2 with 63% yield after chromatography (ethyl acetate). This compound was found to have following characteristics: melting point (M.P.) >300°C; ^{1}H-NMR (DMSO-d6): δ 10.66 (s, 2H, NH), 9.23 (s, 2H, H-pyridine), 9.00 (s, 1H, H-pyridine), 7.92 (d, 4H, arom, J=10 Hz), 7.5 ppm (d, 4H, arom, J=10 Hz); IR (KBr): 3500 (NH), 2230 cm^{-1} (CN), 1690 (CO); elemental analysis, $C_{21}H_{13}N_5O_2$ (367.36) with calculated results of C, 68.66; H, 3.57; N, 19.06, and found results of C, 68.61; H, 3.43; N, 19.15.

N, N'-bis(4-cyanophenyl)pyrrazol -3,5-dicarboxamide (8a)

Following the general procedure described for 4a, it was prepared from 6 in 58% yield after chromatography (ethyl acetate). This compound was found to have following characteristics: M.P.>300°C; ^{1}H-NMR (DMSO-d6): 8.2-7.6 (m, 10H, arom); IR (KBr): 3500 (NH), 2230 cm^{-1} (CN), 1690 (CO); elemental analysis, $C_{19}H_{12}N_6O_2$ (356.34) with calculated results of C, 64.04; H, 3.39; N, 23.58, and found results of C, 64.10; H, 3.28; N, 23.71.

General procedure for the synthesis of diamidines (4b, 8b)

A solution of the appropriate acid dichloride (2.3 mmol) in DMA (5 mL) was added stepwise to a stirring solution of arylamine (4.6 mmol) in DMA (25 mL) at 0°C**.** After 24 h, the mixture was treated with concentrated ammonium hydroxide. The product was collected by filtration.

N, N'-bis(4-amidinophenyl)pyridine-3,5-dicarboxamide (4b)

Following the general procedure described above, 4a was prepared from 2 in 87% yield. This compound was found to have following characteristics: M.P. 219-225°C; ^{1}H-NMR (CD_3OD): δ 9.26 (s, 2H, H-pyridine), 9.00 (s, 1H, H-pyridine), 7.86 (d, 4H, arom, J=10 Hz), 6.97 ppm (d, 4H, arom, J=10 Hz); IR (KBr):

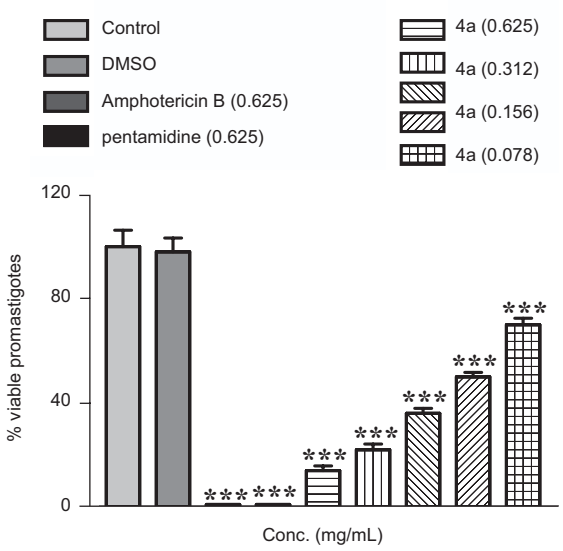


Figure 1. Effects of different concentrations of **4a** against *L. major* promastigotes after 2 days of incubation. Each bar represents the mean ± SEM of percent of viable promastigotes in 5 wells. ***P≤0.01, Tukey-Kramer test.

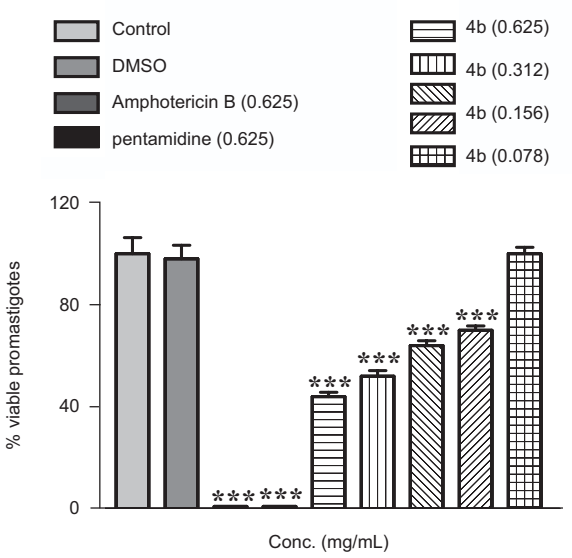


Figure 2. Effects of different concentrations of **4b** against *L. major* promastigotes after 2 days of incubation. Each bar represents the mean ± SEM of percent of viable promastigotes in 5 wells. ***P≤0.01, Tukey-Kramer test.

3328 (NH), 1670 cm^{-1} (CO); elemental analysis, $C_{21}H_{19}N_7O_2$ (401.42) with calculated results of C, 62.83; H, 4.77; N, 24.42, and found results of C, 62.94; H, 4.897; N, 24.31.

N, N'-bis(4-amidinophenyl)pyrrazol -3,5-dicarboxamide (8a)

Following the general procedure described for 4b, it was prepared from 6 in 83% yield. This compound was found to have following characteristics: M.P. >300°C; ^{1}H-NMR (DMSO-d6): 8.2-7.4 (m, 8H, arom), 7.1 ppm (s, 1H, H-pyrrazole); IR (KBr): 3300 (NH), 1680 (CO); elemental analysis, $C_{19}H_{18}N_8O_2$ (390.40) with calculated results of C, 58.45; H, 4.65; N, 28.70, and found results of C, 58.25; H, 4.81; N, 28.63.

Leishmania parasites

Leishmania major strain MRHO/IR75/ER was maintained with passage in BALB/c female mice. The amastigotes were isolated from lesions of infected BALB/c mice and transformed to promastigotes on NNN medium then subcultured in RPMI 1640 (Sigma) containing 10% v/v heat inactivated FCS, 2 Mm glutamine, 100 U/mL of penicillin and 100 mg/mL of streptomycin sulfate (RPMI-FCS) at 25°C. Leishmanicidal assays were conducted using stationary-phase promastigotes.

Assay for leishmanicidal activity

The antileishmanial activity against promastigotes was determined as described elsewhere (13, 14). Briefly, *L. major* promastigotes in stationary phase were seeded at 50.000 parasites/200µL/well in 24-well plate in RPMI-FCS. Samples **4a,b** and **8a,b** were dissolved in DMSO and added further 200 µL/well to give final concentrations of 1 mg/mL and serial two fold dilutions thereof. Promastigotes were incubated over a period of 2 days at 25°C and after that percent of viable parasite in each well determined spectrophotometrically using MTT assay (15). Amphotericin B and pentamidine was used as positive control, culture media was used as negative control and DMSO alone was used as solvent control.

Statistical analysis

Statistical analysis was carried out using one-way ANOVA and multiple comparison Tukey-Kramer test was used to compare the means of different treatment groups. The EC_{50} was determined by Litchfield and Wilcoxon method.

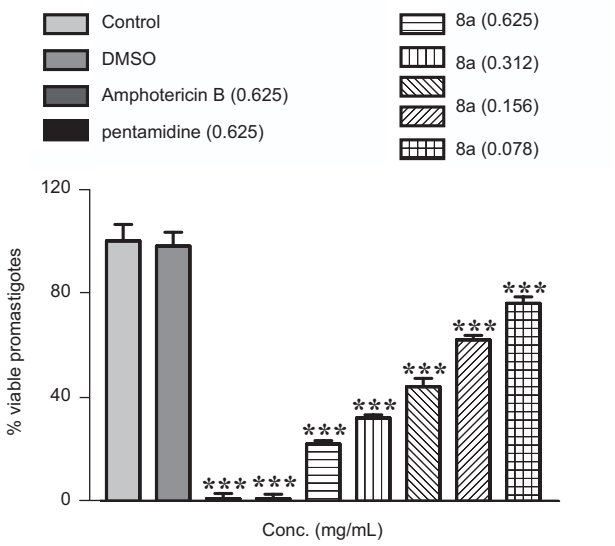


Figure 3. Effects of different concentrations of **8a** against *L. major* promastigotes after 2 days of incubation. Each bar represents the mean ± SEM of percent of viable promastigotes in 5 wells. ***P≤0.01, Tukey-Kramer test.

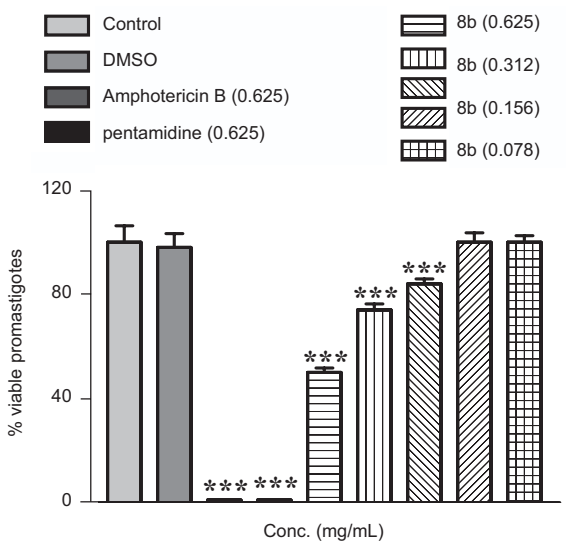


Figure 4. Effects of different concentrations of **8b** against *L. major* promastigotes after 2 days of incubation. Each bar represents the mean ± SEM of percent of viable promastigotes in 5 wells. ***P≤0.01, Tukey-Kramer test.

Results and Discussion

Amphotericin B (0.625 mg/mL) and pentamidine (0.625 mg/mL) in DMSO killed all of the *L. major* promastigotes (Figures 1-4). All synthesized compounds **4a,b** and **8a,b** in DMSO killed *L. major* promastigotes dose-dependently (Figures 1-4). Dinitriles **4a**, **8a** showed higher activity (EC_{50} 0.098 and 0.150 mg/mL, respectively) in comparison to diamidines **4b**, **8b** (EC_{50} 0.410 and 0.720 mg/mL, respectively) (see Table 1 for EC_{50} values). The control solvent had no significant effect on *L. major* promastigotes. All compounds were weaker in comparison to positive controls pentamidine and amphotericin B (EC_{50} 0.062 and 0.026 mg/mL, respectively).

Acknowledgement

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Reference

(1) Croft SL, Sundar S and Fairlamb AH. Drug resistance in leishmaniasis. *Clin. Microbiol. Rev.* (2006) 19: 111-126.

(2) Natera S, Machuca C, Padron-Nieves M, Romero A, Diaz E and Ponte-Sucre A. *Leishmania* spp.: proficiency of drug-resistant parasites. *Int. J. Antimicrob. Agents* (2007) 29: 637-642.

(3) Guerin PJ, Olliaro P, Sundar S, Boelaert M, Croft SL, Desjeux P, Wasunna MK and Bryceson AD. Visceral leishmaniasis: current status of control, diagnosis, and treatment, and a proposed research and development agenda. *Lancet Infect. Dis.* (2002) 2: 494-501.

(4) Banuls AL, Hide M and Prugnolle F. *Leishmania* and the leishmaniases: a parasite genetic update and advances in taxonomy, epidemiology and pathogenicity in humans. *Adv. Parasitol.* (2007) 64: 1-109.

(5) Van Zandbergen G, Bollinger A, Wenzel A, Kamhawi S, Voll R, Klinger M, Muller A, Holscher C, Herrmann M, Sacks D, Solbach W and Laskay T. *Leishmania* disease development depends on the presence of apoptotic promastigotes in the virulent inoculum. *Proc. Natl. Acad. Sci. USA* (2006) 103: 13837-13842.

(6) Perez-Victoria JM, Di Pietro A, Barron D, Ravelo AG, Castanys S and Gamarro F. Multidrug resistance phenotype mediated by the P-glycoprotein-like transporter in *Leishmania*: a search for reversal agents. *Curr. Drug Targets* (2002) 3: 311-333.

(7) Berman JD. Human leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last 10 years. *Clin. Infect. Dis.* (1997) 24: 684-703.

(8) Croft SL and Yardley V. Chemotherapy of leishmaniasis. *Curr. Pharm. Des.* (2002) 8: 319-342.

(9) Lira R, Sundar S, Makharia A, Kenney R, Gam A, Saraiva E and Sacks D. Evidence that the high incidence of treatment failures in Indian kala-azar is due to the emergence of antimony-resistant strains of *Leishmania*

donovani. *J. Infect. Dis.* (1999) 180: 564-567.

(10) Amsden GW, Kowalsky SF and Morse GD. Trimetrexate for *Pneumocystis carinii* pneumonia in patients with AIDS. *Ann. Pharmacother.* (1992) 26: 218-226.

(11) Wharton JM, Coleman DL, Wofsy CB, Luce JM, Blumenfeld W, Hadley WK, Ingram-Drake L, Volberding PA and Hopewell PC. Trimethoprim-sulfamethoxazole or pentamidine for *Pneumocystis carinii* pneumonia in the acquired immunodeficiency syndrome. A prospective randomized trial. *Ann. Intern. Med.* (1986) 105: 37-44.

(12) Tao B, Huang TL, Zhang Q, Jackson L, Queener SF and Donkor IO. Synthesis and anti-*Pneumocystis carinii* activity of conformationally restricted analogues of pentamidine. *Eur. J. Med. Chem.* (1999) 34: 531-538.

(13) Tempone AG, Andrade HF, Spencer PJ, Lourenco CO, Rogero JR and Nascimento N. *Bothrops moojeni* venom kills *Leishmania* spp. with hydrogen peroxide generated by its L-amino acid oxidase. *Biochem. Biophys. Res. Commun.* (2001) 280: 620-624.

(14) Jaafari MR, Hooshmand S, Samiei A and Hossainzadeh H. Evaluation of leishmanicidal effect of *Perovskia abrotanoides* Karel. Root extract by *in vitro* leishmanicidal assay using promastigotes of *Leishmania major*. *Pharmacologyonline* (2007) 1: 299-303.

(15) Tada H, Shiho O, Kuroshima K, Koyama M and Tsukamoto K. An improved colorimetric assay for interleukin 2. *J. Immunol. Methods* (1986) 93: 157-165.

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