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ANTITUMOR AND ANTI-*PNEUMOCYSTIS CARINII* ACTIVITIES OF NOVEL BISBENZAMIDINES

Jean Jacques Vanden Eynde[†], Annie Mayence[†], Melissa T. Johnson[†], Tien L. Huang[†]*, Margaret S. Collins[‡], Sandra Rebholz[‡], Peter D. Walzer[‡], Melanie T. Cushion[‡], Isaac O. Donkor[±]

[†] *Xavier University of Louisiana, College of Pharmacy, Division of Basic Pharmaceutical Sciences, 1 Drexel drive, New Orleans, LA 70125, USA*

[‡] *Division of Infectious Diseases, Department of Internal Medicine, University of Cincinnati, Cincinnati, OH 45267-0560, USA*

[‡] *Research Service, Veterans Affairs Medical Center, Cincinnati, OH 45220, USA*

[±] *The University of Tennessee, College of Pharmacy, Memphis, TN 38163, USA*

Abstract: Among a library of 17 bisbenzamidines connected with various linkers, compounds with a flexible pentanediamide (**10**) or hexanediamide (**12**) linker were the most potent derivatives against rat *Pneumocystis carinii* (IC_{50} values of 3 and 2 nM, respectively) and had the highest selectivity index ratios (GI_{50} of human tumor cells/IC_{50} of rat *P. carinii* cells) of $>10^4$. Seven compounds caused 50% growth inhibition (GI_{50}) of tumor cells at concentrations of <100 μM while the remaining ten were not cytotoxic. DNA binding affinity (ΔT_m) of the tested compounds did not correlate with either their anti-*P. carinii* activity or cytotoxicity.

*Corresponding author e-mail: thuang@xula.edu

Aromatic dicationic molecules, and more specifically bisbenzamidines, remain exciting potential drug candidates since the discovery of their antimicrobial properties in the late 1930s.[1,2] They exert their activities against numerous microbial pathogens including *Cryptococcus neoformans*,[3] *Candida albicans*,[4] *Cryptosporidium parvum*,[5] *Aspergillus* sp.,[4] *Giardia lamblia*,[6] *Plasmodium* sp.,[2,7-9] *Leishmania* sp.,[10,12] *Trypanosoma* sp.,[13,14] *Pneumocystis carinii*,[15-21] *Toxoplasma* gondii, [22] and *Mycobacterium tuberculosis*.[23] These compounds have also been reported to display antiviral [24] and antitumor properties.[25-28]

Despite the broad range of activities displayed by the bisbenzamidines, pentamidine (**6**) is the only drug in this class that is clinically used for the treatment of *Pneumocystis jirovecii* pneumonia, leishmaniasis and trypanosomiasis. Other representatives of this group of compounds include terephthalamidine (**1**), diminazene (berenil, **2**), stilbamidine (**3**), hydroxystilbamidine (**4**), and imidocarb (**5**). Although the detailed mechanism of action of this class of drugs is not well understood, it has long been hypothesized that their biological activity is related to their ability to bind to the minor groove of DNA at AT-rich sites. Such interactions could lead to the inhibition of one or more of several DNA dependent enzymes

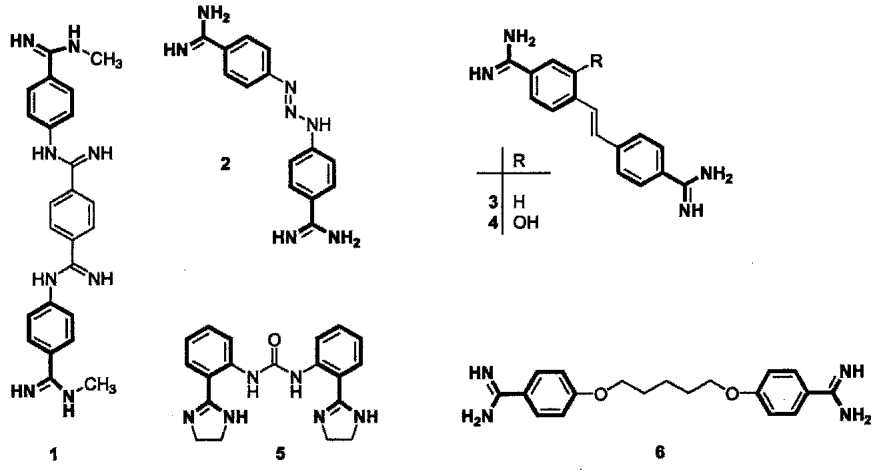


Figure 1. Structure of selected bisbenzamidines.

(e.g. topoisomerases [29] and nucleases [30]) or possibly by direct inhibition of the transcription process.[31] Another plausible mode of action is the ability of bisbenzamidines to form complexes with ferriprotoporphrin IX as reported recently. [7-9] Although the structures of the complexes have not been

determined, a sandwich type structure for the complex was suggested.[9] The results indicated that heme-containing macromolecules such as cytochrome bc_1 complex in the mitochondria could be another potential target site for this class of compounds.[19]

In previous works, [17-21] we have described the synthesis and the anti-*Pneumocystis carinii* activity of various congeners of pentamidine. In order to further investigate the potential clinical application of these compounds, we decided to study the effects of some of those compounds on the growth of human tumor cells. Results from our earlier studies suggested that the nature of the linker (i.e. electronic and conformational effects) between the bisbenzamidine moieties plays an important role in influencing their biological properties. Thus, the first set (Figure 2) of pentamidine congeners we considered contains flexible or rigid linkers bearing either strong or weak electron-donating heteroatoms. The rigid linker in **7** is comprised of a sequence of seven atoms and linked to the benzamidine groups by ether functions, just like in pentamidine **6**. Reduced flexibility in **7**, when compared to **6**, is due to the incorporation of the 3 central carbon atoms of the linker in a phenylene ring. In the isomer **8**, the benzamidine groups are no longer anchored to the linker by the strong electron-donating ether functions; the length of the linker is reduced by one carbon atom and the *ortho* substitution constrains the arylamidines in closer proximity. Derivative **9** is structurally related to the diether **7** but weak electron-donating amide groups have now replaced the methyleneoxy groups. Substances **10** and **12** are the flexible analogs of **9** and **11** respectively. Compound **13** is structurally related to **11** but in **13** the *para*-substituted phenylene core is linked to the benzamidine rings by ester functions.

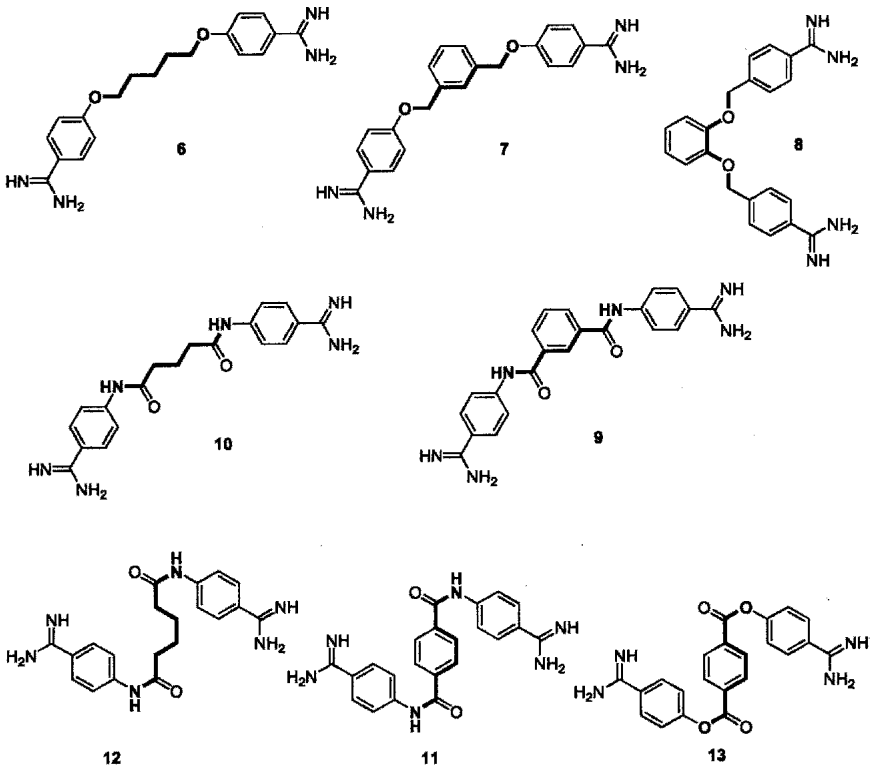


Figure 2. First set of analogs of pentamidine **6**

The second set (Figure 3) of compounds evaluated was constituted by a few members of the libraries of homopiperazine- and piperazine-linked bisbenzamidines we recently prepared.[8,12,14,17,19,21,23] It includes the parent derivatives **14** and **15**, compounds substituted by one alkyl group on each amidine function (**16** – **19**), and products in which the amidine moieties are included in a cyclic structure (**20** and **21**). To determine the influence of the end groups on the eventual biological activities of those constrained substances, the dinitrile analogs **22** and **23** were also included.

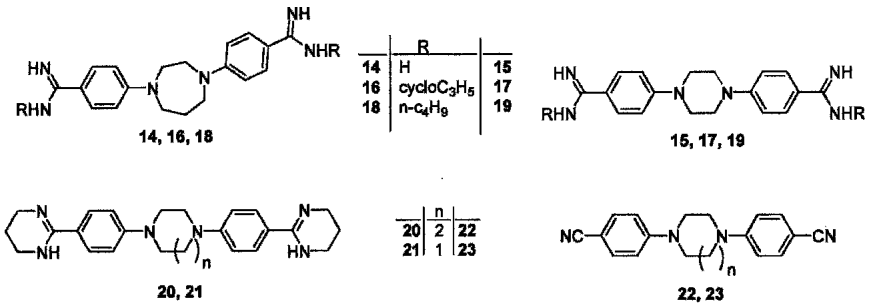
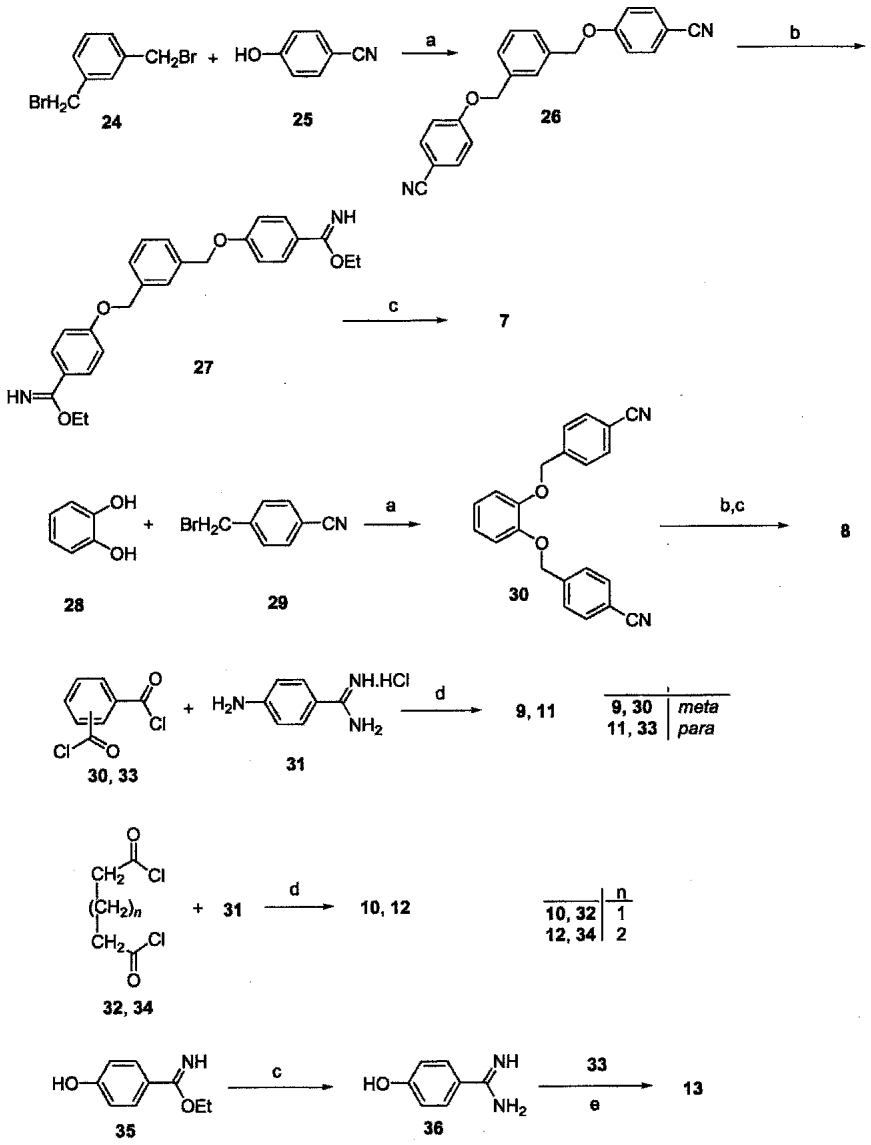


Figure 3. Second set of analogs of pentamidine **6**

Chemistry

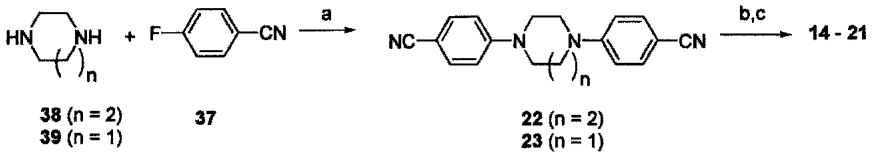
Scheme 1 depicts the synthetic sequences used to obtain compounds **7** to **13**. Nucleophilic substitution of the bromine atoms in α,α'-dibromo-*m*-xylene (**24**) by 4-hydroxybenzonitrile (**25**) in aqueous sodium hydroxide yielded the bisbenzonitrile **26**. The latter was transformed, by treatment with hydrochloric acid and ethanol in dichloromethane, into the corresponding imidate **27**, which was isolated, but further used without purification. Aminolysis of **27** with ethanolic ammonia afforded the targeted ether **7**.[32,33] In a similar way, **8** was obtained from catechol (**28**) and 4-bromomethylbenzonitrile (**29**). Diamide **9** was prepared by action of isophthaloyl dichloride (**30**) on 4-aminobenzamidine monohydrochloride (**31**). Diamides **10**, **11**, and **12** were synthesized following the same procedure, but from glutaryl (**32**), terephthaloyl (**33**), or adipoyl (**34**) dichlorides, respectively. Reaction of the commercially available ethyl 4-hydroxybenzimidate (**35**) with ammonia in ethanol afforded 4-hydroxybenzamidine (**36**), which was readily converted into **13** by interaction with the acid dichloride **33**.



Reagents: (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) pyridine, DMF, reflux, 1 h; (e) pyridine, dioxane, reflux, 8 h.

Scheme 1. Synthesis of compounds **7 – 13**.

As outlined in Scheme 2, the key step for the synthesis of compounds **14 – 21** involved two consecutive aromatic nucleophilic substitutions [34-35] between 4-fluorobenzonitrile (**37**) and homopiperazine (**38**) or piperazine (**39**) to yield the dinitriles **22** and **23**. Those dinitriles were further derivatized by the Pinner reaction.[32,33]



Reagents: (a) K_2CO_3, DMF, reflux, 7 h; (b) HCl(g), EtOH, CH_2Cl_2, rt, 4 d; (c) NH_3(g) or amine, EtOH, reflux, 1 h.

Scheme 2. Synthesis of compounds **14 – 23**.

Pharmacology

Anti-Pneumocystis carinii activity

Compounds **6 – 21** and **23** were evaluated against rat *P. carinii* in an ATP detection assay based on the release of bioluminescence driven by ATP in a luciferin-luciferase mediated reaction. [16,19] Results are expressed as the concentration of compound needed to reduce the ATP content of the exposed *P. carinii* populations by 50% versus the untreated control organisms (IC_{50}). Pentamidine **6**, the drug of reference for that assay, was characterized by an IC_{50} value of 500 nM. The results for the other compounds are shown in the Table.

Antitumor activity

The antitumor activity of the compounds was determined by the National Cancer Institute (Bethesda, MD). Their protocols start with a primary antitumor assay at a concentration of 100 μM against a 3-cell line panel consisting of NCI-H460 cell line of lung tumor, SF-268 cell line of CNS tumor, and MCF7 cell line of breast tumor. Results for each test agent are reported as the percent of growth of the treated cells compared to untreated cells. For NCI, compounds that reduce the growth of any one of the three cell lines to approximately 32 %, or less, are passed on for evaluation in a panel of 60-cell lines over a 5-log dose range (10^{-4} to 10^{-8}). Data collected from the screening of our derivatives in the primary antitumor assay at 100 μM against the 3-cell line are gathered in the Table.

Following the initial screen, derivatives **7**, **8**, **14** (for comparison purpose), **15**, **18**, **19**, **20**, and **21** were further evaluated in the human tumor 60-cell lines derived from leukemia, lung tumor, colon tumor, CNS tumor, melanoma, ovarian tumor, renal tumor, prostate tumor, and breast tumor. A 48 hour continuous drug exposure protocol is followed by NCI and a sulforhodamine B (SRB) protein assay was used to estimate viability or growth.[36-38]

The antitumor activity of the compounds was reported for each cell line by three parameters: log GI_{50} (log of the molar concentration that inhibits 50 % cell growth), log TGI (log of the molar concentration leading to a total inhibition of cell growth), and log LC_{50} (log of the molar concentration leading to 50 % cell death). A meangraph midpoint (MG_MID) is calculated for each mentioned parameters (i.e. log GI_{50}, log TGI, and log LC_{50}), giving an averaged activity parameter over 60 cell lines (for the calculation of MG_MID, insensitive cell lines are included with the highest concentration tested). The MG_MID-GI_{50} reported in the Table is the meangraph midpoint for the GI_{50} values. The selectivity indices of the compounds shown in the Table were calculated from the ratio between the MG_MID-GI_{50} values and the IC_{50} values of the anti-*P. carinii* activity. The binding affinities of the compounds, as estimated by the ΔT_m values,[12,17] for calf thymus DNA and poly(dA-dT) are also shown in the Table.

Results and Discussion

Of the 17 compounds tested against *P. carinii* (Table), four compounds, **10**, **12**, **18** and **19** were more potent than pentamidine **6**. Compounds **10** and **12** in which the benzamidine groups are linked by flexible linkers bearing poor electron-donating amide groups (pentanediamide or hexanediamide) were exceptionally potent with IC_{50} values of 3 and 2 nM respectively, whereas the IC_{50} value for the reference compound **6** was 500 nM. On the other hand, the benzamidine groups in compounds **18** and **19** are linked either with a rigid homopiperazine or piperazine ring and are characterized by IC_{50} values of 357 and 91 nM respectively. It is interesting to note that compounds **9** and **11**, which are rigid analogs of **10** and **12** respectively, were even less potent than pentamidine. Among the homopiperazine or piperazine-linked bisbenzamidines, the anti-*P. carinii* activity can further be modulated by attaching either a n-alkyl or cycloalkyl group to one of the nitrogens of the amidine moieties (compare **18** and **19** with the parent unsubstituted diamidines **14** and **15**). A clear correlation between the anti-*P. carinii* activity and DNA binding affinity for these compounds was not observed. For example, some of the more potent compounds such as **10**, **12** and **18** showed moderate binding to DNA, whereas, the less potent compounds such as **7**, **14-17**, **20** and **21** showed stronger binding to DNA.

Table. Anti-*Pneumocystis carinii* activity, antitumor activity in the primary antitumor assay, meangraph midpoint calculated for the 50% growth inhibition of human tumor cells, selectivity index, and DNA binding for compounds **6 – 23**.

Cpd	Anti-*P. carinii* activity IC_{50} (μM)	Antitumor activity				SI[a]	DNA binding (ΔT_m, °C)	
		% of control growth at 100 μM			MG_MID-GI_{50} (μM)		Calf thymus DNA	Poly (dA-dT)
		Non-small cell lung cancer NCI-H460	CNS cancer SF-268	Breast cancer MCF7				
6	0.5	NA[b]	NA[b]	NA[b]	10	20	11.6	20.6
7	2.6	0	0	0	8	3	14.0	23.9
8	1.7	5	27	42	40	24	0.1	- 0.1
9	1.2	58	68	68	> 100	> 83	8.0	9.9
10	0.003	105	121	122	> 100	> 33,333	5.6	11.4
11	22.8	99	100	115	> 100	> 4	9.7	7.7
12	0.002	75	52	34	> 100	> 50,000	8.7	14.4
13	13.4	96	94	79	> 100	> 8	9.9	10.4
14	2.0	44	94	87	63	32	15.0	23.1
15	2.6	2	13	45	40	15	17.0	23.8
16	1.4	71	104	87	> 100	> 71	15.3	23.6
17	5.4	74	115	108	> 100	> 19	14.5	23.9
18	0.357	17	101	39	13	36	11.8	13.3
19	0.091	2	1	6	13	143	15.2	23.9
20	4.5	28	55	42	80	18	15.5	21.3
21	3.0	18	93	77	> 100	> 33	12.2	17.9
22	ND[c]	92	101	70	> 100	ND[c]	ND[c]	ND[c]
23	> 125	106	105	102	> 100	ND[c]	0	-0.2

[a] SI = ratio between the meangraph midpoint calculated in μM for the 50% growth inhibition of tumor cells (MG_MID-GI_{50}) and the IC_{50} value, in μM, for the anti-*Pneumocystis carinii* activity.

[b]NA = Data not available from NCI.

[c]ND = Not determined.

A large number of the bisbenzamidines studied reached the criteria required for evaluation in the 60-cell lines panel. One of the conformationally restricted congeners of pentamidine, namely **7**, appeared to totally inhibit the growth of the 3 cell lines in the primary screen (Table). Its isomer, **8**, also exhibited noticeable antitumor activity. Both compounds **7** and **8** contain benzamidine groups, which are linked to the rigid spacer by ether functions. Several of the homopiperazine- and piperazine-linked bisbenzamidines, namely, **15, 18, 19, 20** and **21** also showed marked antitumor activity (Table), the rod-like piperazine derivatives, **15, 19, 21** being the most potent.

With the exception of compound **8**, compounds such as **7, 15, 18, 19, 20** and **21**, which inhibited the growth of any one of the three cell lines to 32% or less when compared to the control growth, showed significant binding affinity to calf thymus DNA and poly (dA-dT). However, it is noted that non-cytotoxic compounds such as **14, 16,** and **17** were also strong DNA binders. The poor DNA binding of **8** is probably due to the shape of the molecule not complementing with the helicity of the minor groove in DNA. Compounds that showed moderate binding to DNA, e.g. **9-13**, were generally not cytotoxic. Therefore, a direct correlation between DNA binding and cytotoxicity was not observed for these compounds.

A detailed analysis of the data based on the expanded panel of 60 cell lines indicated that bisbenzamidines linked by a 1,2-phenylenbis(methyleneoxy) (**8**), a 1,4-homopiperazinediyl (**14**), or a 1,4-piperazinediyl (**15**) group exerted only minimal antitumor activity, except against some renal tumor cell lines (A498, CAKI-1 and SN12C) where the log GI_{50} values were in the range –5.7 to –5.1. Their behavior can be compared to that of stilbamidine **3**. On the other hand, Compound **7**, a conformationally constrained analog of pentamidine (**6**), exhibited significant antitumor activity. It inhibited 50 % cell growth (log GI_{50}) at micromolar (-5.8 - -5.0) concentrations in most types of tumor cell lines, with the exception of some of the ovarian tumor cell lines. A total inhibition of the growth (log TGI) in many cell lines was also observed at the micromolar range, but lethal doses (log LC_{50}) exceeded the micromolar level in all cases except in one line of melanoma (SK-MEL-5). Globally, the antitumor profile of **7** is similar to pentamidine (**6**), and therefore the cytotoxicity profile of **6** is not affected by restricting the flexibility of the linker through the introduction of a phenylene moiety between the ether functional groups.

In the series of the bisbenzamidines linked by a 1,4-homopiperazinediyl or a 1,4-piperazinediyl core, substitution pattern of the amidine functions seemed to play a crucial role on the cytotoxicity of the substances. Indeed the unsubstituted amidine derivatives (**14** and **15**) and the products **20** and **21** in

which the amidine groups are part of a 6-membered ring showed poor antitumor activity except in two of the renal cell lines (A498 and SN12C). On the other hand, the growth of most susceptible tumors was significantly inhibited by both derivatives bearing a N-butyl amidine moiety (**18** and **19**). The antitumor activity (based on GI_{50} values) of **18** and **19** was most notable against leukemia (SR: -6.2), melanoma (MALME-3M: -6.4), and renal (A498: -6.8 and –6.7) cell lines. The meangraph midpoint of the GI_{50} values of the 60 cell lines for both compounds was 13 μM; the meangraph midpoint of the TGI and LC_{50} values were about 80 μM and >100 μM, respectively.

On the basis of the above data, we defined a selectivity index (SI) as the ratio between the meangraph midpoint calculated in μM for the 50% growth inhibition of tumor cells (MG_MID-GI_{50}) and the IC_{50} value, in μM, for the anti-*Pneumocystis carinii* activity. The SI value calculated for the drug of reference, pentamidine **6**, was 20, a number that could tentatively be attributed to the well-known toxicity profile of that substance. Some of the bisbenzamidines selected for this study were even less selective. This includes compounds **7**, **15**, and **20** but these derivatives were relatively poor inhibitors of *Pneumocystis carinii*. Compound **18**, a homopiperazine ring system 1,4-disubstituted by *N*-butyl benzamidine groups, was more potent than **6** against *Pneumocystis carinii* and its SI of 36 is slightly higher than **6**. The piperazine analog **19** has a better SI because of its greater inhibitory effect on *P. carinii*. The most exciting results were obtained for the bisbenzamidines **10** and **12**, in which the benzamidine moieties are separated by flexible alkyldiamide linkers. These two compounds were highly potent against the fungal pathogen and showed little or no toxicity in the primary screen against the 3 human tumor cell lines. They were characterized by exceptionally high selectivity indices exceeding 10^4. The excellent in vitro profile of these two compounds remains to be confirmed in in vivo models but it clearly constitutes an encouraging basis for the development of novel bisbenzamidines that are more potent and less toxic than pentamidine as potential drug candidates for the treatment of *Pneumocystis* pneumonia.

Conclusions

The present study enabled us to evaluate the anti-*Pneumocystis carinii* and the antitumor activities of a series of derivatives structurally related to pentamidine **6**. Among the compounds we tested, two bisbenzamidines (**10** and **12**) linked by an alkyldiamide group emerged as highly potent drug leads against *Pneumocystis* pneumonia because they were 167-250 fold more active than pentamidine in vitro and they exhibited little or no cytotoxicity against several human tumor cells at concentrations of 100

μM. This novel class of compounds is under intensive study in our laboratories and the progress of our investigations will be reported in due course.

Experimental

Chemistry

^{1}H NMR spectra were obtained using a Varian Inova instrument (500 MHz), chemical shifts (δ) are given in ppm using TMS as internal reference. IR spectra were recorded on a Perkin-Elmer Spectrum One instrument operating in the diffuse reflectance mode. Solvents and reagents are commercially available (Aldrich Co, Acros Organics, Fisher Scientific, Sigma Chemical Co) and were used without further purification. All the compounds reported in this study were synthesized in the laboratory of Dr. Tien L. Huang at Xavier University of Louisiana. The structures and purity of the tested compounds were confirmed with proton nuclear magnetic resonance, infrared, and elemental analyses.
Compounds **7**,[39] **8**,[39] **9**,[17,18] **10**,[9] **11**,[18,40] **12**,[18] **13**,[18] **14**,[17,21] **15**,[17] **16**,[21] **17**,[23] **18**,[21] **19**,[8] **20**,[21] **21**,[8] **22**,[17,21] **23**,[17] **26**,[39] **30**,[39] and **36**[41] have been described in the literature.

Inhibition of P. carinii using the ATP assay[16,19]

The anti-*P. carinii* activity of the compounds were evaluated in an ATP detection assay based on the release of bioluminescence driven by ATP in a luciferin-luciferase mediated reaction. *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).[42] *P. carinii* were extracted and purified from the lungs of rats after 8- 12 weeks of immunosuppression, enumerated, cryopreserved and stored in liquid nitrogen as previously described.[43,44] Typically, infected rat lungs yield up to 2 x 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.[43]

Inhibition of the tumor cell growth assay[36-38]

The antitumor activity of the compounds was determined by the National Cancer Institute using an in vitro model consisting of a panel of 60 human tumor cell lines. The entire set of antitumor data (log GI_{50}, log TGI and log LC_{50}) in the 60 cell lines of compounds **3**, **6**, **7**, **8**, **14**, **15**, **18**, **19**, **20** and **21** can be obtained from the corresponding author.

DNA binding affinity measurements [12,17]

The procedure to determine the binding affinity is based on measurements of the change in midpoint (T_m) of the thermal denaturation curves for a 1:5 compound to DNA base pair ratio. Each ΔT_m value reported in the tables represents the mean of at least two experimental determinations.

Acknowledgments

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