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[c008]

Bisbenzamidines as antifungal agents. Are both amidines functions required to observe an anti-*Pneumocystis carinii* activity ?

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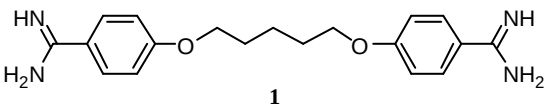
Abstract

A library of 19 novel 4-(4-phenylpiperazine-1-yl)benzamidines has been synthesized and evaluated *in vitro* against *Pneumocystis carinii*. Among these compounds, N-ethyl and N-hexyl 4-(4-phenylpiperazine-1-yl)benzamidines emerged as the most promising compounds with inhibition percentages at 0.1µg/ml of 61% and 56% respectively.

Introduction

Pneumocystis jirovecii pneumonia is a fungal disease that affects immunodeficient individuals and remains an important cause of mortality in AIDS infected persons [1] The parasite does not respond to classical antifungal therapy, but it is sensitive to some antiprotozoal medicines.[2] Classical treatments to cure the disease include the well-known trimethoprim-sulfamethoxazole association (TMP-SMX; Bactrim ™, Septra®), dapsone (Avlosulfon®), atovaquone (Mepron®), and pentamidine (NebuPent®, Pentacarinat®) (**1**, scheme 1).

The structural simplicity of pentamidine **1** and its efficacy have encouraged some laboratories to prepare original analogues with the hope to design more efficient and less toxic agents. In previous works [3,4] we demonstrated that 4, 4'-(piperazine-1,4-diyl)bisbenzamidine (**2,** scheme 1) is a promising candidate characterized by an *in vitro* IC_{50} of 2.61 µM against *P. carinii*. In addition, we observed that introduction of alkyl substituents on nitrogen atoms of the amidine functions of **2** could increase up to 1000 fold the activity of the parent compound. [3, 4]



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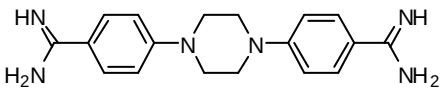
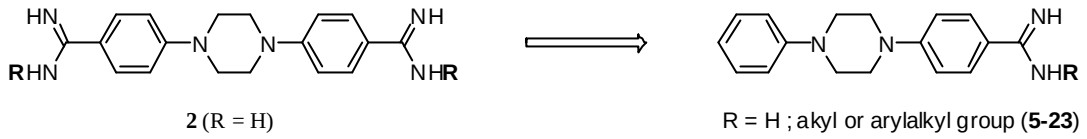


2

Scheme 1: Structure of pentamidine **1** and piperazine-1, 4-bisbenzamidine **2**.

In order to gain insight into the mode of action of those bisbenzamidines, we decided to prepare a library of monobenzamidines structurally related to **2** and to evaluate their biological behavior against *P. carinii.*



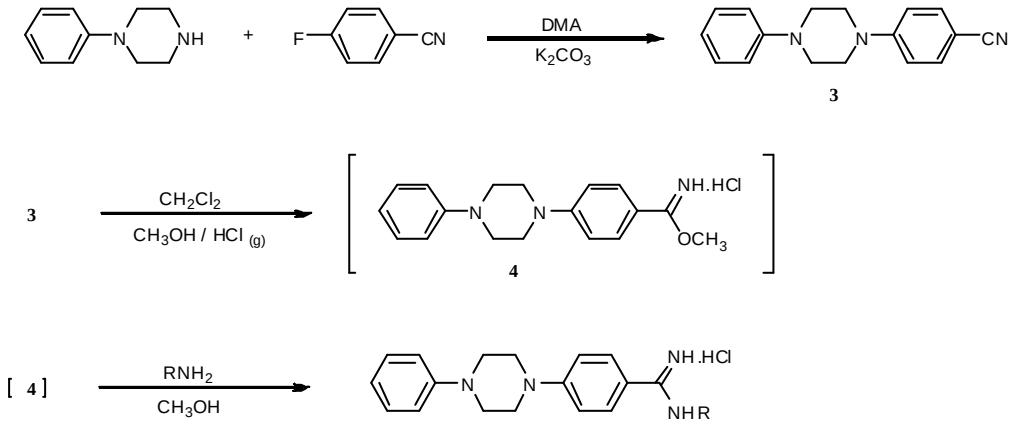
2 (R = H) R = H ; akyl or arylalkyl group (**5-23**)

Scheme 2: General structure of the monobenzamidines prepared in this study

Chemistry

The first step in the preparation of such compounds is the nucleophilic displacement of the fluorine atom in 4-fluorobenzonitrile by the secondary amine function of 4-phenylpiperazine in refluxing DMA in the presence of K_2CO_3 as a base (scheme 3). This step could advantageously be performed in a laboratory microwave oven so that reaction time can be reduced from 5 hours to 60 minutes.

Conversion of the nitrile compound **3** into the amidines **5-23** was effected by the Pinner[5] reaction where a solution of **3** in dichloromethane saturated with gaseous hydrochloric acid was treated with methanol to afford the imidate intermediate (scheme 3). This compound was finally reacted with the appropriate amine to give analytically pure amidines.



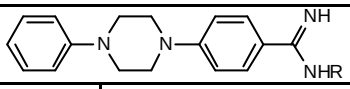
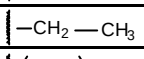
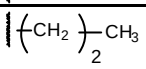
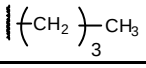
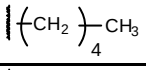
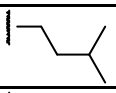
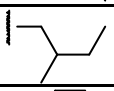
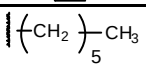
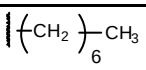
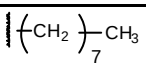
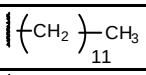
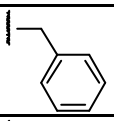
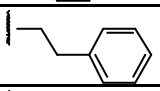
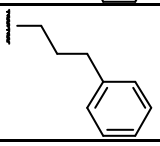
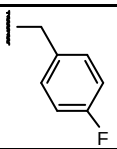
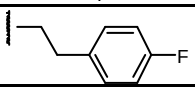
Scheme 3 : Synthesis of compounds **3-23**.

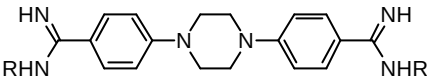
Biological evaluation

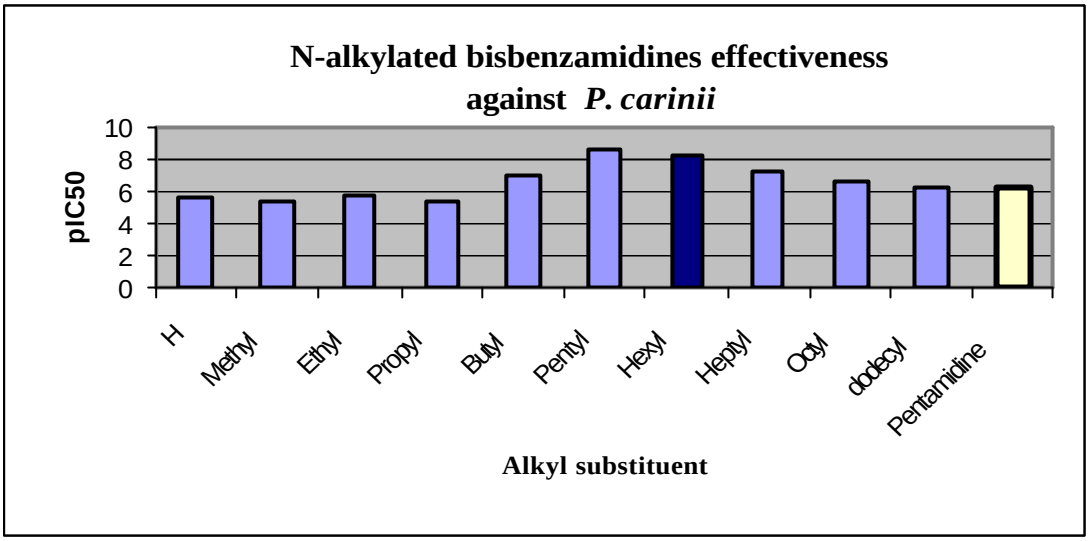
Results and discussion

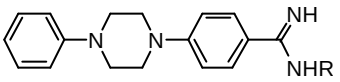
Table 1 contains the results of the *in vitro* evaluation of pentamidine **1**, compound **2**, and the benzamidines **5**-**23** against *Pneumocystis carinii*. Analysis of the data indicated that all compounds retain a (high) antifungal activity at a concentration of 10 µg/ml, with inhibition percentage ranging from 56% to 96%.

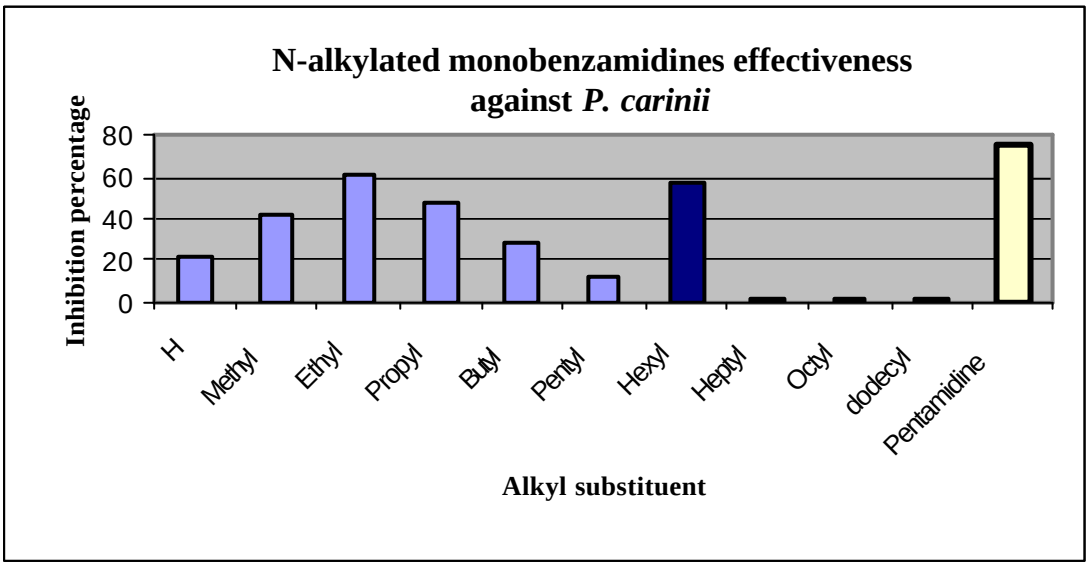
At a concentration of 0.1 µg/ml, the results are more contrasted. At that concentration, the data suggest that the activity was dramatically dependent on the nature of the substituent on the amidine function, as previously described in the bisbenzamidine series. [3, 4] In particular, we observed that the presence of an arylalkyl substituent on the amidine function (**19**-**23**) led to a loss of the antifungal activity. Among the other derivatives, the most active compounds were those bearing a linear alkyl group (**6**-**8**; **15**). Whereas piperazine-1, 4-bisbenzamidine (**2**) and most of its N-alkyl substituted congeners previously studied were at least as efficient as pentamidine (**1**), the situation is a little bit different in the monobenzamidine series described in the present work. Indeed, all derivatives are less active than pentamidine. Starting from the unsubstituted compound, introduction of an alkyl chain of increasing length on the amidine function leads to a modulation of the anti-*Pneumocystis* activity, with a maximum of activity observed in the case of ethyl and hexyl substituents. Interestingly, compounds bearing an alkyl chain constituted by 7, 8, or 12 carbon atoms are not active against the fungus. Mention should also be made that in the bisbenzamidine series as well as in the monobenzamidine series, the N-hexyl substituted candidates emerged among the most promising substances. That can tentatively be attributed to a favorable compromise between the hydrophilic properties of the amidine function(s) and the lipophilic character of the alkyl chain(s).

				
Compound Number	R	Inhibition percentage at		
		50 µg/ml (%)	10 µg/ml (%)	0.1 µg/ml (%)
Pentamidine **1**		92 ± 2.5	88 ± 8	78 ± 3
5	H	87 ± 4.4	80 ± 10	23 ±5
6		62 ± 16.5	56 ± 27.6	42 ± 12.1
7		83 ± 8.6	87 ± 27.22	61 ± 15.56
8		88 ± 7	72 ± 18.33	48 ± 31.28
9		88 ± 4.7	90 ± 6.9	No activity
Pentamidine **1**		98 ± 1	94 ± 1	76 ± 8.7
10		94 ± 1.15	92 ± 0.6	28 ± 14.57
11		90±0.63	94±0.3	13±16.46
12		96±0.6	91±0.6	No activity
13		97±0.6	92±1.1	No activity
14		97±1.5	93±4.9	43±3.2
15		91±7.5	96±0.58	56±2.6
Pentamidine **1**		93±3.5	91±8	53±6.5
16		92±2.6	90±4	No activity
17		80±16	93±4.3	No activity
18		85±12.5	79±9.7	No activity
19		93±4.1	91±1.4	No activity
20		95±2.08	94±1.15	No activity
21		95±2.47	96±2.3	No activity
Pentamidine **1**		97±1.5	95±1	96±1.5
22		73±4.5	70±9	No activity
23		96±1.5	69±7.6	No activity
2		59±4.5	66±7.5	32±7









Scheme 4 : Comparison of the antifungal profiles of N-alkylated bisbenzamidines and N-alkylated monobenzamidines

Conclusion

In summary, a library of 19 monobenzamidines linked on a 4-phenylpiperazine-1-yl scaffold has been synthesized and evaluated *in vitro* against *Pneumocystis carinii.* As in a series of bisbenzamidine analogues, the antifungal activity can easily be modulated by the introduction of appropriate alkyl substituents on the amidine function. Derivatives **7** and **15** emerged as promising candidates since they exhibited an inhibition effect on the growth of the fungus in the same range of concentrations than pentamidine. Further studies on those compounds are actually under investigation in our laboratories.

Experimental Protocols

^{1}H NMR spectra were obtained using a Brucker AMX instrument (300 MHz), chemical shifts (d) are given in ppm using TMS as internal reference.
IR spectra were recorded on a Perkin-Elmer FTIR 1760K.
Microwave synthesis were performed in Milestone Multisynth® oven.

Solvents, reagents, and pentamidine (**1**) were commercially available (Aldrich, Alfa Aesar, Acros Organics) and were used without further purification.
Compounds **2** [6] and **3** [7] have been described in the litterature
Elemental analyses were performed at the Centre Wallon de Recherches Agronomiques (Libramont-Chevigny, Belgium) or at the Laboratoire de Microanalyse Organique of the Institut des Sciences Appliqués de Rouen (France).

General procedure for the preparation of compound **3** *under microwave irradiation*

A mixture of 4-fluorobenzonitrile (2.5 mmol ; 0.3 g) and 1-phenylpiperazine (2.5 mmol ; 0.38 ml ; 0.41 gr) in DMA in the presence of K_2CO_3 (2.5 mmol ; 0.35g) was heated 60 minutes at 140 °C in a Multisynth® oven (Milestone) operating at 300 watts. After cooling, the solution was poured into ice cold water and the precipitate was filtered and washed with water and ethanol.

General procedure for the preparation of compounds **4-23**

A mixture of 4-(4-phenylpiperazine-1-yl)benzonitrile (**3**) (10 mmol; 2.66 g) in dichloromethane (250 ml) and methanol (25 ml) was saturated with HCl gas and the reaction medium was left at room temperature for 24 hours. The precipitate was filtered and thoroughly washed with ether. The crude imidate (**4**) was used without further purification and treated with the appropriate amine in refluxing methanol for 1 hour. A precipitate was obtained either by cooling or by addition of ether.

Biological evaluation

The *in vitro* anti-PcPactivity was tested using axenic culture of *P. carinii*. Parasites were incubated with (0.1, 10, or 50 µg/ml) or without drugs in DMEM + 10% of fetal calf serum for 4 days in an atmosphere of 5% CO2 at 37ºC. *P. carinii* was quantitated on air dried smears stained with a rapid panoptic methanol-Giemsa stain (RAL-555), which stains trophic forms, sporocytes and cysts of *Pneumocystis* sp.

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