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ARTICLE

Development of highly active anti-*Pneumocystis* bisbenzamidines: Insight into the influence of selected substituents on the *in vitro* activity†

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Here we describe the potency of 21 pentamidine analogues against the fungal pathogen, *Pneumocystis carinii*, in an ATP bioluminescent assay with toxicity profiles in 2 mammalian cell lines. Reduction of two 5-methyl-1,2,4-oxadiazole rings was applied to the synthesis of acid-labile bisamidines. Anti-*Pneumocystis* activity is discussed in the context of 3 groups of compounds depending on the main structural changes of the pentamidine lead structure. The groups include: 1) 1,4-bis(methylene)piperazine derivatives **1–5**; 2) alkanediamide derivatives **6–10**; 3) alkane-derived bisbenzamidines **11–21**. IC_{50} values of 18 compounds were lower than the IC_{50} of pentamidine. Four bisamidines were active at nanogram concentrations. Introduction of sulfur atoms in the alkane bridge, replacement of the amidino groups with imidazoline rings, or attachment of nitro or amino groups to the benzene rings is responsible for remarkable activity of the new leading structures. The vast majority of compounds, including four highly active ones, can be classified as mild or nontoxic to host cells. These compounds show promise as candidates for new anti-*Pneumocystis* agents.

Introduction

Immunocompromised humans and other mammals are potential targets for opportunistic infections (OI).[1–5] In humans with debilitated immune systems, infection with *Pneumocystis jirovecii* and development of *Pneumocystis* pneumonia (PCP) remains a major cause of morbidity and mortality in these susceptible populations.[6,7] Although the incidence of PCP has decreased in HIV-positive individuals after introduction of Highly-Active Antiretroviral Therapy (HAART), it remains a principal AIDS-defining illness.[8] Infection and colonization with *P. jirovecii* continue to be serious problems in patients with other types of immunodeficiencies as well. For example, widespread use of immunosuppressive medications plays a crucial role in the development of PCP in patients with autoimmune diseases (rheumatoid arthritis, Crohn's disease) and following transplantations.[9]

While PCP is a significant life-threatening OI, there are still many problems hindering the development of modern and dedicated therapies for its treatment and prevention. *P. jirovecii* is naturally resistant to the majority of antifungal drugs including azoles and amphotericin B, due to the lack of an active ergosterol synthesis pathway.[10] Only a few therapeutic choices are effective for treatment of PCP; primarily trimethoprim-sulfamethoxazole, followed by secondary regimens such as pentamidine, atovaquone, or clindamycin-primaquine. Their application is limited respectively by potential resistance and toxicity.[5,11,12] Sustainable, long term *in vitro* cultivation of any *Pneumocystis* species remains elusive, hampering serious development of alternative therapies and prophylactic agents.[13] Moreover, the mechanism of action of pentamidine on molecular level seems to be complex. Several molecular targets may be involved in the overall anti-*Pneumocystis* activity.[14,15] Despite current searches for the next generation of anti-PCP therapy, no compound has passed all of the phases of clinical trials.[16] Therefore, there is an urgent need for development of new potent molecules for both prophylaxis and treatment of PCP. Pentamidine provides an effective treatment of PCP but is considered a second-line treatment, primarily due to associated toxicities in treatment populations.[6,9]

It is our goal to identify analogues of the parent compound that are equally or more efficacious with reduced toxicity in the pre-clinical setting. Previous reports of new classes of bisamidines showed notable *in vitro* anti-*Pneumocystis* efficacy and low toxicity to the mammalian host cell lines. Leading structures contained alkane[17], alkanediamide[18,19] or a piperazine linker.[20,21] Based on the outstanding properties of these pentamidine analogues, we decided to investigate further selected structural modifications of them.

Among the new bisamidines described herein, compounds **1–5** have piperazine linker with loosened rigidity by adding two

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† Electronic Supplementary Information (ESI) available: details of synthetic procedures, characterization data and NMR spectra of newly reported compounds. See DOI: 10.1039/x0xx00000x

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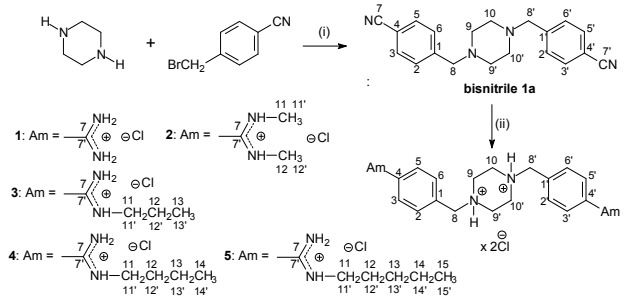
methylene groups between the piperazine and phenyl groups. We wondered if elongation of the distance between the benzamidine moieties would increase anti-PCP activity. This hypothesis was based on the examples of highly active pentamidine analogues with linkers containing more than 4 atoms.[19] Analogues **6–10** were modified alkanediamides. Recently we tested compounds with reversed manner of amide group substitution[17], but this resulted in compounds with decreased activity. In this studies we tested other modifications in the alkyl linker. The last group of pentamidine analogues included compounds **11–21** that have various heteroatoms in the aliphatic linker (*O*, *S*, *N*). The influence of phenyl ring substituents was also studied taking into account electrodonating and electrowithdrawing groups. These modifications expand diversity of the alkane-linked pentamidine analogues library, and add new data to the previously reported results.[17] In summary, we built a library of 21 unique compounds for a comprehensive structure-activity relationship study and comparison with the previously published results.

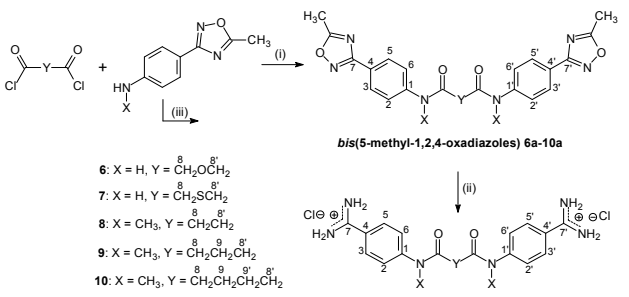
Results and discussion

Chemistry

The new bisamidines were obtained following known methods by the modified Pinner reaction[17,22] of appropriate bisnitriles (**1a, 11a–21a**) in case of compounds **1–5** (Scheme 1) and compounds **11–21** (Scheme 3) or by catalytic reduction[23] of bis(5-methyl-1,2,4-oxadiazoles) **6a–10a** in case of acid-labile diamide-linked derivatives **6–10** (Scheme 2). We report a successful preparation and deprotection strategy for compounds with two 5-methyl-1,2,4-oxadiazole moieties. This strategy seems to be a serious alternative to the Pinner reaction, used widely in the preparation of pentamidine analogues, especially in case of compounds with sensitive functional groups.

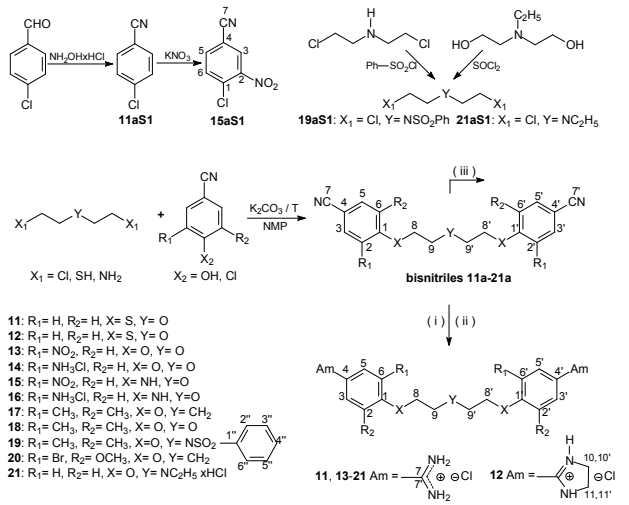


Scheme 1 Synthetic route to 1,4-bis(methylene)piperazine-linked bisamidines **1–5** with atom numbering of the newly synthesized compounds. Reagents and conditions: (i) K_2CO_3, DMF; (ii) 1) HCl/EtOH, 2) RNH_2/EtOH, 3) HCl/EtOH.



Scheme 2 Synthetic route to diamide-linked bisamidines **6–10** with atom numbering of the newly synthesized compounds. Reagents and conditions: (i): *N*-methylmorpholine, CH_2Cl_2; (ii): 1) H_2, 10% Pd/C, AcOH, MeOH, 2) HCl/MeOH; (iii) 4-(5-methyl-1,2,4-oxadiazol-3-yl)aniline was treated with: 1) paraformaldehyde, MeOH, 2) $NaBH_4$, MeOH; to give appropriate *N*-methyl derivative.

Synthesis of compounds **1** and **1a** have been published by our research group together with their structural analysis.[22] Intermediate bis(5-methyl-1,2,4-oxadiazoles) **6a–10a** were obtained by substitution of appropriate dicarboxylic acid chlorides with 4-(5-methyl-1,2,4-oxadiazol-3-yl)anilines. *N*-Methyl-4-(5-methyl-1,2,4-oxadiazol-3-yl)aniline was obtained by reductive amination of paraformaldehyde with 4-(5-methyl-1,2,4-oxadiazol-3-yl)aniline[24] (see Scheme 2). Bisnitriles **11a–21a** were prepared following well established methods, including S_NAr reactions of various 4-chlorobenzonitriles (when X = S or NH), or S_N2 reactions of several 4-hydroxybenzonitriles (when X = O) (Scheme 3).[25–33]



Scheme 3 Synthetic route to bisamidines **11–21** with atom numbering of the newly synthesized compounds. Reagents and conditions: (i) HCl/EtOH; (ii) NH_3/EtOH, or $NH_2CH_2CH_2NH_2$/EtOH in case of **12**; (iii) 1,5-bis(4-cyanophenoxy)-3-oxapentane[33] was treated with HNO_3/AC_2O/TFA to give **13a**; NO_2 groups in **13a** and **15a** were reduced with H_2/Pd to obtain **14a** and **16a**.

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Anti-*Pneumocystis* activity

Compounds **1–21** were first tested at a single high dose to screen out ineffective products. With the exception of compound **2**, all studied pentamidine derivatives were effective at reducing the viability of *P. carinii in vitro* at 100 μg/ml screening concentration. In our discovery pipeline, these compounds would then be selected for *in vitro* evaluation using *P. carinii* organisms and the ATP bioluminescent assay to identify the 50% inhibitory concentration (IC_{50}) as a means to determine efficacy (Table 1). IC_{50} values of 18 bisamidines were lower than 0.30 μg/ml (0.50 μM) given for pentamidine. Compound **1** which showed "very marked" activity is related to the piperazine linked derivatives described before[20,21]. Inserting methylene groups between piperazine and benzene rings increased anti-*Pneumocystis* activity only in case of compounds without bulky groups at amidine part: **1** (IC_{50} = 0.062 μg/ml, 0.109 μM), **3** (IC_{50} = 1.10 μg/ml, 1.685 μM). However we also observed an increase of activity when the longest alkyl chains were introduced (compounds **4** and **5**). For the first time, an acyclic *N*,*N*'-disubstitued bisamidine was evaluated in this assay (compound **2**), but this resulted in total loss of activity. The loss of activity was likely due to a steric hindrance, where the two methyl groups which are apart from each other form an unfavourable trans conformation. This is in opposition to the cyclic analogue (compound **12**) with fixed *cis* conformation and high anti-*Pneumocystis* activity.

The alkanediamide linked bisamidines previously have been tested[18,19], with an IC_{50} of 0.001 μg/ml (0.003 μM) for a compound with a propylene linker. Replacement of the middle methylene group in the connecting propylene chain with heteroatoms *O* and *S* (compounds **6** and **7**) decreased activities against *P. carinii*, though significant activities were retained. This is consistent with data obtained earlier for alkane-linked bisamidines[17]. Substitution of amide nitrogen atoms with methyl groups (giving new compounds **8–10**) modulated the potency of these compounds. The high activity at nanogram concentration of **10** was retained only for the butylene chain (0.006 μg/ml, 0.012 μM). This probably indicates that additional steric hindrance is not desirable in case of bisamidines with short alkanediamide linker.

The best results were obtained for compounds **11–21** with variations of bridge heteroatoms and additional substituents at phenyl rings. They demonstrated higher potency than that of the parent compound, pentamidine, apart from compound **19**, which had an aromatic substituent at central *N* atom. All other structural changes caused improvement of activity. Introducing two *S* atoms at the benzene ring and *O* atom in the middle of chain (compounds **11** and **12**) yielded very active compounds. Remarkably, closing both amidine groups of **11** into imidazoline rings resulted in the new leading structure **12**, with IC_{50} = 0.005 μg/ml. This is in consent with similar results.[35–38] Addition of two nitro or two amino groups *ortho* to the linker in benzene rings is also favourable (compounds **13–16**). Among them, compounds with oxygen atoms in the linker (**13** and **14**), showed better activity than aza analogues (**15** and **16**). Similar observations have been made previously.[17,38] Compounds **13** and **14**, with their activity at nanogram concentration, should be considered as next two leading structures. With the exception of bisamidine **19** mentioned above, introduction of four methyl substituents at *ortho* positions (never studied before) produced compounds: **17** and **18**. Methyl substituents at the aromatic rings seems to be superior to methoxy groups, because all respective bisamidines with methoxy substituents are less active than pentamidine.[17] When bromine atoms are introduced together with methoxy groups, as in compound **20**, activity increased significantly in comparison to activity of bisamidines with only methoxy[17] or only bromine[38] substituents. Perhaps, heteroatoms in amino, nitro, methoxy and bromo substituents in compounds **13, 14, 15** and **20** favourably interact with the molecular target increasing activities of these compounds, and only combination of different types of the substituents improves anti-*Pneumocystis* activity. Compound **21** with *N*-ethyl group in the central part of the linker shows better activity (IC_{50} < 0.1 μg/ml, < 0.209 μM) than the analogue with *N*-methyl group (IC_{50} = 0.82 μg/ml, 1.73 μM)[17], what means that increasing length of alkyl group in the middle of linker can be favourable. Introducing *N* instead of *O* atoms have pronounced consequences. A nitrogen atom in the centre of the pentamidine bridge introduces a positive charge (compounds **1–5** and **21**), while at the benzene ring it does not (compounds **15** and **16**). Further studies are necessary to validate the impact of additional positive charges on anti-*Pneumocystis* activity.

Toxicity

Mammalian cell toxicity testing was assessed in 2 cell lines. The A549 cell line is a human-derived adenocarcinomic alveolar basal epithelial cell line and the L2 was derived from rat lung and is an adherent epithelial cell line. For compounds 1–21 the 50% inhibitory concentration (IC_{50}) was determined and pentamidine isethionate was run as a positive control (see Table 2). Only compounds **11** (with bridge *S* atoms), **17** and **18** (with methyl groups), and **20** (with bromine and methoxy substituents) were found to have mild to moderate toxicity to both A549 and L2 cells. Compounds **12** (with bridge *S* atoms) and **13** (with nitro groups) were found to be mildly toxic to only one cell line, A549 or L2 respectively. All piperazine and alkanediamide derivatives were found to be non-toxic. Similar observations were made in previous studies.[17–21] Closing of both amidine groups into imidazoline ring decreased the toxicity of compound **11** with heteroatoms *O* and *S* in the linker, and compound **12** became only mildly toxic to A549 cells. Toxicity of **13** with two nitro groups at benzene ring disappeared without decreasing anti-*Pneumocystis* activity after reducing the nitro to amino groups. Introducing benzenesulfonyl group in the middle of linker (compound **19**) decreased toxicity of compounds **17** and **18** with four methyl groups at benzene ring, but had an adverse impact on anti-*P. carinii* activity. In general, substituents at the benzene rings make bisamidines more toxic, with exception of amino substituents. Tidwell et al. reported the same relationship.[38]

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Table 1 Anti-*Pneumocystis* activity (IC_{50}) and activity ranking of compounds **1–21**

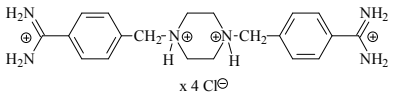
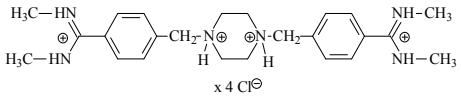
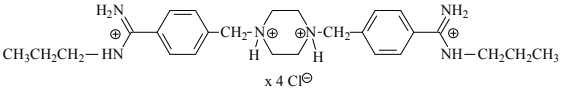
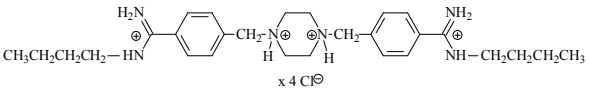
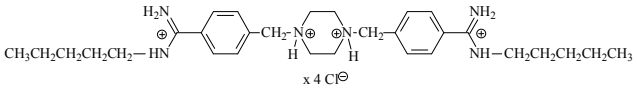
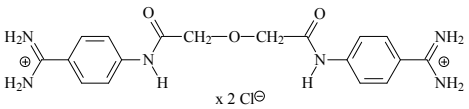
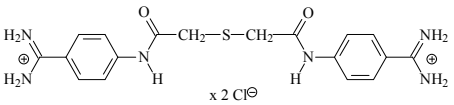
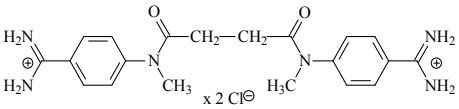
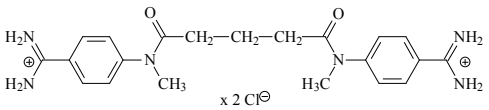
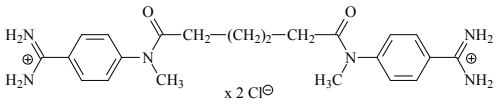
No.	Chemical formula	IC_{50} in µg/ml (µM)	Activity ranking
	Pentamidine	0.30 (0.50)	Marked
1		0.062 (0.109)	Very Marked
2		–	–
3		1.10 (1.685)	Moderate
4		0.052 (0.083)	Very Marked
5		0.027 (0.041)	Very Marked
6		0.066 (0.146)	Very Marked
7		0.27 (0.568)	Marked
8		0.035 (0.076)	Very Marked
9		0.15 (0.303)	Marked
10		0.006 (0.012)	Highly Active

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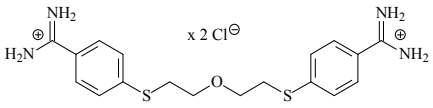
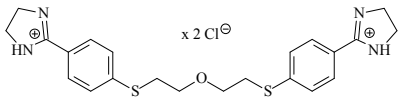
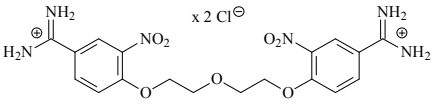
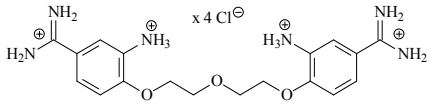
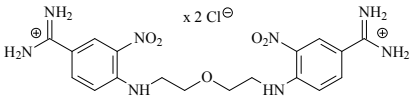
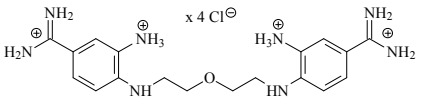
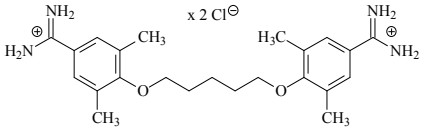
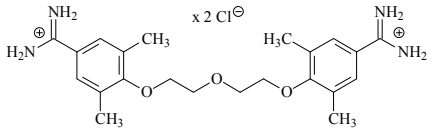
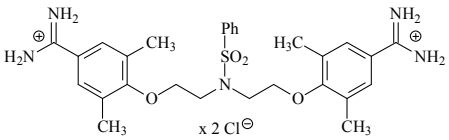
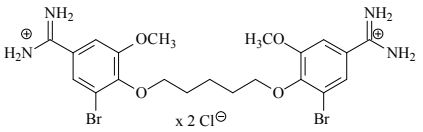
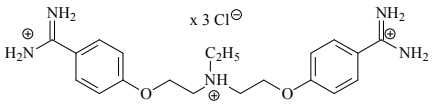
Table 1 (continued)

No.	Chemical formula	IC_{50} in µg/ml (µM)	Activity ranking
11		< 0.1 (< 0.223)	Very Marked
12		0.005 (0.010)	Highly Active
13		0.007 (0.014)	Highly Active
14		0.006 (0.011)	Highly Active
15		0.031 (0.062)	Very Marked
16[a]		< 0.1 (< 0.175)	Very Marked
17		< 0.1 (< 0.213)	Very Marked
18		0.084 (0.175)	Very Marked
19		1.2 (1.519)	Moderate
20		0.025 (0.036)	Very Marked
21		< 0.1 (< 0.209)	Very Marked

[a] Quench effect of 15% at 100 µg/ml.

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Table 2 Toxicity IC_{50} results in A549 and L2 cell lines[a]

No.	Average IC_{50} [μg/ml ± SD, (μM)]		Toxicity
	A549	L2	
Pentamidine	13.9 ± 2.4 (23.5±4.3)	20.0 ± 7.9 (33.8±13.3)	Mild
1	> 100	> 100	None
2	ND[b]	ND[b]	—
3	> 100	> 100	None
4	> 100	> 100	None
5	> 100	> 100	None
6	> 100	> 100	None
7	> 100	> 100	None
8	> 100	> 100	None
9	> 100	> 100	None
10	> 100	> 100	None
11	22.8 ± 4.6 (50.9±10.3)	11.8 ± 3.3 (26.4±7.4)	Mild
12	15.3 ± 5.1 (30.6±10.2)	> 100	Mild
13	> 100	89 ± 19 (176±38)	Mild
14	> 100	> 100	None
15	> 100	> 100	None
16	> 100	> 100	None
17	15.1 ± 2.5 (32.2±5.3)	8.9 ± 2.8 (19.0±6.0)	Mild to moderate
18	36.4 ± 2.3 (75.8±4.8)	8.0 ± 2.7 (16.7±5.6)	Mild to moderate
19	> 100	> 100	None
20	18.0 ± 3.6 (26.3±5.2)	20.2 ± 5.6 (29.5±8.2)	Mild
21	> 100	> 100	None

[a] IC_{50} – average of 3 separate assays.
[b] ND – not done due to lack of efficacy against *P. carinii*.

Experimental

Chemistry

Materials and methods. All chemicals were purchased from 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 apparatus. 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 VNMRS spectrometer 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) precoated plates Merck Kieselgel 60 F_{254} were used. For column chromatography Merck Silica gel 60, 230–400 mesh ASTM (0.040–0.063 mm) was used.

General procedure for the preparation of bisamidines 1–5. Procedure is based on the previously published synthesis of **1**.[15] 19.6 g (0.1 mol) 4-cyanobenzyl bromide, 13.8 g K_2CO_3 (0.1 mol), 4.3 g piperazine (0.05 mol) and 235 ml DMF were stirred for 1 h at room temperature (RT) and then for 5 h at 80–90°C. The reaction mixture was cooled to RT, 700 ml ice-water was added, and stirring was continued for 0.5 h at 0–5 °C. The white precipitate was filtered off, washed with cold water and dried *in vacuo*. Crystallization from acetone yielded 27.2 g (86%) of fine colourless crystals of 1,4-bis(4-cyanobenzyl)piperazine (**1a**). 1.26 g (4 mmol) of 1,4-bis(4-cyanobenzyl)piperazine **1a** was suspended in 40 ml of anhydrous ethanol. To this mixture dry hydrogen chloride was passed at 0–5 °C until saturation. The reaction vessel was stoppered tightly and stirred for 2 weeks at RT. After 1 week, additional re-saturation of HCl at 0–5 °C has been done. The solvent was evaporated near to dryness under reduced pressure below 40 °C. To the residue 100 ml of dry diethyl ether was added with stirring. A white hygroscopic precipitate of crude bisimidate was formed, quickly filtered off and stored for 24 h in a vacuum desiccator over sodium hydroxide granules. It was then added to a solution of an appropriate aliphatic amine in absolute ethanol at RT. Reagents were stirred in tightly stoppered flask at RT for 24 h. Volatiles were evaporated *in vacuo* almost to dryness. The residue was suspended in the solution of 1.0 g NaOH in 40 ml of water, and stirred for 15 min. The formed precipitate was filtered, washed thoroughly with water and dried under reduced pressure over NaOH granules. Obtained dry powder was mixed with 10 ml of anhydrous EtOH, acidified with 5 ml of saturated ethanolic HCl and refluxed for 0.5 h. After cooling, 30 ml of dry Et_2O was added slowly with stirring. The formed precipitate was filtered off, washed with 10 ml of dry Et_2O and dried *in vacuo* over NaOH granules. The crude product was purified by crystallization or column chromatography. In the case of the synthesis of compound **2**, 40 ml of 33% methylamine solution in absolute ethanol was used in the second step of Pinner reaction. In other cases (compounds **3–5**), 4 eq. of aliphatic amine dissolved in anhydrous ethanol was used. Representative data for compound **5** are given below. Characterizations of compounds **2–5** are given in ESI. Bis[4-(*N*-pentylamidino)benzyl]piperazine tetrahydrochloride (**5**). Following the general procedure, after column chromatography (CH_2Cl_2/MeOH/AcOH/H_2O – 70/20/5/5), a light beige powder was obtained with yield 23%; MP = 279.5–280.5 °C (decomp.). ^{1}H NMR (299.87 MHz, D_2O) δ in ppm: 0.91–0.96 (t; *J* = 6.9 Hz; 6H; H-15, H-15'), 1.37–1.47 (m; 8H; H-13, H-14, H-13', H-14'), 1.74–1.84 (p; *J* = 6.9 Hz; 4H; H-12, H-12'), 3.48–3.52 (m; 12H; H-9, H-10, H-11, H-9', H-10', H-11'), 4.46 (br s; 4H; H-8, H-8'), 7.71–7.73 (pd; *J* = 7.8 Hz; 4H; H-2, H-6, H-2', H-6'), 7.82–7.84 (pd; *J* = 7.8 Hz; 4H; H-3, H-5, H-3', H-5'). ^{13}C NMR (75.40 MHz, D_2O) δ in ppm: 13.1 (C-15, C-15'), 21.6 (C-14, C-14'), 26.4 (C-13, C-13'), 28.2 (C-12, C-12'), 43.0 (C-11, C-11'), 48.7 (C-9, C-10, C-9', C-10'), 59.6 (C-8, C-8'), 128.5 (C-3, C-5, C-3', C-5'), 130.7 (C-4, C-4'), 131.8 (C-2, C-6, C-2', C-6'), 133.5 (C-1, C-1'), 163.5 (C-7, C-7'). ^{1}H NMR (299.87 MHz, DMSO-d_6) δ in ppm: 0.87–0.92 (t; *J* = 6.9 Hz; 6H; H-15, H-15'), 1.28–1.39 (m; 8H; H-13, H-14, H-13', H-14'), 1.60–1.69 (p; *J* = 6.9 Hz; 4H; H-12, H-12'), 3.29 (br s; 8H; H-9,H-10,

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H-9', H-10'), 3.38–3.44 (q; *J* = 6.6 Hz; 4H; H-11, H-11'), 4.29 (br s; 4H; H-8, H-8'), 7.83 (br s; 8H; H-2, H-3, H-5, H-6, H-2', H-3', H-5', H-6'), 9.16 (br s; 2H; 2 × NH), 9.54 (br s; 2H; 2 × NH–C_5H_{11}), 9.89 (br s; 2H; 2 × NH). Element. Anal. $C_{30}H_{46}N_6$ × 4HCl × 1.5H_2O (663.59 g/mol) calcd. (%): C = 54.30, H = 7.99, N = 12.67, Cl = 21.42; found (%): C = 54.24, H = 7.79, N = 12.73, Cl = 21.15.

General procedure for the preparation of bisamidines 6–10. Bis(5-methyl-1,2,4-oxadiazoles) **6a–10a** as intermediates were obtained as follows (see Scheme 2). 5 mmol of an appropriate aliphatic dicarboxylic acid chloride in 20 ml of dichloromethane was cooled to 0–5 °C. Then it was added dropwise to a stirred, ice-cooled solution of 4-(5-methyl-1,2,4-oxadiazol-3-yl)aniline or *N*-methyl derivative (10 mmol) and of *N*-methylmorpholine (10 mmol) in 20 ml of CH_2Cl_2. After addition, the reaction mixture was stirred at RT for 12 h. The solvent was evaporated under reduced pressure to dryness and the residue was washed consecutively with 1 M $NaHCO_3$, water, 1 M HCl and again with water. The rinsed precipitate was dried under reduced pressure over anhydrous $CaCl_2$ yielding pure bis(5-methyl-1,2,4-oxadiazoles). *N*-Methyl-4-(5-methyl-1,2,4-oxadiazol-3-yl)aniline was synthesized by reductive amination of paraformaldehyde with 4-(5-methyl-1,2,4-oxadiazol-3-yl)aniline[24] before preparation of intermediates **8a–10a**. Representative data for compound **10a** are given below. Characterizations of compounds **6a–10a** are given in ESI. N^1,N^6-Dimethyl-N^1,N^6-bis[4-(5-methyl-1,2,4-oxadiazol-3-yl)phenyl]-hexanediamide (**10a**). Following the general procedure, a white powder of pure **10a** was obtained. Yield 99%; MP = 143.5–145.5 °C. ^{1}H NMR (299.87 MHz, DMSO-d_6) δ in ppm: 1.39 (br s; 4H; H-9, H-9'), 2.04 (br s; 4H; H-8, H-8'), 2.68 (s; 6H; 2 × H_3C–C), 3.18 (s; 6H; 2 × H_3C–N), 7.44–7.46 (pd; *J* = 8.4 Hz; 4H; H-2, H-6, H-2', H-6'), 7.99–8.02 (pd; *J* = 8.4 Hz; 4H; H-3, H-5, H-3', H-5'). ^{13}C NMR (75.40 MHz, DMSO-d_6) δ in ppm: 12.0 (2 × H_3C–C), 24.2 (C-9, C-9'), 33.0 (C-8, C-8'), 36.7 (2 × H_3C–N), 124.8 (C-4, C-4'), 127.8 (C-2, C-6, C-2', C-6'), 128.0 (C-3, C-5, C-3', C-5'), 146.5 (C-1, C-1'), 167.0 (C-7, C-7'), 171.3 (2 × C=O), 177.5 (2 × C–CH_3). 2.5 mmol of the appropriate bis(5-methyl-1,2,4-oxadiazole) was dissolved in 25 ml of glacial acetic acid. In some cases quick heating was needed for complete dissolution. The resultant clear solution was diluted with 100 ml of methanol and cooled. Pd/C (10% Pd, 25% w/w) was then added. The suspension was vigorously stirred at RT under hydrogen atmosphere (4 bar) for 12 h. After completion of the reaction the catalyst was filtered through thick filter paper. Filtrate was evaporated nearly to dryness and dissolved in 10 ml of methanol. 10 ml of saturated methanolic HCl was added to this solution. After 1 h of stirring at RT, the solvent was evaporated. The residue was triturated with dry acetone. The formed suspension was quickly filtered, washed with dry diethyl ether and dried immediately under reduced pressure over NaOH granules yielding pure (TLC) bisamidines as dihydrochlorides. Representative data for compound **10** are given below. Characterizations of compounds **6–10** are given in ESI. N^1,N^6-Dimethyl-N^1,N^6-bis(4-amidinophenyl)hexanediamide dihydrochloride (**10**). After the general procedure, a white powder was obtained with yield 88%; MP = 233.5–235.5 °C (decomp.). ^{1}H NMR (299.87 MHz, DMSO-d_6) δ in ppm: 1.40 (br s; 4H; H-9, H-9'), 2.15 (br s; 4H; H-8, H-8'), 3.21 (s; 6H; 2 × H_3C–N), 7.55–7.58 (pd; *J* = 8.6 Hz; 4H; H-2, H-6, H-2', H-6'), 7.87–7.90 (pd; *J* = 8.6 Hz; 4H; H-3, H-5, H-3', H-5'), 9.18 (br s; 4H; 4 × NH), 9.41 (br s; 4H; 4 × NH). ^{13}C NMR (75.40 MHz, DMSO-d_6) δ in ppm: 24.3 (C-9, C-9'), 33.3 (C-8, C-8'), 36.7 (2 × H_3C–N), 125.8 (C-4, C-4'), 127.1 (C-2, C-6, C-2', C-6'), 128.6 (C-3, C-5, C-3', C-5'), 147.5 (C-1, C-1'), 164.7 (C-7, C-7'), 171.3 (2 × C=O). Element. Anal. $C_{22}H_{28}N_6O_2$ × 2HCl (481.42 g/mol) calcd. (%): C = 54.89, H = 6.24, N = 17.46, Cl = 14.76; found (%): C = 54.74, H = 5.96, N = 17.40, Cl = 14.67.

General procedure for the preparation of bisamidines 11–21. Bisbenzonitriles **11a–21a** were obtained with two types of reactions: *O*-alkylation of appropriate 4-hydroxybenzonitriles with bis(2-chloroethyl) derivates or by nucleophilic aromatic substitution of appropriate 4-chlorobenzonitriles and bis(2-mercaptoethyl)ether or bis(2-aminoethyl)ether. Synthetic procedures were based on previously published papers[17,28–33], but several modifications were implemented. Characterizations of compounds **11a–21a** are given in ESI. The appropriate bisbenzonitrile **11a–21a** (1–5 mmol) was stirred in a sealed flask with a saturated solution of HCl in anhydrous ethanol (15–25 ml) for 24 h at RT. Dry diethyl ether was added until a solid was precipitated or solvent was evaporated *in vacuo*. The solid was filtered, washed with diethyl ether and dried under vacuum over anhydrous $CaCl_2$ for 2–6 h. The crude imidate obtained in high yield was added to a saturated solution of NH_3 in anhydrous ethanol (15–30 ml). The mixture was stirred at RT for 24–48 h in a sealed vessel. The solvent was evaporated *in vacuo*, and the solid residue was mixed with 2–4% aq NaOH (10–20 ml). The precipitate of free bisamidines was filtered, washed with water, and dried under reduced pressure over anhydrous $CaCl_2$. The solid of free bisamidine was suspended in anhydrous ethanol (5–10 ml) and saturated solution of HCl in anhydrous ethanol (0.5–1 ml) was added and boiled for a few min to obtain the appropriate hydrochloride. Representative data for compound **13** are given below. Characterizations of compounds **11–21** are given in ESI. 1,5-Bis(4-amidino-3-nitrophenoxy)-3-oxapentane dihydrochloride (**13**). After the general procedure, the solid residue of was filtered off, washed with ethanol and dried. The crude product was suspended in acetone (25 ml) and intensively stirred under reflux for 10 minutes, then cooled to RT, filtered off and dried to give a pale yellow solid of **13**; yield 41%; MP. 237.0–238.5 °C (decomp.). ^{1}H NMR (299.87 MHz, DMSO-d_6) δ in ppm: 3.88 (t; *J* = 4.2 Hz; 4H; H-9, H-9'), 4.44 (t; *J* = 4.2 Hz; 4H; H-8, H-8'), 7.62 (d; *J* = 4.5 Hz; 2H; H-6, H-6'), 8.16 (dd; J_1 = 2.1 Hz, J_2 = 8.4 Hz; 2H; H-5, H-5'), 8.45 (d; *J* = 2.1 Hz; 2H; H-3, H-3'), 9.31 (br s; 4H; 4 × NH), 9.54 (br s; 4H; 4 × NH). ^{13}C NMR (75.40 MHz, DMSO-d_6) δ in ppm: 68.6 (C-9,C-9'), 69.9 (C-8, C-8'), 115.6 (C-6,C-6'), 119.6 (C-4, C-4'), 125.5 (C-3, C-3'), 134.2 (C-5, C-5'), 138.9 (C-2, C-2'), 154.9 (C-1, C-1'), 163.3 (C-7, C-7'). Element. Anal. $C_{18}H_{20}N_6O_7$ × 2HCl (505.31 g/mol) calcd. (%): C = 42.78, H = 4.39, N = 16.63; found (%): C = 42.63, H = 4.51, N = 16.72.

Anti-*Pneumocystis* activity

Organism sources. *Pneumocystis carinii* organisms were obtained from male CD rats (125–145 g) (Charles River, Portage, MI). Animals were housed under barrier conditions at the Cincinnati Veteran's Affairs Veterinary Medical Unit. After a week of acclimation, the immune systems of the rats were suppressed by weekly subcutaneous injections of methylprednisolone (Pharmacia & Upjohn, Kalamazoo, MI)

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20 mg/kg body weight. After the second injection, rats were inoculated with 2 × 107 cryopreserved *P. carinii* by intranasal inoculation. Seven to nine weeks after inoculation, moribund animals were sacrificed by CO_2 inhalation. Lungs were aseptically removed, homogenized, strained, and centrifuged at 2,400 × g, 10 min., 4 °C. Red blood cells were lysed using 0.85% NH_4Cl pH 6.8, 37 °C, 10 min. Cells were washed with PBS pH 7.0 and prepared for cryopreservation in RPMI-1640 (Invitrogen-Gibco, Grand Island, NY) with 10% Fetal Bovine Serum (Atlanta Biologicals, Lawrenceville, GA) and 7.5% dimethylsulfoxide (Sigma-Aldrich, St. Louis, MO) and stored in liquid nitrogen. Nuclei were enumerated by microscopic analysis of HEMA-3 (Fisher Scientific, Kalamazoo, MI) stained slides. Isolates were screened for bacterial contamination using Luria-Bertani agar, Columbia Blood Agar (Fisher Scientific, Cincinnati, OH), and MacConkey II plates (Becton-Dickinson, Sparks, MD).

ATP assay. Cryopreserved *P. carinii* was thawed and suspended in RPMI-1640 media containing 20% donor horse serum (Atlanta Biologicals, Lawrenceville, GA), 1% Non-essential amino acids solution (NEAA), 1% MEM vitamin solution, 2% penicillin/streptomycin (Invitrogen-Gibco, Grand Island, NY) to 1 × 10^8 nuclei/ml. 0.25 ml of the cell suspension was distributed into 48-well tissue culture plates (Corning, Inc. Corning, NY). Experimental and control compounds ampicillin and pentamidine isethionate (Sigma-Aldrich, St. Louis, MO) were suspended in the RPMI 1640 media at 2 × concentration and added to the *P. carinii* at 0.25 ml/well. Plates were incubated at 36 °C, 100% humidity, 5% CO_2 for 24, 48, and 72 h. At each time point, plates were agitated and 10% volume transferred to 96-well opaque white plates (USA Scientific, Ocala, FL). ATP content of samples was determined using ATP-lite-M (Perkin-Elmer Inc., Waltham, MA) and evolved light was measured with a PolarStar Optima luminometer (BMG Labtechnologies, Durham, NC). For each group, background luminescence was subtracted and triplicate well readings of duplicate assays were averaged. For each day's readings, % reduction in ATP for all groups was calculated: media control – experimental / media control × 100. 50% inhibitory concentration (IC_{50}) was calculated using INSTAT linear regression program. Data are shown after 3 days of exposure to the test compounds. Prior to testing, all compounds, except compounds **12**, **15**, and **16**, were solubilized in sterile deionized water for a 20 mg/ml stock solution. Compounds **12**, **15**, and **16** were solubilized in sterile DMSO. Dilutions of 100 µg/ml were made for each compound in RPMI-1640 containing 20% calf serum, 1% MEM vitamin solution, 1% MEM non-essential amino acids, 2,000 units/ml penicillin-streptomycin, and 50 µg/ml vancomycin. Negative controls were media alone, DMSO vehicle control, and 10 µg/ml ampicillin. Positive control was 1 µg/ml pentamidine isethionate. Compounds **1–21** were tested for luciferin/luciferase reaction interference at 100 µg/ml concentration. Compound **16** resulted in a 15% quench effect, which may have accounted for a slight decrease in the overall lost in ATP. All other compounds did not exhibit a quenching effect.

Data analysis. 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} values of 0.011 to 0.099 µg/ml), "Marked" (IC_{50} values from 0.10 to 0.99 µg/ml), "Moderate" (IC_{50} values from 1.0 to 9.99 µg/ml), "Slight" (IC_{50} values from 10.0 to 49.9 µg/ml), and "None" (i.e., inactive; IC_{50} values of ≥ 50 µg/ml).[8]

A549 and L2 toxicity assay. A549 and L2 cell lines were purchased from American Type Culture Collection, Manassas, Virginia, USA. The A549 cells were provided with DMEM media and L2 cells with F12 media for mammalian cell toxicity testing. Both were supplemented with 10% fetal calf sera, 1% MEM vitamins, and 1% NEAA. Cultured cells were plated at 2 × 10^5/ml and grown to confluent monolayers. Media was removed and replaced with fresh media containing controls and test compound dilutions. First, all compounds were tested at a single high dose, 100 µg/ml. Assays of 3 time points (24, 48, 72 hours) with duplicate wells were tested for viability. Media was aspirated from the wells, adherent cells were lysed with 0.1 M NaOH, and a portion of the lysate was assayed for ATP using the Perkin Elmer ATP-liteM luciferin-luciferase assay as described above. Background luminescence was subtracted and replicate well readings of were averaged. For each day's readings, % reduction in ATP for all groups was calculated: media control – experimental / media control × 100. The compounds that were found to be toxic in the initial screen (that reduced the ATP content by > 50% after 72 h) were tested at 100, 10, 1, and 0.1 µg/ml and the 50% inhibitory concentration (IC_{50}) was calculated by linear regression (InSTAT and GraphPad Prism v.6 software). Based on the scale using 72-hour IC_{50} values, mammalian cell toxicity of tested compounds was classified as non-toxic (compounds with IC_{50}s > 100µg/ml), as mildly toxic (compounds with IC_{50} values of 10.11 to 99.9 µg/ml), moderately toxic (compounds with IC_{50} values from 1.1–10.0 µg/ml), highly toxic (compounds with IC_{50} values < 1µg/ml).

Conclusions

A detailed SAR study with 21 pentamidine analogues was successfully undertaken. High potency against *Pneumocystis carinii* was identified for most of the analogues using an assay to measure the effects on viability (ATP levels) after exposure to the compounds. The highest activities were found for each group of compounds as follows: among *N*,*N*'-dimethylenepiperazine analogues of pentamidine, compound **5**, with pentyl substituents at amidine groups (IC_{50}=0.027 µg/ml, 0.041 µM); from the series with alkanediamide bridges, compound **10** with a butyl chain (IC_{50}= 0.006 µg/ml, 0.012 µM); and from the series with variation of heteroatoms, compound **12** with *S* atoms (IC_{50}= 0.005 µg/ml, 0.010 µM). The majority of the compounds can be classified as non-toxic or much less toxic than the parent compound

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pentamidine, apart from compounds **17** and **18** with four methyl substituents at the *ortho* positions in benzene rings, which had mild to moderate toxicity as evidenced by the IC_{50} values between 8.0 and 36.4 μg/ml (16.7 – 75.8 μM).

Presence of amide groups or sulfur atoms in the linker, imidazoline rings instead the amidine groups, and amino groups at the benzene rings are factors responsible for high activity of tested bisamidines. Four new leading structures (**10** and **12–14**) were identified. Their structural properties should be taken into account in further lead optimization.

Conflicts of interest

The authors declare no competing interests.

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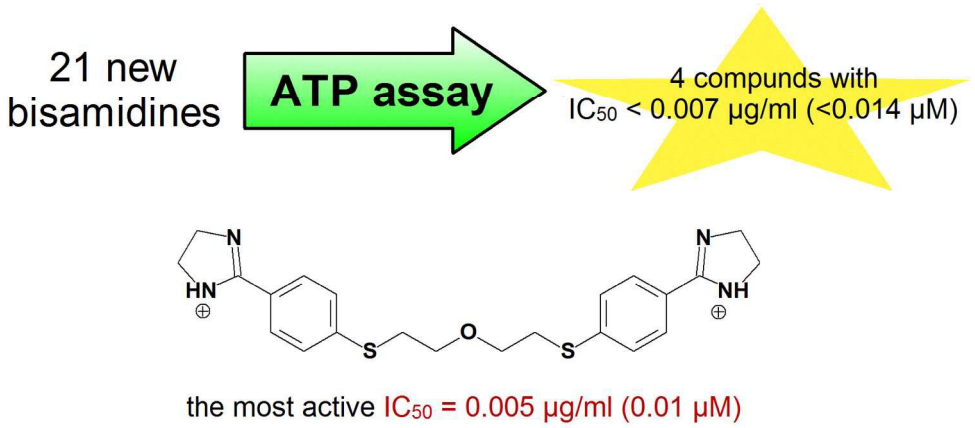

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