Published in final edited form as:
Eur J Med Chem. 2012 February ; 48: 164–173. doi:10.1016/j.ejmech.2011.12.010.

Analogs of Pentamidine as Potential Anti-Pneumocystis Chemotherapeutics

Dorota Maciejewska[a,*], **Jerzy Żabinski**[a], **Pawel Kaźmierczak**[a], **Mateusz Rezler**[a], **Barbara Krassowska-Świebocka**[a], **Margaret S. Collins**[b], and **Melanie T. Cushion**[b,**]

[a]Medical University of Warsaw, Faculty of Pharmacy, Department of Organic Chemistry, Banacha 1 Str., 02 097 Warsaw, Poland. [b]University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati, OH 45267-0560 and VAMC, Cincinnati, OH 45220, USA.

Abstract

A series of 20 pentamidine analogs were prepared using 2 general Schemes that evaluated heteroatoms, sulfobenzene and alkanediamide groups in the aliphatic linker and methoxy substituents attached to the benzene rings for efficacy against the fungal pathogen, *Pneumocystis carinii* in an ATP bioassay. All but one of the 20 bisamidines reduced the ATP content of the *P. carinii* over the 72 hr of the assay period. The highest activities were associated with the lack of methoxy groups and the presence of the O, N and S heteroatoms. Activity (IC_{50}) for compounds **1**, **5**, **6**, **10** ranged from 1.1 to 2.13 μM. The compound **11** with similar activity (1.33 μM), bears a sulfobenzene group at a nitrogen in the aliphatic linker. The alkanediamide-linked bisbenzamidines showed a moderate inhibition of ATP. Generally, the inclusion of a heteroatom in the aliphatic linker and absence of methoxy groups at the benzene rings were associated with higher activities in this assay. Of note, most of the compounds had little to no cytotoxicity in mammalian cell cultures. Although not quite as potent as other pentamidine derivatives, these compounds hold promise for decreased side effects within the mammalian host.

Keywords

Pentamidine analogs; anti-*Pneumocystis carinii* activity; in vitro ATP bioluminescent assay

1. Introduction

Treatment of Pneumocystis pneumonia (PCP) remains a challenge due to limited therapeutic choices, potential evolving mutations in the targets of standard anti-Pneumocystis compounds including trimethoprim-sulfamethoxazole and atovaquone, and toxicity associated with second line therapies such as pentamidine isethionate [1]. Few drugs are in the development pipeline due to elimination of programs supported within the pharmaceutical industry and shifting priorities of national research foundations.

*Corresponding author. Tel./fax:+48 22 5720 643. dmaciejewska@wum.edu.pl (D. Maciejewska). **Co-corresponding author. University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati, OH 45267-0560; cushiomt@ucmail.uc.edu (M. T. Cushion).

Concomitantly, infection with *Pneumocystis jirovecii* (the species infecting humans) is expanding into new patient populations besides the frankly immunocompromised host. PCP is a significant cause of morbidity and mortality in patients with rheumatoid arthritis or other chronic conditions requiring anti-TNF alpha therapies [2,3], while colonization with *P. jirovecii* is associated with a poorer outcome and more severe disease in patients with Chronic Obstructive Pulmonary Disease (COPD) [4]. Standard antifungal therapies such as the azoles or amphotericin B are not effective against PCP, likely due to the lack of ergosterol biosynthesis by these fungi [5]. Therefore, a common approach to identify new effective therapies for PCP has been to use compounds with known efficacies, such as trimethoprim, sulfamethoxazole or pentamidine, and chemically modify these parent compounds to increase efficacy and reduce toxicity [6–9].

In the present report, we have undertaken an analysis of linear pentamidine analogs for the purpose of identifying candidate anti-*Pneumocystis* therapy. The antimicrobial activity of aromatic bisamidines is well known, but only pentamidine is clinically used. Its high activity is associated with toxicity and low bioavailability, indicating a need for new derivatives that provide increased efficacy with no toxicity. The mechanism of biological action of the bisamidines is not clear, but their ability to bind to the AT minor groove has been demonstrated [10–14].

The first group of tested pentamidine analogs include 10 compounds **1** – **10** with different heteroatoms in the aliphatic linker (O, N, and S), and varying numbers of methoxy groups on the benzene rings (0, 2 or 4) (Group I in Figure 1). We have planned to develop derivatives which could combine both, pentamidine and trimethoprim potency. The second group (Group II in Figure 1) of pentamidine analogs were synthesized to include sulfonamide substituents, which were hypothesized to increase anti-*Pneumocystis* activity given the known efficacy of the sulfonamide group against *Pneumocystis* . Group II includes 6 compounds **11** – **16**, with the N atom bearing sulfobenzene substituents in the middle of the aliphatic linker, and with 0-, 2- and 4-methoxy substituents at the benzene rings. The third group includes the bisamide linkers type of Ph-CONH-R_n-NHOC-Ph, with n = 3 to 6 (Group III in Figure 1). Activity of this series of bisamides **17** – **20** was compared to previous studies of similar compounds where the carbonyl groups were switched with amino groups giving compounds of the type of Ph-NHOC-R_n-CONH-Ph [15].

2. Result and Discussion

2.1. Chemistry

The method of synthesis for these bisamidines generally followed established procedures [16–18], which involves the preparation of the bisnitriles and their conversion into bisamidines. Compounds **1** – **3**, **7** – **9**, and **11** – **13**, and **15** were obtained in the course of a three-step synthesis (Schemes 1), which involved O-alkylation of 4-hydroxybenzonitrile with bis(2-chloroethyl)ether, *N*,*N*-bis(2-chloroethyl)-*N*-methylamine or *N*,*N*-bis(2-chloroethyl)sulfonamides, then conversion of the formed bisnitriles **1a** – **3a**, **7a**, **11a** – **13a**, and **15a** to bisamidines by the subsequent reactions with ethanol and ammonia. Only the synthesis of compound **10** required the use of 4-(2-chloroethoxy)benzonitrile and sodium sulfide for preparation of bisnitrile **10a**. Bisnitriles which are essential for preparation of compounds **17** – **20**, were obtained by substitution of 4-cyanobenzoyl chloride with the appropriate alkyldiamines. Their transformation into bisamidines followed the procedure for the rest of the bisnitriles (Scheme 2). Syntheses of the bisamidines **4** – **6**, **14** and **16** and bisnitriles **1a**, **4a** – **6a**, **8a** – **9a, 14a**, **16a** have been published by us [19–23] together with their structural analysis, and synthesis of the bisnitrile **18a** was given in paper [24].

2.2. Anti-Pneumocystis Activity and Cytotoxicity

The biological data are reported in Table 1. This series of compounds was remarkable for 2 reasons. First, there was very little toxicity associated with any of the compounds and all but 1 of the 20 compounds showed some efficacy. This is in contrast to previous studies where efficacy was highly variable and toxicity was common [8]. Four of the 10 compounds in Group I showed marked activity; 5 had moderate activity and only 1 had no activity in the ATP bioassay. While their potency was 2–3 fold less than that of pentamidine, (IC_{50} = 0.50 μM; 0.30μg/ml), there was little to no toxicity associated with their anti-*P. carinii* activity. Within Group I, greater inhibitory activity was associated with the presence of a heteroatom within the aliphatic linker and the absence of methoxy groups at the benzene rings. The introduction of S atom increased the activity of bisamidine **10** two-fold as compared to **6**, in which the O atom is in the middle of the aliphatic chain. While the presence of the N atom in the aliphatic linker with the sulfobenzene group and the absence of methoxy groups in compound **11** correlated with marked activity, addition of methoxy groups to this structure reduced efficacy (compounds **12**, **13**). A lack of activity in sulfonamide group was associated with the lack of methyl group and the addition of *N*-acetyl group to the sulfobenzene group (**14**). Activity could be reinstated with the addition of methoxy groups to the benzene rings (**15**) or elimination of the *N*-acetyl group on the sulfobenzene group (**16**). The alkanediamide-linked bisbenzamidines **17** –**20** were generally less active than the other compounds tested. The compounds **17** and **18** linked with shorter alkanediamide chains were more active than those with longer chains (compounds **19**, **20**). The activities of these compounds were much less than the alkanediamide-linked bisamidines in which the alkane chain was bound to carbonyl groups [15].

3. Conlusions

All but one (**14**) of the 20 bisamidines tested in the ATP assay at the screening concentration of 100 μg/ml reduced the ATP content of the *P. carinii* over the 72 hr of the assay period. After titration to determine the IC_{50} of the remaining compounds, 4 of the 5 compounds with the highest activities were found in the Group I, which explored the role of heteroatoms in the aliphatic linker and the addition of methoxy substituents to the benzene rings of the bisamidine. Activity was associated with the lack of methoxy substituents and the presence of the heteroatoms, O, N and S (**1**, **5**, **6** and **10**). The other compound with similar activity is **11** (from Group II), which added a sulfobenzene substituent to a nitrogen in the aliphatic linker. Addition of methoxy groups to the benzene rings also reduced efficacy in this group as well as in the first scheme. The alkanediamide-linked bisbenzamidines from Group III only showed a moderate inhibition of ATP pools in the *P. carinii* bioassay. These results were in contrast to a previous study for compounds in which carbonyl groups were switched with amino groups. Generally, the inclusion of a heteroatom in the aliphatic linker and absence of methoxy groups on the benzene groups was associated with higher activities in this assay. Most of the compounds had little to no toxicity in the cell line assay typically used to evaluate this characteristic. Although not as quite as potent as other pentamidine derivatives, these compounds hold promise for decreased side effects within the mammalian host and can next be evaluated in a mouse model of Pneumocystis pneumonia.

4. Experimental Section

4.1. Assessment of Anti-Pneumocystis Activity

P. carinii were obtained from the lungs of corticosteroid-immunosuppressed male CD rats (Charles River, Portage, MI housed at the Cincinnati Veteran's Affairs Veterinary Medical Unit under barrier conditions. Their immune system suppression was induced by weekly injections of 20 mg/kg methylprednisolone (Depo-Medrol, Pfizer Pharmacia, New York,

NY). After two weeks of suppression, rats were inoculated by intratracheal or intranasal installation of 2×10^7 cryopreserved *P. carinii* by nuclei count. Immunosuppression continued for 8 weeks and the fungi were purified from rodent lung tissue and cryopreserved as previously described [25]. The assay plates were set up by rapidly thawing of the cryopreserved *P. carinii* at 37°C, which were then resuspended at 5×10^7 nuclei/ml in RPMI-1640 containing 20 % calf serum (Atlanta Biologicals) with or without the bisamidines. Each drug concentration was assayed in triplicate wells in Costar #3548 multiwell plates (Fisher Scientific, Cincinnati, OH) in 3 different suspension assays using two different batches of *P. carinii*. Media without drug, with *P. carinii*, and with 10 μg of ampicillin/ml (Fisher Scientific, FairLawn, NJ) served as negative controls; pentamidine isethionate (Sigma-Aldrich) at 1 μg/ml served as the positive drug activity control. Plates were incubated at 5 % CO_2, 37 °C. At, 6, 24, 48, and 72 hours post inoculation, 50 μl samples were transferred to opaque white plates (USA Scientific, Ocala, FL) and assessed for ATP content using ATPlite-M (Perkin-Elmer, Waltham, MA) [25]. Each of the 20 bisamidines was screened at 100μg/ml in 2 assays and if the ATP was decreased by at least 50 % vs untreated control *P. carinii*, a titered series was then performed.

4.1.1. ATP assay—The in vitro ATP bioluminescent assay to evaluate the efficacy of compounds against *P. carinii* was conducted as previously described [26–28]. The linear range of the ATP assay is 1 μM to 100 fM (~20,000,000 to 2,000 RLU). Samples removed from the suspension cultures were lysed, placed in opaque white plates and the ATP content measured with a luciferin-luciferase kit (ATPlite, Perkin-Elmer, Inc.) for light emission at 562 nm with a FluoSTAR Optima plate reader (BMG Labtechnologies, Inc.). A quench control to evaluate effects on the enzyme-substrate reaction was run for every drug tested and no inhibition was observed with any compound.

4.1.2. Data analysis—The IC_{50} for each bisamidine was calculated using linear regression of the percent decrease in ATP content of compound vs the log drug concentrations (GraphPad Software v2 for Science; GraphPad, San Diego, Calif.). Based on the IC_{50} values, each agent was classified by using an activity scale of 5 rankings ranging from “Highly active” (compounds with an IC_{50} of < 0.010 μg/ml), “Very marked” (IC_{50}s of 0.011 to 0.099 μg/ml), “Marked” (IC_{50}s from 0.10 to 0.99 μg/ml), “Moderate” (IC_{50}s from 1.0 to 9.99 μg/ml), “Slight” (IC_{50}s from 10.0 to 49.9 μg/ml), and “None” (i.e., inactive; IC_{50}s of ≥50 μg/ml) [1].

4.2. Chemistry

All chemicals were purchased from major chemical suppliers as high or highest purity grade and used without any further purification. Melting points were determined with an Electrothermal 9001 Digital Melting Point. The chemical structure of the synthesized compounds were confirmed by their spectral data (^{1}H NMR and ^{13}C NMR 1D and 2D spectra in solution were recorded with Varian 300 V NMR S or with a Bruker Avance DMX 400, and chemical shifts δ (ppm) in solutions were referenced to TMS). Their purity was verified by elemental analyses using C, H, N, S Elementar GmbH Vario EL III apparatus. For thin layer chromatography (TLC) prepared plates Merck Kieselgel 60 F_{254} were used (toluene/dioxane/ethanol 6.0/3.2/0.5). For column chromatography Merck Silicagel 60, 230–400 mesh ASTM (0.040–0.063 mm) was used.

4.2.1. General procedure for synthesis of bisamidines—An appropriate bisnitrile (2–5 mmol) was stirred in a sealed flask with the saturated solution of HCl in anhydrous ethanol (25–50 ml) for 20–40 h at room temperature. The solvent was evaporated in vacuum or dry diethyl ether was added until complete precipitation was attained. The precipitate was quickly filtered off and dried under reduced pressure over anhydrous $CaCl_2$ for 2–6 hours

giving the very hygroscopic HCl salt in nearly quantitative yield. The crude diiminoester was immediately added to a saturated solution of NH_3 in anhydrous ethanol (25–50 ml). The resulting mixture was stirred at room temperature for 24–48 h in a sealed vessel, the solvent was then evaporated in vacuum or refluxed for 3 h, and subsequently poured into a 10% aqueous solution of NaOH (10–20 ml). The precipitate of free bisamidine was filtered, washed with water and acetone, then dried under reduced pressure over anhydrous $CaCl_2$. The solid of free bisamidine was suspended in anhydrous ethanol (5–10 ml) then an ethanolic solution of HCl (2–5 ml) was added and heated to boiling for a few minutes, to obtain the appropriate hydrochlorides.

The preparation of bisnitrile, the substrate leading to bisamidine, is given before appropriate bisamidine, if it was not published.

4.2.1.1. 1,5-Bis(4-amidinophenoxy)-N-methyl-3-azapentane trihydrochloride (1): A white solid of **1** was obtained (Yield 44 %). M.p.= 262–265 °C (decomp.). ^{1}H NMR (400.13 MHz, DMSO-d_6) δ in ppm: 2.94–2.95 (m; 3H; H-11), 3.60–3.74 (m; 4H; H-9,H-9’), 4.58–4.59 (m; 4H; H-8,H-8’), 7.20–7.22 (m; 4H; H-2,H-6, H-2’, H-6’), 7.91–7.93 (m; 4H; H-3, H-5, H-3’, H-5’), 9.15 (broad s; 4H, 2 × 2NH), 9.36 (broad s; 4H; 2 × 2NH), 11.52 (broad s; 1H; N × HCl). ^{13}C NMR (100.61 MHz, DMSO–d_6) δ in ppm: 40.9 (C-11), 54.2 (C-9, C-9’), 62.8 (C-8, C-8’), 115.0 (C-2, C-6, C-2’, C-6’), 120.2 (C-4, C-4’), 130.2 (C-3, C-5, C-3’, C-5’), 161.8 (C-1, C-1’), 164.7 (C-7, C-7’). $C_{19}H_{25}N_5O_2 \times 3HCl \times ½H_2O$ (473.82 g/mol). Calcd. (%) C=48.16; H= 6.12; N=14.77; Cl=22.45; found (%): C=47.80; H=6.20; N=15.10; Cl=22.46.

4.2.1.2. 1,5-Bis(4-cyano-2-methoxyphenoxy)-N-methyl-3-azapentane (2a): *N*,*N*-bis(2-chloroethyl)-*N*-methylamine hydrochloride (0.96 g; 5 mmol), 3-methoxy-4-hydroxybenzonitrile (1.49 g; 10 mmol), anhydrous K_2CO_3 (4.14 g; 30 mmol) and N-methyl-2-pyrrolidone (20 ml) was stirred together at 115 °C for 3 hours (progress of the reaction was followed by TLC) and then poured into ice water (200 ml). The precipitated solid was filtered, washed with water, and dried to obtain 1.86 g (Yield: 98 %) of a white solid of **2a**. M.p.= 84.5–88 °C. ^{1}H NMR (299.86 MHz, $CDCl_3$) δ in ppm: 2.56 (s; 3H; H-11), 3.09 (t; J=5.7Hz; 4H; H-9, H-9’), 3.85 (s; 6H; H-10, H-10’), 4.23 (t; J=5.7Hz; 4H; H-8, H-8’), 6.92 (d; J=8.4Hz; 2H; H-6, H-6’), 7.07 (d; J=1.5Hz; 2H; H-3, H-3’), 7.25 (dd; J_1=8.4Hz J_2=1.5Hz; 2H; H-5, H-5’). ^{13}C NMR (75.40 MHz, $CDCl_3$) δ in ppm: 43.73 (C-11), 56.3 (C-10, C-10’), 56.3 (C-9, C-9’), 67.2 (C-8, C-8’), 104.5 (C-4, C-4’), 112.9 (C-6, C-6’), 114.5 (C-3, C-3’), 119.3 (C-7, C-7’), 126.5 (C-5, C-5’), 149.7 (C-2, C-2’), 151.2 (C-1, C-1’). $C_{21}H_{23}N_3O_4$ (381.44 g/mol): calcd (%): C=66.13; H=6.08; N=11.02; found (%): C=65.87; H=6.24; N=10.91.

4.2.1.3. 1,5-Bis(4-amidino-2-methoxyphenoxy)-N-methyl-3-azapentane (2): A light yellow solid of **2** (Yield: 58 %) was obtained. M.p.= 255.5–257,5 °C (decomp). ^{1}H NMR (299.86 MHz, DMSO-d_6) δ in ppm: 3.00 (broad s, 3H; H-11), 3.63–3.81 (m; 4H; H-9, H-9’), 3.87 (s; 6H; H-10, H10’), 4.59 (t; J=4.8Hz; 4H; H-8, H-8’), 7.26 (d; J=8.4Hz; 2H; H-6, H-6’), 7.55–7.58 (m; 4H; H-3, H-5, H-3’, H-5’), 9.13 (broad s; 4H; 2 × 2NH), 9.40 (broad s; 4H; 2 × 2NH), 11.62 (broad s; 1H; N × HCl). ^{13}C NMR (75.40 MHz, DMSO-d_6) δ in ppm: 41.5 (C-11), 54.2 (C-9, C-9’), 56.1 (C-10, C10’), 63.7 (C-8, C-8’), 111.7 (C3, C3’), 113.0 (C6, C6’), 120.3 (C4, C4’), 121.7 (C5, C5’), 148.7 (C2, C2’), 151.4 (C1, C1’), 164.6 (C7, C7’). $C_{21}H_{29}N_5O_4 \times 3HCl \times 5H_2O$ (614.98 g/mol): calcd. (%): C=41.02; H=6.88; N=11.39 %; found (%): C=40.89; H=6.87; N=11.28.

4.2.1.4. 1,5-Bis(4-cyano-2,6-dimethoxyphenoxy)-N-methyl-3-azapentane (3a): To *N*,*N*-*bis* (2-chloroethyl)-*N*-methylamine hydrochloride (0.48 g, 2.5 mmol) and anhydrous K_2CO_3 (2.07 g, 15 mmol) in *N*-methyl-2-pyrrolidone (15 ml), 3,5-dimethoxy-4-hydroxybenzonitrile

(0.90 g, 5 mmol) was added and the entire reaction was stirred together at 80 °C for 90 minutes (progress of the reaction was followed by TLC), then poured into water (400 ml). The precipitated solid was filtered, washed with water and purified using column chromatography (eluent CH_2Cl_2/MeOH 99.5/0.5) to give a light beige solid of **3a** (Yield: 74%). M.p.= 186.0–189.0 °C (decomp). ^{1}H NMR (400.13 MHz, $CDCl_3$) δ in ppm: 2.45 (s; 3H; H-11), 2.92 (t; J=6 Hz; 4H; H-9, H-9'), 3.85 (s; 12H; H-10, H-10'), 4.14 (t; J=6 Hz; 4H; H-8, H-8'), 6.85 (s; 4H; H-3, H-5, H-3', H-5'). ^{13}C NMR (100.61 MHz, $CDCl_3$) δ in ppm: 42.9 (C-11), 56.5 (C-10, C10'), 57.3 (C-9, C-9'), 71.3 (C-8, C-8'), 106.9 (C-4, C-4'), 109.5 (C-3, C-5, C-3', C-5'), 119.2 (C-7, C-7'), 141.7 (C-1, C-1'), 153.9 (C-2, C-6, C-2', C-6'). $C_{23}H_{27}N_3O_6$ (441.49 g/mol): calcd (%): C=62.57, H=6.16, N=9.52; found (%): C=62.28, H=6.19, N=9.26.

4.2.1.5. 1,5-Bis(4-amidino-2,6-dimethoxyphenoxy)-N-methyl-3-azapentane trihydrochloride (3): A white solid of **3** was obtained (Yield: 72%). M.p.= 206.5–208 °C (decomp). ^{1}H NMR (299.87 MHz, DMSO-d_6) δ in ppm: 3.06–3.08 (m; 3H; H-11), 3.57–3.77 (broad m; 4H; H-9, H-9'), 3.92 (s; 12H; H-10, H10'), 4.37 (t; J=4.2Hz; 4H; H-8, H-8'), 7.34 (s; 4H; H-3, H-5, H-3' ,H-5'), 9.26 (broad s; 4H; 2 × 2NH), 9.56 (broad s; 4H; 2 × 2NH), 10.81 (broad s; 1H; N × HCl). ^{13}C NMR (75.40 MHz, DMSO-d_6) δ in ppm: 40.2 (C-11), 54.9 (C-9, C-9'), 56.5 (C-10, C-10'), 67.0 (C-8, C-8'), 105.9 (C-3, C-5, C-3', C-5'), 123.3 (C-4, C-4'), 139.4 (C-1, C-1'), 152.7 (C-2, C-6, C-2', C-6'), 164.6 (C-7, C-7'). $C_{23}H_{33}N_5O_6 \times 3HCl \times 4H_2O$ (657.01 g/mol): calcd. (%): C=42.05, H=6.75, N=10.66; found (%): C=41.76, H=6.28, N=10.45.

4.2.1.6. 1,5-Bis(4-cyano-2-methoxyphenoxy)-3-oxapentane (7a): To a solution of 4-hydroxy-3-methoxybenzonitrile 1.49 g (10 mmol) in N-methyl-2-pyrrolidone (15 ml) with anhydrous K_2CO_3 2.76 g (20 mmol), 0.72 g (5 mmol) of bis(2-chloroethyl)ether was added and stirred at 130 °C for 3 hours (progress of the reaction was followed by TLC). The hot reaction mixture was poured into ice water (300 ml), and the precipitated solid was filtered, washed with water and dried at 60 °C. Recrystalisation from ethanol gave 1.64 g (Yield: 89 %) of a white solid of **7a**. M.p.= 137–139 °C. ^{1}H NMR (400.13 MHz, $CDCl_3$) δ in ppm: 3.86 (s; 6H; H- 10, H-10'), 3.98 (t; J=4.7Hz; 4H; H-8, H-8'), 4.24 (t; J=4.7Hz; 4H; H-9, H-9'), 6.93 (d; J=8.4Hz; 2H; H-6, H-6'), 7.08 (broad s; 2H; H-3, H-3'), 7.24 (dd; J_1=1.2Hz J_2=8.4Hz; 2H; H-5, H-5'). ^{13}C NMR (100.61 MHz, DMSO-d_6) δ in ppm: 56.3 (C-10, C-10'), 68.8 (C-9, C-9'), 69.9 (C-8, C-8'), 104.5 (C-4, C-4'), 113.2 (C-6, C-6'), 114.6 (C-3, C-3'), 119.4 (C-7, C-7'), 126.5 (C-5, C-5'), 149.8 (C-2, C-2'), 152.4 (C-1, C-1'). $C_{20}H_{20}N_2O_5$ (368.39 g/mol): calcd. (%): C=65.21, H=5.47, N=7.60; found (%): C=65.38, H=5.52, N=7.37.

4.2.1.7. 1,5-Bis(4-amidino-2-methoxyphenoxy)-3-oxapentane dihydrochloride (7): A yellow solid was obtained, filtered, dissolved in water (25 ml) and the insoluble residue was filtered. Dry acetone was added to the water solution until the solid started to precipitate. The resultant solid was filtered and dried to give a pure beige solid of **7** (Yield: 67 %). M.p.=280–282 °C (decomp.). ^{1}H NMR (299.87 MHz, DMSO-d_6) δ in ppm: 3.86 (broad s; 10H, H-9, H-10, H-9', H-10'), 4.23 (t; J=4.5Hz; 4H; H-8, H-8'), 7.20 (d; J=8.4Hz; 2H; H-6, H-6'), 7.48–4.51 (m; 4H; H-3, H-5, H-3', H-5'), 8.95 (broad s; 4H; 2 × 2NH), 9.24 (broad s; 4H; 2 × 2NH). ^{13}C NMR (75.40 MHz, DMSO-d_6) δ in ppm: 55.9 (C-10, C-10'), 68.2 (C-8, C-8'), 68.8 (C-9, C-9'), 111.4 (C-3, C-3'), 112.4 (C-6, C-6'), 119.4 (C-4, C-4'), 121.8 (C-5, C-5'), 148.6 (C-2, C-2'), 152.5 (C-1, C-1'), 164.6 (C-7, C-7'). $C_{20}H_{26}N_4O_5 \times 2HCl$ (475.37 g/mol): calcd. (%): C=50.53, H=5.94, N=11.79; found (%): C=50.49, H=6.06, N=11.61.

4.2.1.8. 1,5-Bis(4-amidino-2,6-dimethoxyphenoxy)-3-oxapentane dihydrochloride (8): Ethanol was evaporated *in vacuo*. The resultant brown crystals were dissolved in a small amount of water and dry acetone was added until the solid started to precipitate. The

resultant solid was filtered, washed with acetone, and dried at room temp. to give a beige solid of **8** (Yield: 75%). M.p.= 142.5–145 °C (decomp). ^{1}H NMR (400.13 MHz, DMSO-d_6) δ in ppm: 3.70 (t; J=5.6Hz, 4H; H-9, H-9'), 3.86 (s; 12H; H-10, H-10'), 4.06 (t; J=5.6Hz, 4H; H-8, H-8'), 7.25 (s; 4H; H-3, H-5, H-3', H-5'), 9.18 (broad s; 4H; 2 × 2NH), 9.44 (broad s; 4H; 2 × 2NH). ^{13}C NMR (100.61 MHz, DMSO-d_6) δ in ppm: 56.4 (C-10, C10'), 69.7 (C-9, C-9'), 71.9 (C-8, C-8'), 106.0 (C-3, C-5, C-3', C-5'), 122.3 (C-4, C-4'), 140.9 (C-1, C-1'), 152.8 (C-2, C-6. C-2', C-6'), 164.8 (C-7, C-7'). $C_{22}H_{30}N_4O_7 \times 2HCl \times 3H_2O$ (589.49 g/mol): calcd. (%): C=44.83, H=6.50, N=9.50; found (%): C=44.72, H=6.35, N=9.07.

4.2.1.9. 1,5-Bis(4-amidino-2,6-dimethoxyphenoxy)pentane dihydrochloride (9): Ethanol was evaporated *in vacuo*. The resultant solid was dissolved in a small amount of water and dry acetone was added until the solid started to precipitate. The resultant solid was filtered, washed with acetone and dried at room temp. to yield a beige solid of **9** (Yield: 58%). M.p.= 225.5–227.0 °C. ^{1}H NMR (400.13 MHz, DMSO-d_6) δ in ppm: 1.57–1.58 (m; 2H; H-11), 1.66–1.68 (m; 4H; H-9, H-9'), 3.86 (s; 12H; H-10, H-10'), 3.95 (t; J=6 Hz; 4H; H-8, H-8'), 7.27 (s; 4H; H-3, H-5, H-3', H-5'), 9.20 (broad s; 4H; 2 × 2NH), 9.47 (broad s; 4H; 2 × 2NH). ^{13}C NMR (100.62 MHz, DMSO-d_6) δ in ppm: 21.7 (C-11), 29.2 (C-9, C-9'), 56.4 (C-10, C-10'), 72.5 (C-8, C-8'), 106.0 (C-3, C-5, C-3', C-5'), 122.2 (C-4, C-4'), 141.0 (C-1, C-1'), 153.0 (C-2, C-6, C-2', C-6'), 164.8 (C-7, C-7'). $C_{23}H_{32}N_4O_6 \times 2HCl \times 1½H_2O$ (560.48 g/mol): calcd. (%): C=49.28, H=6.60, N=9.99; found (%): C=49.07, H=6.72, N=9.74.

4.2.1.10. 1,5-Bis(4-cyanophenoxy)-3-thiapentane (10a): 4-(2-bromoethoxy)benzonitrile (6,78 g; 30 mmol) and $Na_2S \cdot 9H_2O$ (3.60 g; 15 mmol) were stirred with DMSO (30 ml) for 2 h at 115–120 °C. The mixture was poured into ice water (150 ml) and left for 24 h. The precipitate was filtered, washed with cold water, and recrystalized from ethanol to give 3.89 g (Yield: 80 %) of **10a**. M.p.= 106–107 °C. ^{1}H NMR (400.13 MHz, DMSO-d_6) δ in ppm: 3.01 (t; J=6.3 Hz; 4H: H-9, H-9'), 4.26 (t; J=6.3 Hz; 4H: H-8, H-8'), 7.11 (d; J=8.5 Hz; 4H; H-2, H-6, H-2', H-6'), 7.76 (d; J=8.5 Hz; 4H; H-3, H-5, H-3', H-5'). ^{13}C NMR (100.62; DMSO-d_6) δ in ppm: 30.4 (C-9, C-9'), 68.0 (C-8, C-8'), 103.0 (C-4, C-4'), 115.6 (C-2, C-6, C-2', C-6'), 119.1 (C-7, C-7'), 134.2 (C-3, C-5, C-3', C-5'), 161.7 (C-1, C-1').

4.2.1.11. 1,5-Bis(4-amidinophenoxy)-3-thiapentane dihydrochloride (10): The obtained solution was cooled and diluted with an excess of anhydrous diethyl ether. The precipitated solid was filtered, washed with diethyl ether and dried at 100 °C for 3–4 h to obtain an almost anhydrous white solid of **10** (Yield: 65 %). M.p.= 235.5–236.5 °C. ^{1}H NMR (299.86 MHz, DMSO-d_6) δ in ppm: 3.04 (t; J=6.5 Hz; 4H; H-8, H-8'), 4.30 (t; J=6.5 Hz; 4H; H-9, H-9'), 7.16 (m; 4H, H-2,6, H-2'6'), 7.89 (m; 4H, H-3,5, H-3'5'), 9.12 (broad s; 4H; 2 × 2NH), 9.30 (broad s; 4H; 2 × 2NH). ^{13}C NMR (75.40 MHz, DMSO-d_6) δ in ppm: 30.5 (C-9, C-9'), 68.0 (C-8, C-8'), 114.8 (C-2, C-6, C-2', C-6'), 119.6 (C-4, C-4'), 130.2 (C-3, C-5, C-3', C-5'), 162.6 (C-1, C-1'), 164.7 (C-7, C-7').

4.2.1.12. N,N-Bis[2(4-cyanophenoxy)ethyl]benzenesulfonamide (11a): 4-hydroxybenzonitrile 1.19 g (10 mmol), *N,N-bis* (2-chloroethyl)benzenesulfonamide 1.41 g (5 mmol), and anhydrous K_2CO_3 2.76 g (20 mmol) were heated to 130 °C with stirring in N-methyl-2-pyrrolidone (15 ml). Progress of the reaction was followed by TLC and after 14 h the hot reaction mixture was poured into ice water (200 ml) and extracted with ethyl acetate. The combined ethyl acetate layers were dried ($MgSO_4$) and the solvent was evaporated *in vacuo*. Crude product was crystallized from ethanol to obtain 0.78 g of crystals of **11a** (Yield: 35 %). M.p.= 136–137 °C. ^{1}H NMR (299.86 MHz, $CDCl_3$) δ in ppm: 3.68 (t; J=5.7 Hz; 4H; H-9, H-9'), 4.23 (t; J=5.7 Hz; 4H; H-8, H-8'), 6.83–6.86 (m; 4H;H-2, H-6, H-2', H-6'), 7.48–7.62 (m; 7H; H-3, H-5, H-3', H-5', H-3", H-4", H-5"), 7.82–7.85 (m; 2H;

H-2”-, H-6”). ^{13}C NMR (75.40 MHz, $CDCl_3$) δ in ppm: 49.2 (C-9, C-9’), 67.6 (C-8, C-8’), 104.9 (C-4, C-4’), 115.3 (C-2, C-6, C-2’, C-6’), 119.0 (C-7, C-7’), 127.2 (C-2”, C-6”), 129.5 (C-3”, C-5”), 133.2 (C-4”), 134.3 (C-3, C-5, C-3’, C-5’), 139.3 (C-1”), 161.5 (C-1, C-1’). $C_{24}H_{21}N_3O_4S$ (477.52 g/mol): calcd. (%): C=64.41, H=4.73, N=9.39, S=7.16; found (%): C=64.16, H=4.87, N=9.11, S=7.02.

4.2.1.13. N,N-Bis[2(4-amidinophenoxy)ethyl]benzenesulfonamide dihydrochloride (11): The solvent was evaporated *in vacuo* to give a yellow solid of **11** (Yield: 82 %). M.p.= 246–249 °C (decomp.). ^{1}H NMR (299.86 MHz, DMSO-d_6) δ in ppm: 3.67 (t; J=5.3 Hz; 4H; H-9, H-9’), 4.27 (t; J=5.3 Hz; 4H; H-8.H-8’), 7.03–7.06 (m; 4H; H-2, H-6, H-2’, H-6’), 7.58–7.63 (m; 2H; H-3”, H-5”), 7.66–7.71 (m; 1H; H-4”), 7.84–7.90 (m; 6H; H-3, H-5, H-3’, H-5’, H-2”, H-6”), 9.10 (broad s; 4H; 2 × 2NH), 9.30 (broad s; 4H; 2 × 2NH). ^{13}C NMR (75.40 MHz, DMSO-d_6) δ in ppm: 47.7 (C-9, C-9’), 66.8 (C8, C-8’), 114.6 (C-2, C-6, C-2’, C-6’), 119.7 (C-4, C-4’), 126.9 (C-2”, C-6”), 129.4 (C-3”, C-5”), 130.2 (C-3, C-5, C-3’, C-5’), 133.0 (C-4”), 138.8 (C-1”), 162.3 (C-1, C-1’), 164.6 (C-7, C-7’). $C_{24}H_{27}N_50_4S$ × 2HCl × $3H_2O$ (608.56 g/mol): calcd. (%): C=47.37, H=5.80, N=11.51, S=5.27; found (%): C=47.79, H=5.88, N=11.45, S=5.50.

4.2.1.14. N,N-Bis[2(4-cyano-2-methoxyphenoxy)ethyl]benzenesulfonamide (12a): 3-methoxy-4-hydroxybenzonitrile (1.49 g, 10 mmol), *N*,*N-bis* (2-chloroethyl)benzenesulfonamide (1.41 g, 5 mmol) and anhydrous K_2CO_3 (2.76 g, 20 mmol) in *N*-methyl-2-pyrrolidone (15 ml) were stirred together at 120 °C for 10 h (progress of the reaction was followed by TLC). The hot reaction mixture was poured into ice water (250 ml), and the precipitated solid was filtered, washed with water and dried. After recrystalisation from ethanol with hot filtering, the white solid of **12a** was obtained (Yield: 54%). M.p.= 151.5–153.5 °C. ^{1}H NMR (400.13 MHz, $CDCl_3$) δ in ppm: 3.63 (t; J=6.0 Hz; 4H; H-9, H-9’), 3.69 (s; 6H; H-10, H10’), 4.25 (t; J=6.0 Hz; 4H; H-8, H-8’), 6.81–6.83 (ps d; 2H; H-6, H-6’), 6.98 (d; J=1.2Hz; 2H; H-3, H-3’), 7.17–7.19 (m; 2H; H-5, H-5’), 7.41–7.44 (m; 2H; H-3”, H-5”), 7.50–7.53 (m; 1H; H-4”), 7.77–7.79 (m; 2H; H-2”, H-6”). ^{13}C NMR (100.62 MHz, $CDCl_3$) δ in ppm: 49.9 (C-9, C-9’), 56.1 (C-10, C-10’), 68.3 (C-8, C-8’), 104.7 (C-4, C-4’), 112.7 (C-6, C-6’), 114.4 (C-3, C-3’), 119.2 (C-7, C-7’), 126.5 (C-5, C-5’), 127.3 (C-2”, C-6”), 129.5 (C-3”, C-5”), 133.2 (C-4”), 139.0 (C-1”), 149.5 (C-2, C-2’), 151.8 (C-1, C-1’). $C_{26}H_{25}N_3O_6S$ (507.57 g/mol): calcd. (%): C=61.53, H=4.96, N=8.28, S=6.32; found (%): C=61.28, H=5.14, N=8.51, S=6.12.

4.2.1.15. N,N-Bis[2(4-amidino-2-methoxyphenoxy)ethyl]benzenesulfonamide dihydrochloride (12): Ethanol was evaporated *in vacuo* to give a yellow solid of **12** (Yield: 78). M.p.= 166,0–170,0 °C. ^{1}H NMR (299.87 MHz, DMSO-d_6) δ in ppm: 3.68 (t; J=5.7Hz; 4H; H-9, H-9’), 3.79 (s, 6H; H-10, H-10’), 4.28 (t; J=5.7Hz; 4H; H-8, H-8’), 7.14 (d; J=8.1Hz; 2H; H-6, H-6’), 7.49–7.52 (m; 4H; H-3, H-5, H-3’, H-5’), 7.56–7.61 (m; 2H; H-3”, H-5”), 7.65–7.70 (m; 1H; H-4”), 7.88–7.91 (m; 2H; H-2”, H-6”), 8.98 (broad s; 4H; 2 × 2NH), 9.28 (broad s; 4H; 2 × 2NH). ^{13}C NMR (75.40 MHz, DMSO-d_6) δ in ppm: 48.4 (C-9, C-9’), 55.9 (C-10, C-10’), 67.6 (C-8, C-8’), 111.4 (C-3, C-3’), 112.2 (C-6, C-6’), 119.6 (C-4, C-4’), 121.8 (C-5, C-5’), 126.9 (C-2”, C-6”), 129.5 (C-3”, C-5”), 133.1 (C-4”), 138.5 (C-1”), 148.5 (C-2, C-2’), 152.0 (C-1, C-1’), 164.5 (C-7, C-7’). $C_{26}H_{31}N_5O_6S$ × 2HCl × 3 ½ H_2O (677.62 g/mol): calcd. (%): C=46.08, H=5.90, N=10.33, S=4.73; found (%): 46.08, H=5.80, N=10.25, S=4.68.

4.2.1.16. N,N-Bis[2(4-cyano-2,6-dimethoxyphenoxy)ethyl]benzenesulfonamide (13a): 3,5-dimethoxy-4-hydroxybenzonitrile (1.79 g, 10 mmol), *N*,*N-bis* (2-chloroethyl)benzenesulfonamide (1.41 g, 5 mmol), anhydrous K_2CO_3 (2.76 g, 20 mmol) and *N*-methyl-2-pyrrolidone (30 ml) were heated at 120 °C for 3 h while stirring (progress of the reaction was followed by TLC) and then poured into ice water (250 ml). The resultant solid

was filtered, washed with water and dried at 60 °C. Recrystalisation from a large volume of ethanol gave a white solid of **13a** (Yield: 64 %). M.p.= 194–195 °C. ^{1}H NMR (299.86 MHz, $CDCl_3$) δ in ppm: 3.71 (t; J=8.0 Hz, 4H; H-9, H-9’), 3.80 (s; 12H; H-10, H-10’), 4.22 (t; J=8.0 Hz, 4H; H-8, H-8’), 6.82 (s; 4H; H-3, H-5, H-3’, H-5’), 7.44–7.48 (m; 2H; H-3”, H-5”), 7.52–7.57 (m; 1H; H-4”), 7.83–7.85 (m; 2H; H-2”, H-6”). ^{13}C NMR (75.40 MHz, $CDCl_3$) δ in ppm: 49.2 (C-9, C-9’), 56.4 (C-10, C-10’), 72.4 (C-8, C-8’), 107.1 (C-4, C-4’), 109.5 (C-3, C-5, C-3’, C-5’), 119.0 (C-7, C-7’), 127.4 (C-2”, C-6”), 129.2 (C-3”, C-5”), 132.7 (C-4”), 140.1 (C-1”), 141.4 (C-1, C-1’), 153.7 (C-2, C-6, C-2’, C-6’). $C_{28}H_{29}N_3O_8S$ (567.62 g/mol): calcd. (%): C=59.25, H=5.15, N=7.40, S=5.65; found (%): C=59.06, H=5.23, N=7.16, S=5.89.

4.2.1.17. N,N-Bis[2(4-amidino-2,6-dimethoxyphenoxy)ethyl]-benzenesulfonamide (13): The solvent was evaporated *in vacuo* and the resultant brown solid was recrystalised from a small amount of ethanol to give a light brown solid of **13** (Yield: 51%). M.p.=207.5–210.0 °C (decomp). ^{1}H NMR (299,86 MHz, DMSO-d_6) δ in ppm: 3.62 (t; J=5.7Hz; 4H; H-9, H-9’), 3.81 (s; 12H; H-10, H-10’), 4.08 (t; J=5.7Hz; 4H; H-8, H-8’), 7.21 (s; 4H; H-3, H-5, H-3’, H-5’), 7.56–7.61 (m; 2H; H-3”, H-5”), 7.63–7.68 (m; 1H; H-4”), 7.79–7.82 (m; 2H; H-2”, H-6”), 9.11 (broad s; 4H; 2 × 2NH), 9.39 (broad s; 4H; 2 × 2NH). ^{13}C NMR (75.40 MHz, DMSO-d_6) δ in ppm: 48.3 (C-9, C-9’), 56.3 (C-10, C-10’), 71.4 (C-8, C-8’), 105.8 (C-3, C-5, C-3’, C-5’), 122.5 (C-4, C-4’), 126.8 (C-2”, C-6”), 129.3 (C-3”, C-5”), 132.8 (C-4”), 139.2 (C-1”), 140.5 (C-1, C-1’), 152.6 (C-2, C-6, C-2’, C-6’), 164.7 (C-7, C-7’). $C_{28}H_{35}N_5O_8S \times 2HCl \times 2H_2O$ (710.64 g/mol): calcd. (%): C=47.33, H=5.82, N=9.86, S=4.51; found (%): C=47.48, H=6.11, N=9.66, S=4.60.

4.2.1.18. N,N-Bis[2(4-cyano-2-methoxyphenoxy)ethyl]-4-acetamidobenzenesulfonamide (15a): 3-methoxy-4-hydroxybenzonitrile (1.49 g, 10 mmol), *N,N-bis* (2-chloroethyl)-4-acethyloaminobenzenesulfonamide (1.69 g, 5 mmol), and anhydrous K_2CO_3 (3.46 g, 25 mmol) in N-methyl-2-pyrrolidone (20 ml) were stirred together at 130 °C for 7 h (progress of the reaction was followed by TLC). The hot reaction mixture was poured into ice water (250 ml), the resultant solid was filtered, washed with water and dried at room temp. After recrystalisation from ethanol, a white solid of **15a** was obtained (50%). M.p.= 106.5–108.0 °C. ^{1}H (299.86 MHz, $CDCl_3$) δ in ppm: 2.23 (s; 3H; H-13), 3.71 (t; J=6.0 Hz, 4H; H-9, H-9’), 3.78 (s; 6H; H-10, H-10’), 4.30 (t; J=6.0 Hz; 4H; H-8, H-8’), 6.87 (d; J=8.4 Hz; 2H; H-6, H-6’), 7.04 (d; J=1.8Hz; 2H; H-3, H-3’), 7.24 (dd; J_1=8.4Hz J_2=1.8Hz; 2H; H-5. H-5’), 7.48 (broad s; 1H; NH-11), 7.58–7.61 (m; 2H; H-3”, H-5”), 7.75–7.78 (m; 2H; H-2”, H-6”). ^{13}C NMR (75.40 MHz, $CDCl_3$) δ in ppm: 24.9 (C-13), 49.7 (H-9, H-9’), 56.2 (C-10, C-10’), 68.3 (C-8, C-8’), 104.6 (C-4, C-4’), 112.8 (C-6, C-6’), 114.5 (C-3, C-3’), 119.3 (C-7, C-7’), 119.5 (C-3”, C-5”), 126.5 (C-5, C-5’), 128.6 (C-2”, C-6”), 133.9 (C-1”), 142.3 (C-4”), 149.6 (C-2, C-2’), 151.9 (C-1, C-1’), 168.8 (C-12). $C_{28}H_{28}N_4O_7S$ (564.62 g/mol): calcd. (%): C=59.56, H=5.00, N=9.92, S=5.68; found (%): C=59.48, H=4.87, N=10.06, S=5.79.

4.2.1.19. N,N-Bis[2(4-amidino-2-methoxyphenoxy)ethyl]-4-acetamidobenzenesulfonamide (15): A light yellow solid of **15** was obtained (Yield: 72%). M.p.=262.0-264.0 °C. ^{1}H (299.86 MHz, DMSO-d_6) δ in ppm: 2.09 (s; 3H; H-13), 3.65 (t; J=5.7Hz; 4H; H-9. H-9’), 3.80 (s; 6H; H-10, H-10’), 4.26 (t; J=5.7Hz; 4H; H-8, H-8’), 7.12 (d; J=8.7Hz; 2H; H-6, H-6’), 7.46–7.52 (m; 4H; H-3, H-5, H-3’, H-5’), 7.75–7.81 (m; 4H; H-2”, H-3”, H-5”, H-6”), 9.03 (broad s; 4H; 2 × 2NH), 9.32 (broad s; 4H; 2 × 2NH), 10.54 (s; 1H; NH-11). ^{13}C NMR (75.40 MHz, DMSO-d_6) δ in ppm: 23.7 (C-13), 47.9 (C-9, C-9’), 55.5 (C-10, C-10’), 67.2 (C-8, C-8’), 111.0 (C-3, C-3’), 111.8 (C-6, C-6’), 118.3 (C-3”, C5”), 119.2 (C-4, C-4’), 121.4 (C-5, C-5’), 127.7 (C-2”, C-6”), 131.7 (C-1”), 143.0 (C-4”), 148.1 (C-2, C-2’), 151.6 (C-1, C-1’), 164.2 (C-7, C-7’), 168.7 (C-12). $C_{28}H_{34}N_6O_7S \times 2HCl$

× 1½H_2O (698.60 g/mol): calcd. (%): C=48.14, H=5.64, N=12.02, S=4.59; found (%): C=48.13, H=5.71, N=11.72, S=4.31.

4.2.2. General procedure for synthesis of bisnitriles 17a – 20a—A solution of an appropriate aliphatic diamine (10 mmol) in dichloromethane (30 mL) and triethylamine (2.8 mL) was added dropwise to a stirred, ice cooled solution of 4-cyanobenzoyl chloride (3.31 g, 20 mmol) in dichloromethane (30 mL). The reaction mixture was stirred at room temperature for 12 h (progress of the reaction was followed by TLC), then the solvent was evaporated under reduced pressure and the residue was washed with 1 M $NaHCO_3$, 1 M HCl and water, then dried over anhydrous $CaCl_2$. Analytical samples were obtained after recrystalisation from DMSO-water mixtures (50 – 90 % DMSO*)* .

4.2.2.1. N,N'-Propane-1,3-diylbis(4-cyanobenzamide) (17a): A white solid of **17a** was obtained (Yield: 94%). M.p.=212.5–213.5 °C. ^{1}H NMR (400.13 MHz, DMSO-d_6) δ in ppm: 1.81 (quintet; J=6.8; 2H; H-11), 3.34 (quartet; J=6.3 Hz; 4H; H-10, H-10'), 7.97 (m; 8H; H-2, H-3, H-5, H-6, H-2', H-3', H-5', H-6'), 8.73 (m; 2H; H-9, H-9'). ^{13}C NMR (100.62 MHz, DMSO-d_6) δ in ppm: 28.8 (C-11), 37.3 (C-10, C-10'), 113.5 (C-4, C-4'), 118.3 (C-7, C-7'), 128.0 (C-2, C-6, C-2', C-6'), 132.4 (C-3, C-5, C-3', C-5'), 138.5 (C-1, C-1'), 164.8 (C-8, C-8'). $C_{19}H_{16}N_4O_2$ × 0.75H_2O (345,87 g/mol). Calcd. (%) C=65.99; H=5.07; N=16.21; found (%): C=66.05; H=4.84; N=16.00.

4.2.2.2. N,N'-Propane-1,3-diylbis(4-amidinobenzamide) dihydrochloride (17): A beige solid of **17** was obtained (Yield: 37%). M.p.=302.5–304.5 °C. ^{1}H NMR (400.13 MHz, DMSO-d_6) δ in ppm: 1.82 (quintet; J=6.3; 2H; H-11), 3.38 (quartet; J=6.2 Hz; 4H; H-10, H-10'), 7.95 (d; J=8.4 Hz; 4H; H-3, H-5, H-3', H-5'), 8.12 (d; J=8.4 Hz; 4H; H-2, H-6, H-2', H-6'), 9.09 (t; J=5.6 Hz; 2H; H-9, H-9'), 9.40 (s; 4H; H-7, H-7'), 9.58 (s; 4H; H-7, H-7'). ^{13}C NMR (100.62 MHz, DMSO-d_6) δ in ppm: 28.8 (C-11), 37.0 (C-10, C-10'), 127.6 (C-2, C-6, C-2', C-6'), 128.2 (C-3, C-5, C-3', C-5'), 130.2 (C-4, C-4'), 138.9 (C-1, C-1'), 164.9 (C-8, C-8'), 165.2 (C-7, C-7'). $C_{19}H_{22}N_6O_2$ × 2HCl × 4H_2O (511.40 g/mol). Calcd. (%) C=44.62; H=6.26; N=16.44; found (%): C=44.61; H=6.06; N=16.21.

4.2.2.3. N,N'-Butane-1,4-diylbis(4-cyanobenzamide) (18a) [24]: A white solid of **18a** was obtained (Yield: 97%). M.p.=266.5–268.5 °C (Ref. [24] 264–265 °C).

4.2.2.4. N,N'-Butane-1,4-diylbis(4-amidinobenzamide) dihydrochloride (18): A beige solid of **18** was obtained (Yield: 52%). M.p.=295.0–296.0 °C. ^{1}H NMR (400.13 MHz, DMSO-d_6) δ in ppm: 1.61 (m; 4H; H-11, H-11'), 3.32 (m; 4H; H-10, H-10'), 7.92 (d; J=8.4 Hz; 4H; H-3, H-5, H-3', H-5'), 8.05 (d; J=8.7 Hz; 4H; H-2, H-6, H-2', H-6'), 8.82 (t; J=5.6 Hz; 2H; H-9, H-9'), 9.28 (s; 4H; H-7, H-7'), 9.51 (s; 4H; H-7, H-7'). ^{13}C NMR (100.62 MHz, DMSO-d_6) δ in ppm: 26.6 (C-11, C-11'), 39.1 (C-10, C-10'), 127.6 (C-2, C-6, C-2', C-6'), 128.2 (C-3, C-5, C-3', C-5'), 130.2 (C-4, C-4'), 139.0 (C-1, C-1'), 164.8 (C-8, C-8'), 165.1 (C-7, C-7'). $C_{20}H_{24}N_6O_2$ × 2HCl × 3½5H_2O (516.42 g/mol). Calcd. (%) C=46.51; H=6.40; N=16.28; found (%): C=46.67; H=6.56; N=15.95.

4.2.2.5. N,N'-Pentane-1,5-diylbis(4-cyanobenzamide) (19a): White solid of **19a** was obtained from 1,5-diaminopentane dihydrochloride after following the general procedure for synthesis of bisnitriles **17a** – **20a** (Yield: 72%). M.p.=174.0–174.5 °C. ^{1}H NMR (400.13 MHz, DMSO-d_6) δ in ppm: 1.35 (m; 2H; H-12), 1.56 (quintet; J=7.2 Hz; 4H; H-11, H-11'), 3.27 (quartet; J=6.5 Hz; 4H; H-10, H-10'), 7.95 (m; 8H; H-2, H-3, H-5, H-6, H-2', H-3', H-5', H-6'), 8.68 (t; J=5.4 Hz; 2H; H-9, H-9'). ^{13}C NMR (100.62 MHz, DMSO-d_6) δ in ppm: 23.9 (C-12), 28.6 (C-11, C-11'), 39.3 (C-10, C-10'), 113.4 (C-4, C-4'), 118.3 (C-7, C-7'), 128.0 (C-2, C-6, C-2', C-6'), 132.3 (C-3, C-5, C-3', C-5'), 138.6 (C-1, C-1'), 164.7

(C-8, C-8'). $C_{21}H_{20}N_4O_2 \times 0.25H_2O$ (364.91 g/mol). Calcd. (%) C=69.14; H=5.62; N=15.36; found (%): C=68.98; H=5.60; N=15.19.

4.2.2.6. N,N'-Pentane-1,5-diylbis(4-amidinobenzamide) dihydrochloride (19): A beige solid of **19** was obtained (Yield: 44%). M.p.=101.0–104.0 °C. ^{1}H NMR (400.13 MHz, DMSO-d_6) δ in ppm: 1.37 (m; 2H; H-12), 1.61 (quintet; J=7.1 Hz; 4H; H-11, H-11'), 3.30 (m; 4H; H-10, H-10'), 7.94 (d; J=8.1 Hz; 4H; H-3, H-5, H-3', H-5'), 8.06 (d; J=8.4 Hz; 4H; H-2, H-6, H-2', H-6'), 8.83 (t; J=5.4 Hz; 2H; H-9, H-9'), 9.37 (s; 4H; H-7, H-7'), 9.57 (s; 4H; H-7, H-7'). ^{13}C NMR (100.62 MHz, DMSO-d_6) δ in ppm: 24.0 (C-12), 28.7 (C-11, C-11'), 39.3 (C-10, C-10'), 127.6 (C-2, C-6, C-2', C-6'), 128.2 (C-3, C-5, C-3', C-5'), 130.1 (C-4, C-4'), 139.1 (C-1, C-1'), 164.8 (C-8, C-8'), 165.2 (C-7, C-7'). $C_{21}H_{26}N_6O_2 \times 2HCl \times 3H_2O$ (516.42 g/mol). Calcd. (%) C=48.37; H=6.53; N=16.12; found (%): C=48.73; H=6.84; N=15.94.

4.2.2.7. N,N'-Hexane-1,6-diylbis(4-cyanobenzamide) (20a): A white solid of **20a** was obtained (Yield: 98%). M.p.=230.0–231.0 °C. ^{1}H NMR (400.13 MHz, DMSO-d_6) δ in ppm: 1.34 (m; 4H; H-12, H-12'), 1.53 (m; 4H; H-11, H-11'), 3.26 (quartet; J=6.4 Hz; 4H; H-10, H-10'), 7.96 (m; 8H; H-2, H-3, H-5, H-6, H-2', H-3', H-5', H-6'), 8.68 (t; J=5.0 Hz; 2H; H-9, H-9'). ^{13}C NMR (100.62 MHz, DMSO-d_6) δ in ppm: 26.2 (C-12, C-12'), 28.9 (C-11, C-11'), 39.2 (C-10, C-10'), 113.4 (C-4, C-4'), 118.3 (C-7, C-7'), 128.0 (C-2, C-6, C-2', C-6'), 132.4 (C-3, C-5, C-3', C-5'), 138.6 (C-1, C-1'), 164.7 (C-8, C-8'). $C_{22}H_{22}N_4O_2 \times ½H_2O$ (383,44 g/mol). Calcd. (%) C=68.93; H=6.01; N=14.62; found (%): C=68.93; H=5.80; N=14.43.

4.2.2.8. N,N'-Hexane-1,6-diylbis(4-amidinobenzamide) dihydrochloride (20): A beige solid of **20** was obtained (Yield: 83%). M.p.=253.0–256.0 °C. ^{1}H NMR (400.13 MHz, DMSO-d_6) δ in ppm: 1.34 (m; 2H; H-12, H-12'), 1.53 (m; 4H; H-11, H-11'), 3.26 (quartet; J=6.4 Hz; 4H; H-10, H-10'), 7.92 (d; J=8.7 Hz; 4H; H-3, H-5, H-3', H-5'), 8.05 (d; J=8.7 Hz; 4H; H-2, H-6, H-2', H-6'), 8.79 (t; J=5.7 Hz; 2H; H-9, H-9'), 9.32 (s; 4H; H-7, H-7'), 9.52 (s; 4H; H-7, H-7'). ^{13}C NMR (100.62 MHz, DMSO-d_6) δ in ppm: 26.2 (C-12, C-12'), 29.0 (C-11, C-11'), 39.2 (C-10, C-10'), 127.6 (C-2, C-6, C-2', C-6'), 128.2 (C-3, C-5, C-3', C-5'), 130.1 (C-4, C-4'), 139.0 (C-1, C-1'), 164.8 (C-8, C-8'), 165.1 (C-7, C-7'). $C_{22}H_{28}N_6O_2 \times 2HCl \times 3H_2O$ (535.46 g/mol). Calcd. (%) C=49.35; H=6.73; N=15.70; found (%): C=49.32; H=7.12; N=15.61.

Research highlights

- A series of 20 pentamidine analogs were prepared with high yield and purity
- All were evaluated for efficacy against *Pneumocystis carinii* in an ATP bioassay
- Heteroatoms in linker and absence of methoxy groups on benzene were associated with higher activities
- These compounds hold promise for decreased side effects within the mammalian host

Acknowledgments

MTC/MSC would like to recognize the support of the NIH (through the contract mechanism N01-A1-25647) for development of the *in vitro* methods used for the screening of candidate anti-*Pneumocystis* agents.

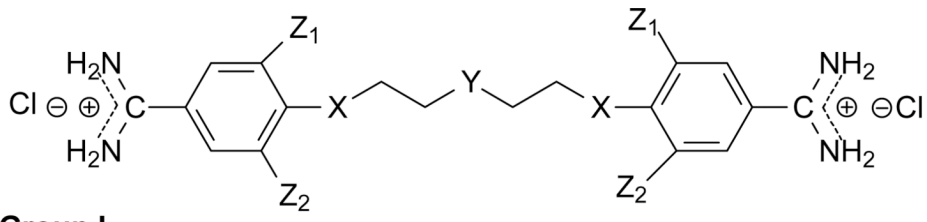
Funding Sources

MTC/MSC were supported by a grant from the National Institutes of Health, NIAID R01 AI076104 and the Department of Veterans Affairs.

References

1. Cushion MT, Walzer PD. Preclinical drug discovery for new anti-neumocystis compounds. Curr. Med. Chem. 2009; 16:2514–2530. [PubMed: 19601796]
2. Kaur N, Mahl TC. Pneumocystis carinii pneumonia with oral candidiasis after infliximab therapy for crohn's disease. Dig. Dis. Sci. 2004; 49:1458–1460. [PubMed: 15481319]
3. Kaur N, Mahl TC. Pneumocystis jiroveci (carinii) pneumonia after infliximab therapy: A review of 84 cases. Dig. Dis. Sci. 2007; 52:1481–1484. [PubMed: 17429728]
4. Morris A, Sciurba FC, Lebedeva IP, Githaiga A, Elliott WM, Hogg JC, Huang L, Norris KA. Association of chronic obstructive pulmonary disease severity and pneumocystis colonization. Am. J. Respir. Crit. Care Med. 2004; 170:408–413. [PubMed: 15117741]
5. Joffrion TM, Cushion MT. Sterol biosynthesis and sterol uptake in the fungal pathogen pneumocystis carinii. FEMS Microbiol. Lett. 2010; 311:1–9. [PubMed: 20528942]
6. Tidwell RR, Jones SK, Geratz JD, Ohemeng KA, Bell CA, Berger BJ, Hall JE. Development of pentamidine analogues as new agents for the treatment of pneumocystis carinii pneumonia. Ann. N. Y. Acad. Sci. 1990; 616:421–441. [PubMed: 2078033]
7. Donkor IO, Tidwell RR, Jones SK. Pentamidine congeners. 2. 2-butene-bridged aromatic diamidines and diimidazolines as potential anti-pneumocystis carinii pneumonia agents. J. Med. Chem. 1994; 37:4554–4557. [PubMed: 7799405]
8. Cushion MT, Walzer PD, Ashbaugh A, Rebholz S, Brubaker R, Vanden Eynde JJ, Mayence A, Huang TL. In vitro selection and in vivo efficacy of piperazine- and alkanediamide-linked bisbenzamidines against pneumocystis pneumonia in mice. Antimicrob. Agents Chemother. 2006; 50:2337–2343. [PubMed: 16801410]
9. Cushion MT, Walzer PD, Collins MS, Rebholz S, Vanden Eynde JJ, Mayence A, Huang TL. Highly active anti-pneumocystis carinii compounds in a library of novel piperazine-linked bisbenzamidines and related compounds. Antimicrob. Agents Chemother. 2004; 48:4209–4216. [PubMed: 15504843]
10. Greenidge PA, Jenkins TC, Neidle S. DNA minor groove recognition properties of pentamidine and its analogs: a molecular modeling study. Mol. Pharmacol. 1993; 43:982–988. [PubMed: 8316226]
11. Simpson IJ, Michael I, Kumar A, Boykin DW, Neidle S. DNA minor groove interactions and biological activity of 2,5-*bis*-[4-(*N*-alkylamidino)phenyl]furans. Bioorg. Med. Chem. Lett. 2000; 10:2593–2597. [PubMed: 11128631]
12. De Oliveira AM, Custodio FB, Donnici CL, Montanari CA. QSAR and molecular modeling studies of B-DNA recognition of minor groove binders. Eur. J. Med. Chem. 2003; 38:141–155. [PubMed: 12620659]
13. Żołek T, Maciejewska D. Theoretical models of pentamidine analogs based on their DNA minor groove complexes. Eur. J. Med. Chem. 2010; 45:1991–1999. [PubMed: 20153561]
14. Nguyen B, Lee MPH, Hamelberg D, Joubert A, Bailly Ch, Brun R, Neidle S, Wilson WD. Strong binding in DNA minor goove by an aromatic diamidine with a shape that does not match the curvature of the groove. J. Am. Chem. Soc. 2002; 124:13680–13681. [PubMed: 12431090]
15. Huang TL, Vanden Eynde JJ, Mayence A, Collins MS, Cushion MT, Rattendi D, Londono I, Mazumder L, Bacchi CJ, Yarlett N. Synthesis and SAR of alkanediamide-linked bisbenzamidines with anti-trypanosomal and antipneumocystis activity. Bioorg. Med. Chem. Lett. 2009; 19:5884–5886. [PubMed: 19736009]
16. Ashley JN, Barber HJ, Ewins AJ, Newbery G, Self ADH. A chemiotherapeutic comparison of the trypanocidal actin of some aromatic diamidines. J. Chem. Soc. 1942:103–116.
17. Berg SS, Newbery G. The search for chemiotherapeutic amidines. Part X. Substituted 4: 4'-diamidino-αω-diphenoxyalkanes and –diphenyl ethers. J. Chem. Soc. 1949:642–648.

18. Francesconi I, Wilson WD, Tanious FA, Hall JE, Bender BC, Tidwell RR, McCurdy D, Boykin DW. 2,4-Diphenyl furan diamidines as novel anti-*Pneumocystis carinii* pneumonia agents. J. Med. Chem. 1999; 42:2260–2265. [PubMed: 10377232]
19. Maciejewska D, Kaźmierczak P, Żabinski J, Wolska I, Popis S. Pentamidine Analogs: Syntheses, structures In solid state by ^{13}C CP/MAS NMR spectroscopy, and X-ray crystallography and their preliminary biological screening against human cancer. Monatsh. f. Chem. 2006; 137:1225–1240.
20. Żabinski J, Maciejewska D, Kaźmierczak P. Structural analysis of bis-nitriles and bisamidines in solid state by combining NMR spectroscopy and molecular modeling. J. Mol. Struct. 2009; 923:132–140.
21. Żabinski J, Maciejewska D, Wolska I. Solid state structural analysis of new pentamidine analogs designed as chemotherapeutics that targed DNA by X-ray diffraction and ^{13}C, ^{15}N CP/MAS NMR methods. J. Mol. Struct. 2010; 984:68–74.
22. Maciejewska D, Wolska I, Żabinski J. Examination of the structure in solid state of amino analogs of 4,4'[1,5-pentanediylbis(oxy)]bisbenzonitrile by means of X-ray diffraction, ^{13}C CP/MAS NMR, and theoretical calculations. J. Mol. Struct. 2008; 879:53–59.
23. Żabiński J, Wolska I, Maciejewska D. Comparsion of structure in solid state of new 1,5-*bis*(4-cyano-2,6-dimethoxyphenoxy)alkanes by means of ^{13}C CP/MAS NMR and X-Ray diffraction. J. Mol. Struct. 2007; 883:74–81.
24. Cui J, Crich D, Wink D, Lam M, Rheingold AL, Case DA, Fu W, Zhou Y, Rao M, Olson AJ, Johnson ME. Desing and synthesis of highly constrained factor Xa inhibitors: amidine-substituted *bis*(benzoyl)-[1,3]-diazepan-2-ones and *bis*(benzylidene)-*bis*(gem-dimethyl)cycloketones. Bioorg. & Med. Chem. 2003; 11:3379–3392. [PubMed: 12878132]
25. Collins MS, Cushion MT. Standardization of an in vitro drug screening assay by use of cryopreserved and characterized pneumocystis carinii populations. J. Eukaryot. Microbiol. 2001; 48 Suppl.:178S–179S. [PubMed: 11906052]
26. Cushion MT, Chen F, Kloepfer N. A cytotoxicity assay for evaluation of candidate anti-pneumocystis carinii agents. Antimicrob. Agents Chemother. 1997; 41:379–384. [PubMed: 9021195]
27. Kaneshiro ES, Collins MS, Cushion MT. Inhibitors of sterol biosynthesis and amphotericin B reduce the viability of pneumocystis carinii f. sp. carinii. Antimicrob. Agents Chemother. 2000; 44:1630–1638. [PubMed: 10817720]
28. Cushion MT, Collins MS, Hazra B, Kaneshiro ES. Effects of atovaquone and diospyrin-based drugs on the cellular ATP of pneumocystis carinii f. sp. carinii. Antimicrob. Agents Chemother. 2000; 44:713–719. [PubMed: 10681344]



Group I

1 X = O, Y = NCH_3, Z_1, Z_2 = H

2 X = O, Y = NCH_3, Z_1 = OCH_3, Z_2 = H

3 X = O, Y = NCH_3, Z_1,Z_2 = OCH_3

4 X = NCH_3, Y =CH_2, Z_1,Z_2 = H

5 X = NH, Y = O, Z_1, Z_2 = H

6 X, Y = O, Z_1, Z_2 = H

7 X, Y = O, Z_1 = OCH_3, Z_2 = H

8 X, Y = O, Z_1, Z_2 = OCH_3

9 X = O, Y = CH_2, Z_1, Z_2 = OCH_3

10 X = O, Y = S, Z_1, Z_2 = H

Group II

11 X = O, Y = NSO_2Ph, Z_1, Z_2 = H

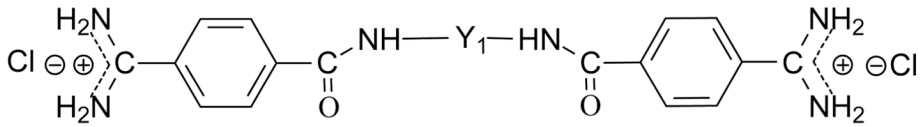
12 X = O, Y = NSO_2Ph, Z_1 =OCH_3, Z_2 = H

13 X = O, Y = NSO_2Ph, Z_1, Z_2 = OCH_3

14 X = O, Y = $NSO_2Ph(4\text{-}NHCOCH_3)$, Z_1, Z_2 = H

15 X = O, Y = $NSO_2Ph(4\text{-}NHCOCH_3)$, Z_1 = OCH_3, Z_2 = H

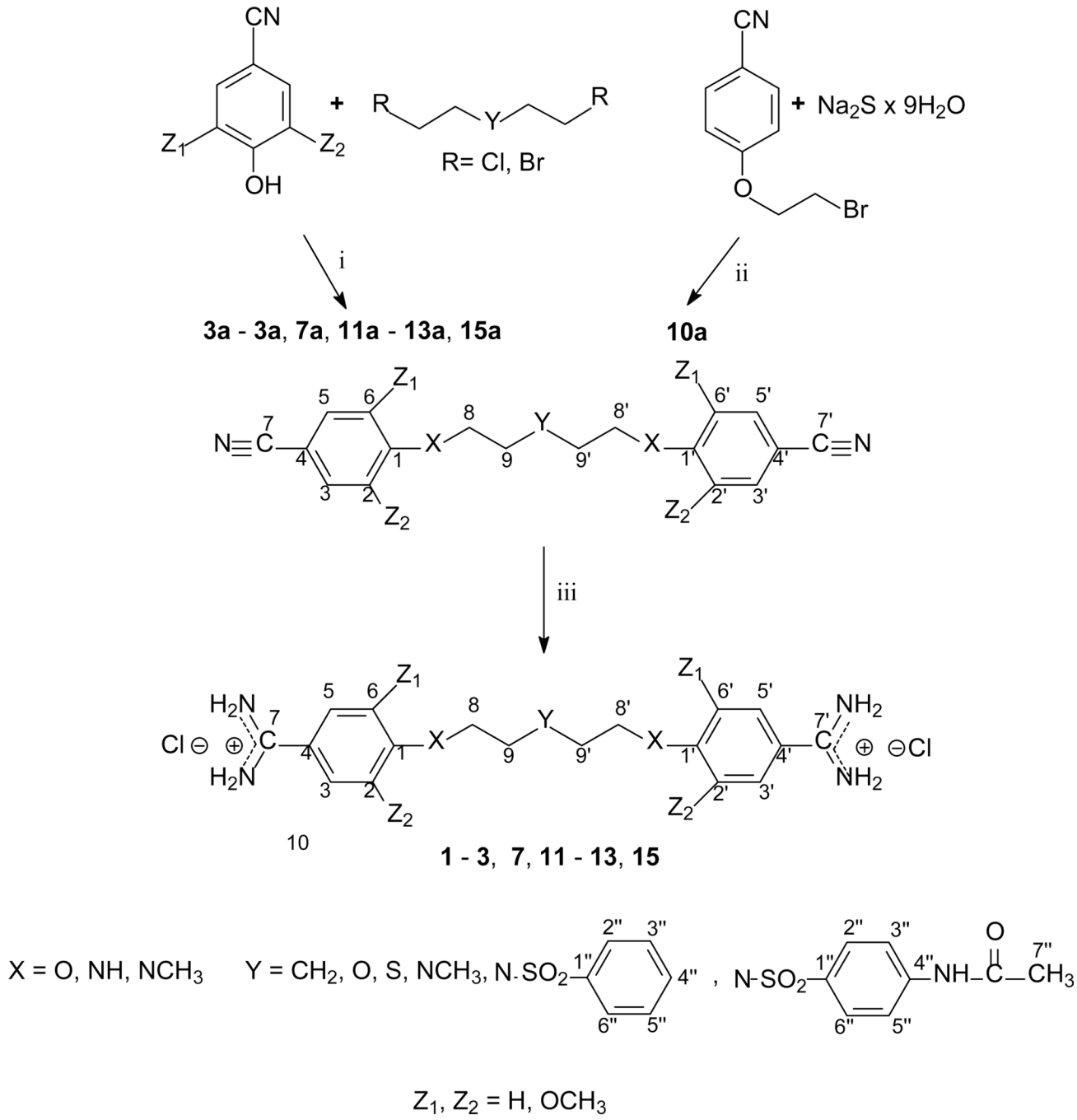
16 X = O, Y = $NSO_2Ph(4\text{-}NH_2)$, Z_1, Z_2 = OCH_3



Group III

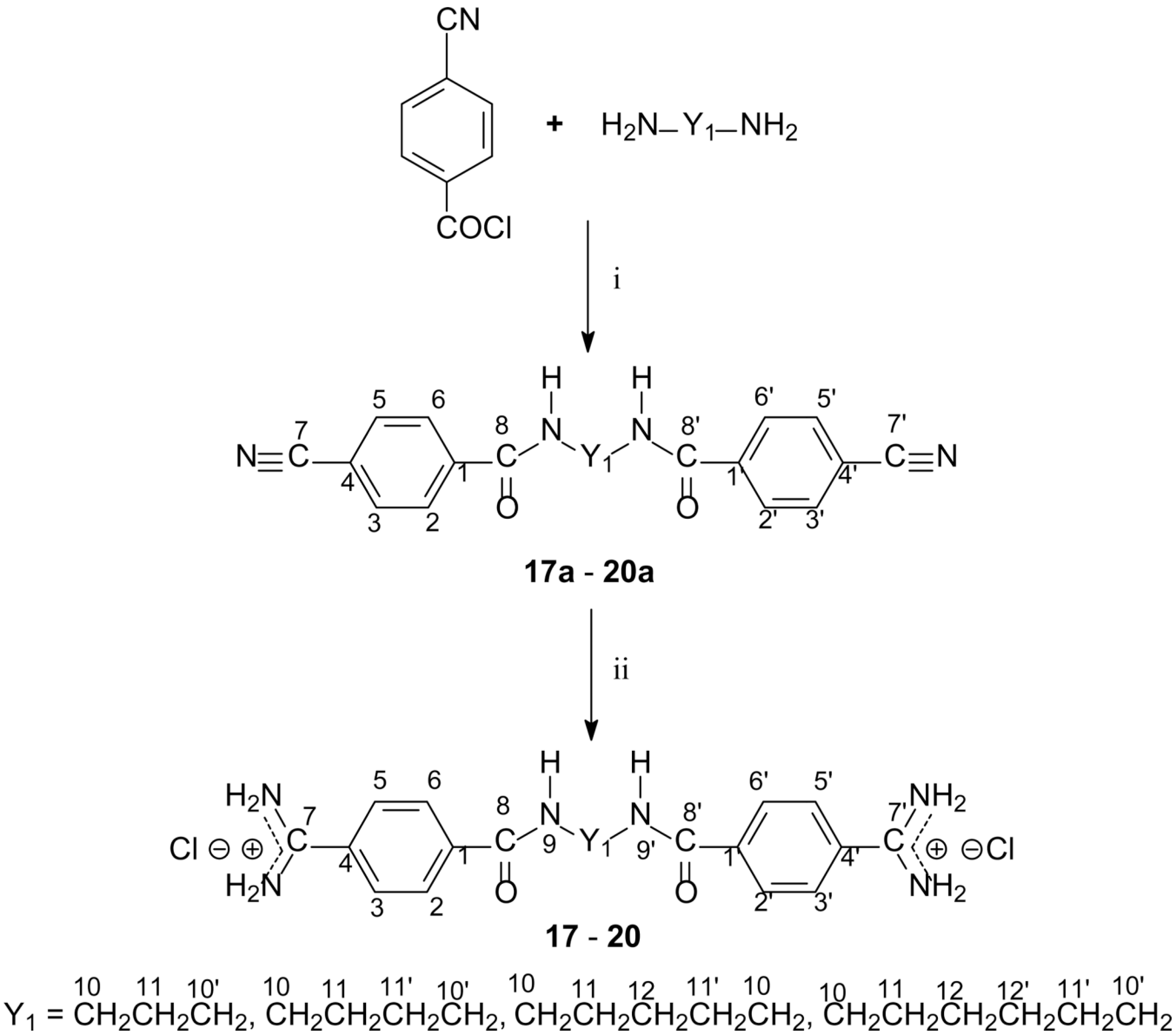
Y_1 = **17** $-(CH_2)_3-$, **18** $-(CH_2)_4-$, **19** $-(CH_2)_5-$, **20** $-(CH_2)_6-$

Figure 1.
Chemical structures of tested pentamidine analogs divided in Group I, II and III.



Scheme 1.

Synthetic routes to bisnitriles **1a** – **3a, 7a**, **11a** – **13a**, **15a** and bisamidines **1** – **3, 7**, **11** – **13**, **15** with atom numbering; i: K_2CO_3, *N*-methyl-2-pyrrolidone; ii: DMSO; iii: 1) HCl/EtOH, 2) NH_3/EtOH.



Scheme 2.

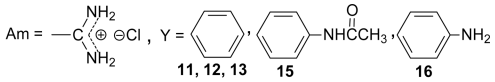
Synthetic routes to bisnitriles **17a** – **20a** and bisamidines **17** – **20** with atom numbering; i: TEA, CH_2Cl_2; ii: 1) HCl/EtOH, 2) NH_3/EtOH.

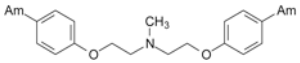
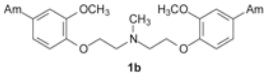
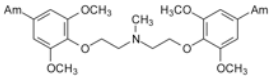
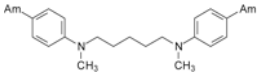
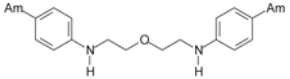
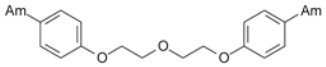
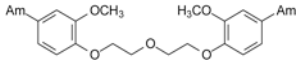
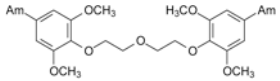
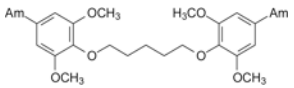
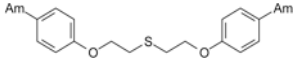
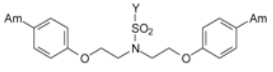
Eur J Med Chem. Author manuscript; available in PMC 2013 February 1.

Table 1

Anti-Pnemocystis Activity (IC_{50}), A549 and L2 toxicity of compounds **1** – **13**, **15** – **20**.

Symbols:



Name	Chemical structure	IC_{50} (μg/ml) ± SD for *P. carinii*	IC_{50} (μM)	Activity ranking	A549 Toxicity IC_{50} (μg/ml)	L2 Toxicity IC_{50} (μg/ml)
1		0.82 ± 0.44	1.73	Marked	>100	>100
2	 **1b**	2.10 ± 1.24	3.41	Moderate	>100	>100
3		45.21 ± 16.9	6.26	Slight	>100	>100
4		1.02 ± 0.51	1.99	Moderate	>100	47.1
5		0.47 ± 0.09	1.11	Marked	>100	36.9
6		0.96 ± 0.32	2.13	Marked	>100	>100
7		2.00 ± 1.65	4.21	Moderate	>100	>100
8		7.66 ± 1.67	12.99	Moderate	>100	>100
9		1.68 ± 0.37	3.00	Moderate	>100	17.8
10		0.52 ± 0.04	1.18	Marked	>100	>100
11		0.81 ± 0.12	1.33	Marked	>100	>100

Name	Chemical structure	IC_{50} (μg/ml) ± SD for *P. carinii*	IC_{50} (μM)	Activity ranking	A549 Toxicity IC_{50} (μg/ml)	L2 Toxicity IC_{50} (μg/ml)
12		1.80 ± 0.76	2.66	Moderate	>100	>100
13		3.13 ± 0.99	4.40	Moderate	>100	>100
15		2.59 ± 0.83	3.71	Moderate	>100	>100
16		3.24 ± 0.88	4.16	Moderate	>100	>100
17		1.53 ± 0.90	2.99	Moderate	>100	>100
18		1.85 ± 0.01	3.58	Moderate	>100	>100
19		7.40 ± 0.22	14.33	Moderate	>100	>100
20		4.90 ± 1.20	9.15	Moderate	>100	>100