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# Novel bisbenzamidines as potential drug candidates for the treatment of *Pneumocystis carinii* pneumonia

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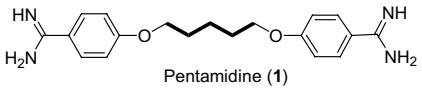
**Abstract**—A series of pentamidine congeners has been synthesized and screened for their in vitro activity against *Pneumocystis carinii*. Among the tested compounds, bisbenzamidines linked by a flexible pentanediamide or hexanediamide chain (**7** and **9**) emerged as exceptionally potent agents that were more effective and less toxic than pentamidine in the assays described in this study.


*Pneumocystis carinii* pneumonia (PCP) is an opportunistic infection that occurs in individuals with weakened immune systems due to cancer, HIV/AIDS, inherited immune deficiencies, or organ transplants as well as in patients receiving corticosteroids.[1] In humans, the infection is caused by the species, *Pneumocystis jirovecii*. PCP does not respond to standard antifungal therapy, but shows pliancy to some antiprotozoal therapies, for example, pentamidine. The most effective treatment for PCP is the well-known trimethoprim–sulfamethoxazole (TMP–SMX; Bactrim™, Septra®) drugs combination. Unfortunately almost half of the HIV-positive patients who take it develop a sulfa allergic reaction necessitating a switch to other less effective medications[2,3] including pentamidine (NebuPent®, Pentacarinat®, **1**, Fig. 1),



Pentamidine (**1**)

**Figure 1.** Structure of pentamidine.

dapsone (Avlosulfon®), and atovaquone (Mepron®). However, deleterious side effects, limited efficacies, and emerging resistance are associated with these drugs, thus justifying the search for more effective and less toxic agents.

Our groups are interested in the design[4–6] and evaluation[7–11] of novel bisbenzamidines and other analogs that are more efficient and less toxic than the parent compound, pentamidine (**1**). With that goal in mind and based on previous works,[12–15] we considered **1** as a bisbenzamidine in which both benzamidine moieties are linked by a flexible pentamethylene chain and activated by electron-donating ether functions. Starting from that simple representation, we first decided to study the influence of the linking chain by reducing its flexibility as in compounds **2**–**4** (Fig. 2). Among these compounds, **2** can be considered to be an analog of pentamidine in which the degrees of freedom of the linker are reduced by incorporating three (of five) carbon atoms into a 1,3-phenylene ring. Derivative **3** is the *para* analog of **2** and is characterized by a sequence of six carbon atoms between the activating ether functions. On the other hand, **4** is the *ortho* analog of **2** and in that molecule, there are only four carbon atoms between the ether functions. Interestingly, such simple structural changes have not been reported as factors that could modulate the anti-*Pneumocystis carinii* effects of bisbenzamidines.

*Keywords*: Bisbenzamidine; Pentamidine; *Pneumocystis carinii* pneumonia; Linker; Conformation; Electronic effect.
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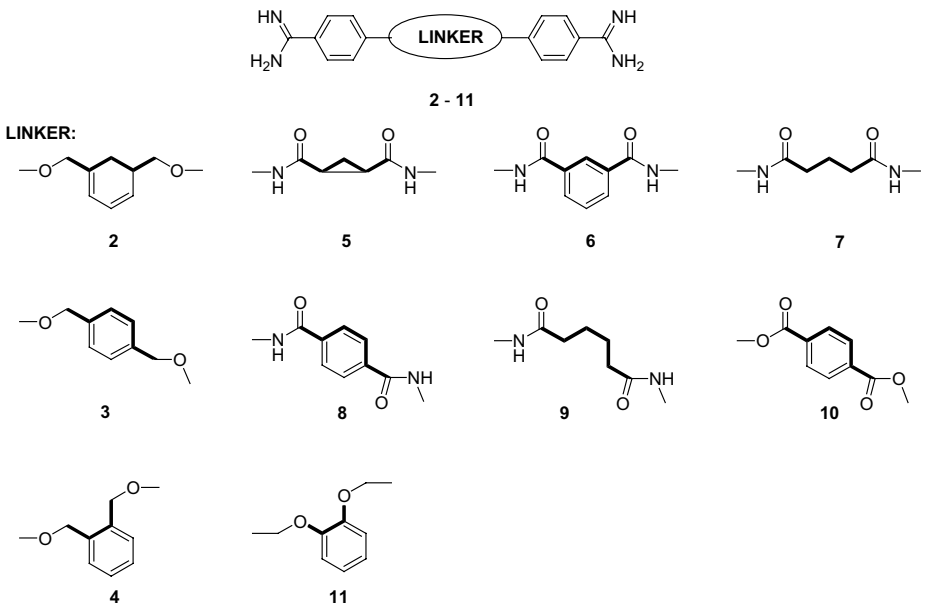
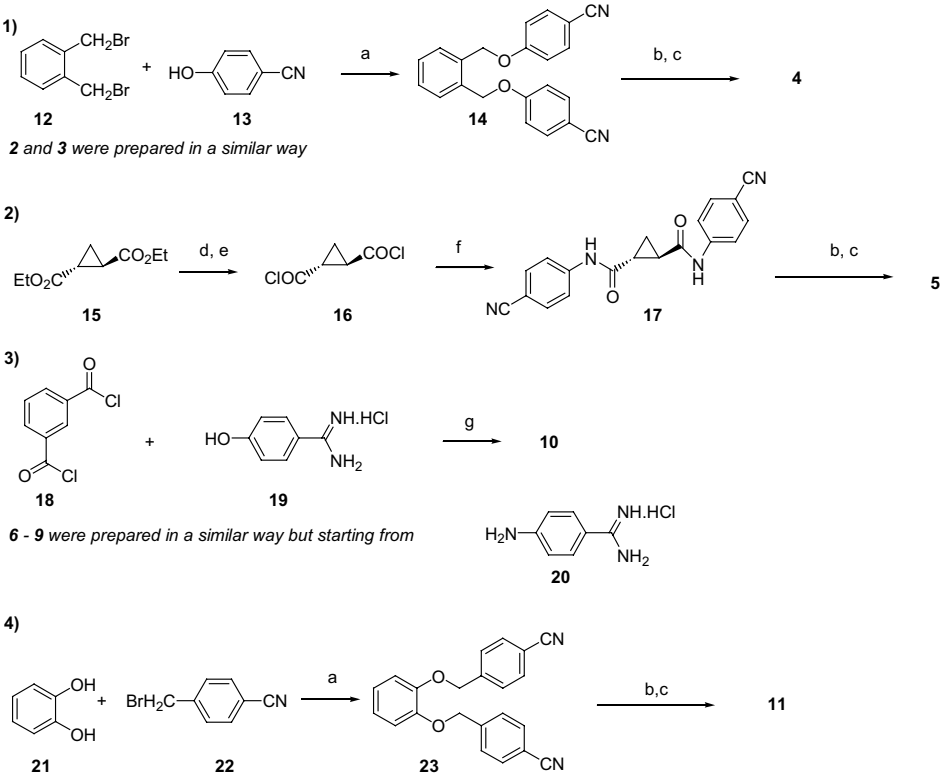



**Figure 2.** Compounds used in this study.

Pentamidine **1** is thought to exert its anti-*P. carinii* effects through binding interactions between the amidine moieties and macromolecular targets such as DNA.[16] The strength of the binding interactions may depend on the chemical nature (e.g., basicity) of the amidine moieties. The nature of the heteroatoms present in the linker may influence the chemical properties of the amidine moieties in the *para* position by conjugation. To assess the electronic effects of the heteroatoms in the linker on the biological activity, we replaced the strong electron-donating ether functions present in **1** with poor electron-donating groups, namely amides as in **5**–**9**, ester as in **10**, or an oxymethylene as in **11**.

Typical procedures[5,11,12,17–19] for the synthesis of **2**–**11** are represented in the Scheme 1. Preparation of **2**–**4**, and **11**




**Scheme 1.** Typical procedures for the preparation of the compounds used in this study. Reagents, solvent, temperature, time: (a) tetrabutylammonium hydrogen sulfate, NaOH 5 M, dioxane, reflux, 1 h; (b) HCl(g), EtOH, $CH_2Cl_2$, rt, 4 d; (c) $NH_3$ (g), EtOH, reflux, 2 h; (d) NaOH 2 M, reflux, 1 h; (e) $SOCl_2$, 60 °C, 30 min; (f) 4-aminobenzonitrile, *N*-diisopropylethylamine, THF, 0 °C, 16 h; (g) pyridine, dioxane, reflux, 8 h.

**Table 1.** Anti-*P. carinii* and cytotoxicity of compounds **1**–**11**[a]

| Compound | Anti-*P. carinii* activity[10,22] | *P. carinii* $IC_{50}$ in μM (in μg/mL) | A549 $IC_{50}$ (μM) |
|---|---|---|---|
| **1** | Marked[b] | 0.5 (0.3) | 24.0 |
| **2** | Moderate | 2.6 (1.2) | 12.1 |
| **3** | Marked | 1.3 (0.6) | 30.2 |
| **4** | Marked | 1.4 (0.8) | 33.1 |
| **5** | Moderate | 5.3 (2.3) | >526 |
| **6** | Marked | 1.2 (0.6) | >120 |
| **7** | Highly active | 0.003 (0.0013) | >0.3 |
| **8** | Slight | 22.8 (10.8) | >2280 |
| **9** | Highly active | 0.002 (0.0009) | >0.2 |
| **10** | Moderate | 13.4 (6.4) | 121 |
| **11** | Marked | 1.3 (0.6) | 44.1 |

[a] All biological activities resulted from the average of at least two determinations.

[b] Drug activity scale: highly active, <0.01 μg/mL; very marked, <0.1 μg/mL; marked, 0.1–0.9 μg/mL; moderate, 1.0–9.9 μg/mL; slight, 10.0–49.9 μg/mL.

was straightforward and involved a nucleophilic substitution of bromine atoms by phenolate anions followed by a Pinner reaction.[20] The key step to obtain the other derivatives is the formation of the diamides **5**–**9**, or diester **10** from the appropriate diacid chlorides and substituted anilines or phenol.

Compounds **1**–**11** were evaluated[21] against *P. carinii* in an ATP detection assay based on the release of bioluminescence driven by ATP in a luciferin–luciferase mediated reaction.[10] The results shown in the Table 1 indicated that the compounds exhibited variable anti-*P. carinii* activity. However partial immobilization of the flexible pentyldioxy linker between the benzamidine functions in **1** was accompanied by a decrease in the activity, irrespective of whether the substituents on the phenylene ring are in the *ortho*, *meta*, or *para* positions (**4**, **2**, or **3**, respectively). Interestingly, the *meta* derivative (**2**), which is the closest conformationally restricted congener of pentamidine, was less active than the *para* isomer (**3**). The situation was reversed when the ether groups are replaced with amides (**2** and **3** vs **6** and **8**) and we noticed that derivatives of terephthalic acid (**8** and **10**) were poor inhibitors of *P. carinii*. Remarkably, relief of any conformational constraint in the diamides series (**5**–**9**) yielded the exceptionally effective lead compounds **7** and **9**. These compounds were characterized by $IC_{50}$ values of 3 and 2 nM, respectively, whereas the value measured for pentamidine reached 500 nM. It is to be noted that replacement of the rigid 1,4-phenylene ring linker in **8** with a flexible butyl chain in **9** led to an increase in potency by 4 orders of magnitude.

All compounds were subsequently tested for toxicity to mammalian cells before consideration for in vivo testing in the mouse model. The ATP assay was used to evaluate the effects of these compounds on the viability of A549 epithelial lung cell monolayers derived from a human carcinoma.[15] It is noteworthy, from the values gathered in the Table 1, that all diamides derivatives (**5**–**9**), including the most potent agents **7** and **9**, showed no cytotoxicity at 100 times the $IC_{50}$ concentration against *P. carinii*. This large selectivity index was not evident for the other compounds in the study including pentamidine.

In conclusion, the compounds evaluated here suggest that both conformational and electronic effects of the linker play an important role in influencing the biological properties of the bisbenzamidines. This study allowed us to identify the simple small molecules **7** and **9** as two very promising therapeutic leads, which deserve further pre-clinical and clinical testing. We are also actively pursuing the synthesis and testing of additional analogs of **7** and **9** for a comprehensive study of their structure–activity relationships.

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18. *N*,*N*′-Bis[4-(aminoiminomethyl)phenyl] hexane-1,6-dicarboxamide, dihydrochloride salt, (**9**) was obtained by heating under reflux for 1 h a mixture of 4-aminobenzamidine monohydrochloride (**20**, 0.86 g, 5 mmol), adipoyl dichloride (0.37 mL, 2.5 mmol), and pyridine (4 mL, 50 mmol) in *N*,*N*-dimethyl formamide (20 mL). The precipitate was filtered and thoroughly washed successively with water and acetone. Yield: 40%. Mp: 294–295 °C (decomp.). $^1$H NMR (DMSO-$d_6$): 10.6 (s, 2H); 9.2 (s, 4H); 9.0 (s, 4H); 7.8 (dd, 8H); 2.4 (br m, 4H); 1.7 (br m, 4H) ppm. IR: 3096, 1671, 1611, 1523, 1483, 1256 cm$^{-1}$. Anal. Calcd for $C_{20}$ $H_{24}N_6O_2$·2HCl (452.15): C, 52.98; H, 5.78; N, 18.54. Found: C, 52.91; H, 5.65; N, 18.31. (M-H-W Laboratories, Phoenix, AZ).
19. Bis(4-aminoiminomethylphenyl) benzene-1,4-dicarboxylate, dihydrochloride salt, (**10**) was obtained by heating under reflux for 8 h a mixture of 4-hydroxybenzamidine hydrochloride (**19**, 1.72 g, 10 mmol), terephthaloyl chloride (**18**, 1.01 g, 5 mmol), and pyridine (1.6 mL, 20 mmol) in dioxane (50 mL). The precipitate was filtered and washed successively with water and ethanol. Yield: 45%. Mp: 273–277 °C. $^1$H NMR (DMSO-$d_6$): 9.5 (s, 4 H); 9.3 (s, 4 H); 8.4 (s, 4 H); 8.0 (d, 4H, $J = 8$ Hz); 7.6 (d, 4H, $J = 8$ Hz) ppm. IR: 3600–2400 (br), 3030, 1743, 1721, 1668, 1607, 1487, 1176, 1071 cm$^{-1}$. Anal. Calcd for $C_{22}H_{18}N_4O_4$·2HCl (475.32): C, 55.59; H, 4.24; N, 11.79. Found: C, 55.34; H, 4.42; N, 11.53. (M-H-W Laboratories, Phoenix, AZ).
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21. *Pneumocystis carinii* were obtained from chronically immunosuppressed Long Evans and Brown Norway rats housed under conventional conditions at the Cincinnati VA medical Center (VAMC) or from CD rats (Charles River Laboratories, Hollister, CA) inoculated intratracheally with *P. carinii* and maintained under barrier conditions at the University of Cincinnati Laboratory Animal Medicine Unit (Cincinnati, OH). *P. carinii* were extracted and purified from the lungs of rats after 8–12 weeks of immunosuppression, enumerated, cryopreserved and stored in liquid nitrogen. Typically, infected rat lungs yield up to $2 \times 10^{10}$ organism nuclei with the vast majority (about 95%) of the life cycle forms present as trophic forms with the remainder (about 5%) being composed of cysts. *P. carinii* preparations were evaluated for microbial contamination, ATP content, karyotype, and host cell content prior to use in the ATP assay. Each concentration of every compound is assayed in triplicate wells and the results expressed as the average relative light units. Triplicate runs for each compound concentration are run using different organism isolation batches.
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