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In vitro screening of pentamidine analogs against bacterial and fungal strains

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ABSTRACT

A series of linear pentamidine analogs exhibiting low cytotoxicity, active against *Pneumocystis carinii*, were evaluated for in vitro activities against bacterial and fungal strains. The majority of the tested bis-amidines exhibited marked activities against Gram-positive strains. In view of the fact that the highest potency was found for 1,5-bis(4-amidinophenoxy)-3-thiapentane dihydrochloride **1j** with the S atom in the middle of the aliphatic linker, four new pentamidines bearing S atoms were synthesized and also evaluated against MRSA strains. *N*,*N*′-Dialkylated pentamidines with S atoms in the linker are the promising lead structures for antimicrobials development.

The antimicrobial activity of aromatic bis-amidines is well known, but only pentamidine is clinically used for the treatment of pneumonia caused by the opportunistic fungus *Pneumocystis jiroveci*, against antimony-resistant leishmaniasis, and in the initial stage of human African trypanosomiasis.[1–9] Pneumocystis pneumonia (PCP) can be life threatening for patients receiving chemotherapy and immunosuppressive medication, including high-dose steroids.[10] Since 1984 pentamidine isethionate has been more commonly used for the treatment of PCP in immunocompromized and immunodeficiency patients. The mechanism of action is unclear and may vary for different microorganisms. Trypanosomes actively transport pentamidine intracellularly, where it may then interfere with DNA biosynthesis, but during interaction with *Pneumocystis* sp. pentamidine appears to kill nonreplicating organisms.[11]

There are several indications that pentamidine may have a wide range of antimicrobial, anti-inflammatory and anti-cancer activities.[12] It was found that in vitro pentamidine inhibits *Tropheryma whipplei*, *Coxiella burnetti*, *Acanthamoeba keratitis* and *Toxoplasma gondii*.[13–16] Aromatic bis-amidines which target the DNA minor groove are intensively tested as anti-infective agents and their significant therapeutic potential was highlighted.[17] Bis-amidines with benzimidazole and imidazole moieties which received much interest as concerns the discovery of antibacterial drugs are also mentioned as possible inhibitors of a bacterial two-component system (a histidine protein kinase and a response regulator) or as targeting bacterial cell-wall synthesis (the inhibitors of undecaprenyl diphosphate synthase).[18,19] To be an effective drug, the cationic bis-amidine compounds which are not lipid soluble, need to reach the molecular target in the cell, and one can suppose that they must be carried into the cell via a dedicated carrier or in non-cationic prodrug forms.[20]

However, it should be taken into account that the activity of bis-amidines is associated with high toxicity and low bioavailability in mammalian host.[21] Recently, we have found that newly synthesized pentamidine analogs had little to no cytoxicity in two in vitro evaluations.[22] These analogs which are shown in Figure 1 (see Supplementary data for their chemical names) exhibited no toxicity in the human epithelial cell line A549 (IC_{50} >100 μg/ml). Moreover, all analogs except **1d**, **1e** and **1i** also exhibited no symptoms of toxicity in the rat cell line L2 (IC_{50} >100 μg/ml).[22] It seemed interesting to assess their activities towards other fungi than *Pneumocystis* sp. and different microorganisms for a comprehensive study on their structure–activity relationship.

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The emergence of microbial resistance to the most commonly used drugs is an increasing problem in medicine,[23] and the earlier mentioned pentamidine analogs with low toxicity in two in vitro assays could be good candidates as alternative substances. Some earlier studies[24–26] supported these predictions because the screening of aromatic bis-amidines identified them as the potent anti methicilin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococccus faecium* agents.

Out of fifteen derivatives (Fig. 1) tested in the first course, the 1,5-bis(4-amidinophenoxy)-3-thiapentane dihydrochloride **1j** with the S atom in the aliphatic linker was the most potent one. Therefore, we synthesized four new pentamidine analogs bearing S atoms in the linker (Fig. 2). In order to determine the influence of alkyl substituents, the *N*,*N*′-dialkyl derivatives **3a**–**3c** of **1j** were prepared, whereas to determine the role of the S atoms localization, the derivative **3d** was synthesized with two S atoms which replaced the O atoms of pentamidine. Compounds **1j**, and **3a**–**3d** were tested additionally against five MRSA strains.

The present study is focused on biological evaluation of low toxic bis-amidines in two in vitro assays. We explored the possibility that linear pentamidine analogs, presented in Figures 1 and 2, have potency against fungal and bacterial strains, and wanted to find out if the selected compounds **1j**, and **3a**–**3d** can also be effective against MRSA strains. Several yeast strains, a mold and

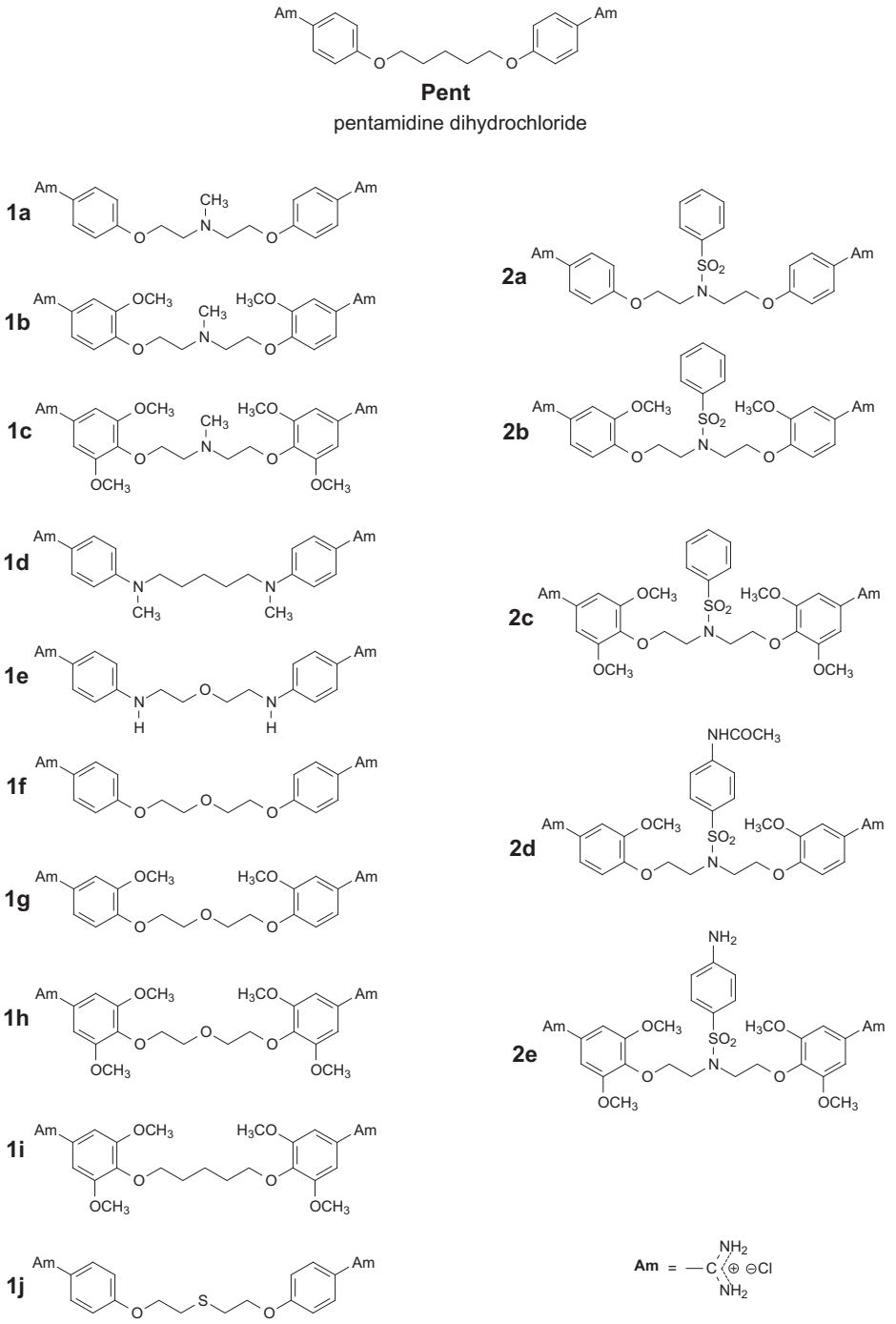


Figure 1. Chemical formulas of tested pentamidine analogs **1a**–**1j** and **2a**–**2e**.

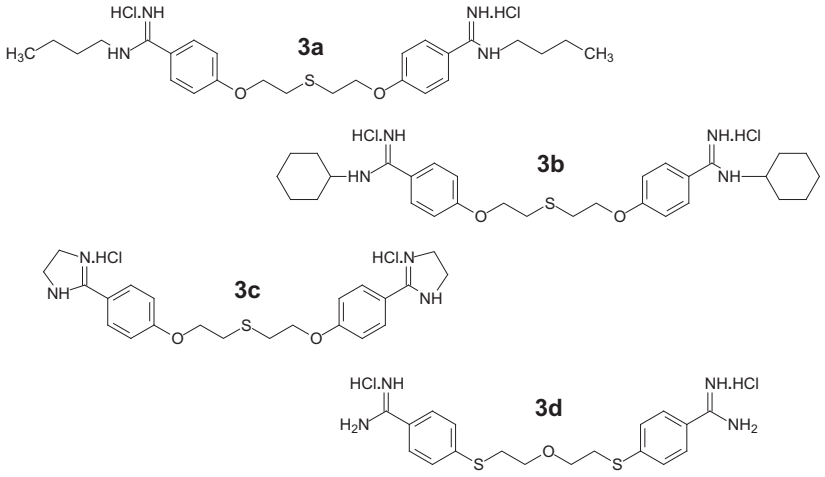


Figure 2. Chemical formulas of tested pentamidine analogs **3a**–**3d**.

Gram-positive and Gram-negative bacteria from American Type Culture Collection (ATCC), and MRSA hospital strains from WUM Museum Collection were chosen for the investigation. Moreover, cytotoxicity assay of new analogs **3a**–**3d** was established on the human lung cell carcinoma cell line A549 (ATCC CCL-185) and the human cervix adenocarcinoma cell line HeLa (ATCC CCL-2).

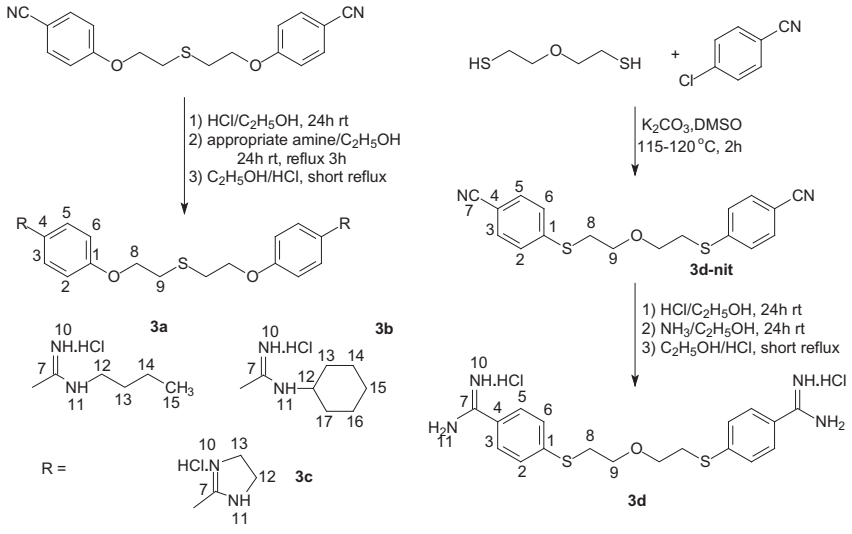
The syntheses and structural studies of 15 compounds were reported previously. For compounds **1a**–**1c**, **1g**–**1j**, **2a**–**2d** data are in,[22] for **1d** in,[27] for **1e** and **1f** in,[28] and for **2e** in.[29] The method of synthesis for new bis-amidines **3a**–**3d** generally followed the established procedures[30–32] which involved the preparation of bis-nitriles and their conversion into bis-amidines. Compounds **3a**–**3c** were synthesized from 1,5-bis(4-cyanophenoxy)-3-thiapentane[22] which was treated with appropriate amines (butylamine, cyclohexylamine, ethane-1,2-diamine) (Scheme 1). The synthesis of compound **3d** required a three-step procedure (Scheme 1). All synthetic details and the spectroscopic data are given in Supplementary data.

From the structural point of view, the tested compounds belong to two series. Series **I** includes 14 compounds **1a**–**1j**, **3a**–**3d** with different heteroatoms in the aliphatic linker (N, O and S), and 0, 2-, and 4-OCH_3 groups on benzamidine moieties. Series **II** includes 5 compounds of **2a**–**2e** bearing the sulfonamide substituents in the middle of the aliphatic linker, and varying numbers of the OCH_3 groups. The methoxy groups were inserted because of their presence in trimethoprim molecule and the sulfonamide groupings were inserted because of their known anti-bacterial efficacy.[33]

All compounds, apart from **1a**–**1c** which were analyzed as trihydrochlorides, were tested as dihydrochlorides. Pentamidine dihydrochloride (**Pent**) was used as a reference compound. The biological assays methods[34] are presented in Supplementary data.

The MIC values (μmol/ml) of pentamidine analogs for the tested bacterial and fungal strains are presented in Tables 1 and 2 and their chemical structures are shown in Figures 1 and 2 to facilitate the discussion about structure–activity relationship.

In the first course (Tables 1 and 2), all pentamidine analogs showed the activity against the tested Gram-positive strains. The analogs **1d**, **1f**, **1i**, **1j**, **2a**, **2b**, **2c**, **2e** showed a broad activity spectrum. They inhibited the growth of all tested strains at a concentration ranging from 0.0028 to 7.4 μmol/ml. In this series, the higher activity was associated with the presence of the sulfonamide group (**2c**) and four methoxy substituents on the benzene rings (**2c** and **1i**). Three active compounds **1d**, **1f**, **1j** have various heteroatoms in the aliphatic linker. The derivatives **1d**, **1j**, **2a** and **2b** also showed considerable activity against the tested yeast (MIC 0.07–0.04 μmol/ml). The introduction of the S atom in the middle of



Scheme 1. Synthesis of new pentamidine analogs **3a**–**3d**. Reagents and conditions together with the atoms numbering.

Table 1
Activities of pentamidine analogs against bacterial strains in MIC [μmol/ml]

Compd no	MIC μmol/ml									
	Micrococcus luteus		*Bacillus subtilis*	*Bacillus cereus*	*Staphylococcus aureus*		*Escherichia Coli*		*Pseudomonas aeruginosa*	
	ATCC 9341	ATCC 10240	ATCC 6633	ATCC 11778	ATCC 6538	ATCC 6538P	ATCC 8739	ATCC 10536	ATCC 15442	ATCC 9027
1a	1.06	1.06	2.11	1.06	21.1	10.6	21.1	21.1	>21.1	>21.1
1b	0.2	0.41	0.81	0.10	8.13	8.13	8.13	16.26	>16.26	>16.26
1c	0.76	1.52	1.52	7.61	7.61	7.61	7.61	15.22	>15.22	>15.22
1d	0.0039	0.0195	0.098	0.059	0.059	0.12	9.78	9.78	>19.55	>19.55
1e	0.024	0.071	0.59	0.59	0.59	0.59	2.37	11.84	>23.68	>23,68
1f	0.0044	0.011	0.55	0.55	0.55	0.55	0.55	0.55	11.08	11.08
1g	2.10	0.52	2.10	1.05	0.26	0.52	2.10	10.52	>21.04	>21.04
1h	0.017	0.051	0.42	16.96	0.21	0.21	16.96	16.96	>16.96	>16.96
1i	0.0035	0.018	0.089	0.11	0.054	0.054	0.22	0.22	4.46	1.78
1j	0.0046	0.023	0.14	0.0695	0.0695	0.14	0.14	0.58	2.32	1.74
2a	0.10	0.016	0.082	0.016	0.049	0.049	0.21	0.21	1.64	1.64
2b	0.015	0.042	0.042	0.042	0.042	0.042	0.37	0.74	7.38	7.38
2c	0.0028	0.007	0.070	0.088	0.070	0.070	7.04	7.04	7.04	7.04
2d	0.072	0.089	0.72	0.072	0.14	0.36	3.58	3.58	7.16	7.16
2e	0.038	0.064	0.32	0.32	0.32	0.32	6.41	6.41	6.41	6.41
Pent	0.0011	0.0044	0.067	0.0011	0.022	0.067	0.28	0.28	1.67	1.11

Table 2
Activities of pentamidine analogs against fungal strains in MIC [μmol/ml]

Compd no	*Candida albicans*		*Candida parapsilosis*	*Aspergillus brasiliensis*	*Zygosaccharo-myces rouxi*	*Saccharomyces cerevisiae*
	ATCC 10231	ATCC 2091	ATCC 22019	ATCC 16404	ATCC 28253	ATCC 9763
1a	>21.11	>21.11	10.6	N.T.	N.T.	N.T.
1b	>16.3	>16.3	>16.3	N.T.	N.T.	N.T.
1c	>15.2	>15.2	>15.2	N.T.	N.T.	N.T.
1d	0.12	0.12	0.020	9.78	0.12	0.24
1e	5.92	5.92	0.071	5.92	5.92	11.84
1f	>22.16	>22.16	0.066	>22.16	>22.16	11.08
1g	10.52	10.52	5.26	21.04	10.52	10.52
1h	>16.96	>16.96	>16.96	N.T.	N.T.	N.T.
1i	0.89	1.78	0.89	8.92	0.11	4.46
1j	0.14	0.14	0.023	0.070	0.070	0.29
2a	0.21	0.21	0.10	8.22	0.01	0.21
2b	0.092	0.37	0.37	7.38	0.18	0.18
2c	7.04	7.04	7.04	7.04	7.04	7.04
2d	0.18	1.43	0.36	7.16	1.43	1.43
2e	>12.83	>12.83	>12.83	>12.83	>12.83	>12.83
Pent	0.11	0.11	0.0044	0.022	0.067	0.28

the aliphatic linker (compound **1j**) resulted in the activity against all tested bacterial and fungal strains including *A. brasiliensis* similar to that of pentamidine. The introduction of the NCH_3 group in the aliphatic linker modified the biological activity of the tested compounds. The analog **1d** in which the O atoms in the aliphatic linker of pentamidine were replaced by NCH_3 groups revealed remarkable antifungal activity against Gram-positive strains whereas the analogs **1a**, **1b**, **1c** with the NCH_3 group in the middle of the aliphatic linker showed no antifungal activity. Moreover, the change of the two distal oxygens to NCH_3 yielded the compound **1d** with the loss of potency against Gram-negative strains, and with the change of cytotoxicity. It exhibited low toxicity on human cell line A549, but some toxic effect was found on rat cell line L2.[22]

The most potent compound **1j**, with limited cytotoxicity, has the S atom in the aliphatic linker and served as the lead compound for the synthesis of four pentamidine analogs **3a**–**3d** reported in this Letter. The synthesized compounds were tested against drug-resistant strains MRSA and compared with MIC values of **1j** and pentamidine. The obtained results are collected in Table 3. In the case of *N*,*N*′-disubstituted **3a**–**3c** derivatives, the transformation of amidine groups in 4,5-dihydro-2-imidazoyl rings (compound **3c**) reduced the anti-MRSA activity as compared with the parent compound **1j** against three MRSA lines (572/12, 19 K/11 and 573/12), or only slightly increased the activity against 15K/11 and 495/11 MRSA strains. Introduction of butyl and cyclohexyl groups had the pronounced consequences, and compounds **3a** and **3b** were found to be more potent by factor ten against all MRSA strains. Compounds **3a** and **3b** showed MIC values in the range 0.012–0.23 μmol/ml, and exhibited the highest activities against the MRSA 573/12 strain. Compound **3d** with the second S atom showed the potency equivalent to that of **1j** and pentamidine.

According to the table 2C from CLSI[35] the susceptibility of *Staphylococcus* spp. to linezoid and vancomycin is defined as $\leqslant$0.012 μmol/ml and $\leqslant$0.0028–0.011 μmol/ml, respectively; whereas the resistance of *Staphylococcus* spp. to linezoid and vancomycin is defined as $\geqslant$0.024 μmol/ml and $\geqslant$0.022 μmol/ml, respectively. *N*,*N*′-Dialkylated pentamidine analogs with the S atoms in the middle of the aliphatic linker constitute a promising structure for further research towards potential medicines.

In order to get a more complete view on the potency of compounds **3a**–**3d** they were screened against other bacterial and fungal strains (see Table 4 for results), and their cytotoxicity was also evaluated (see Supplementary data). The potency of *N*,*N*′-dialkyl substituted compounds **3a** and **3b** approached 2.6–9.8 nmol/ml values for Gram-positive *M. luteus* bacterial strains and *C. albicans* and *C. parapsilosis* yeast strains. Compounds **3a** and **3b** also showed considerable activity against the mold strain *A. brasiliensis*.

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Table 3
Activities of pentamidine analogs against MRSA strains in MIC [μmol/ml]

Compd no	MRSA 15K/11	MRSA 495/11	MRSA 572/12	MRSA 19 K/11	MRSA 573/12
1j	1.16	2.32	0.29	0.14	0.29
3a	0.23	0.23	0.23	0.11	0.014
3b	0.10	0.20	0.10	0.012	0.012
3c	0.98	1.96	1.96	1.96	1.96
3d	1.07	1.07	0.27	0.13	0.13
Pent	1.11	1.11	0.56	0.14	0.14

Table 4
Activities of analogs **3a**–**3d** against bacterial and fungal strains in MIC [μmol/ml]

Compd no	**3a**	**3b**	**3c**	**3d**
Micrococcus luteus ATCC 9341	0.0026	0.0032	0.0098	0.0043
Micrococcus luteus ATCC 10240	0.0036	0.00081	0.0098	0.0043
Bacillus subtilis ATCC 6633	0.014	0.012	0.24	0.13
Bacillus cereus ATCC 11178	0.45	0.10	0.98	0.54
Staphylococcus aureus ATCC 6538	0.014	0.0032	0.98	0.13
Staphylococcus aureus ATCC 6538P	0.027	0.0032	0.98	0.13
Escherichia coli ATCC 8739	0.11	0.10	0.98	0.54
Pseudomonas aeruginosa ATCC 9027	>1.81	>1.63	>1.96	1.07
Pseudomonas aeruginosa ATCC 15422	>1.81	>1.63	>1.96	2.15
Candida albicans ATCC 10231	0.0036	0.0032	0.24	1.07
Candida albicans ATCC 2091	0.0036	0.0032	0.24	1.07
Candida parapsilosis ATCC 22019	0.0036	0.0032	0.029	0.27
Aspergillus brasiliensis ATCC 16404	0.014	0.0032	>1.96	2.15
Saccharomyces cerevisiae ATCC 9763	0.027	0.024	0.98	0.54

Cytotoxicity tests of new analogs **3a**–**3d** showed that three analogs **3a**, **3b** and **3c** exhibited low toxicity against A549 cell line (IC_{50} >100 μg/ml in 48-h and $IC_{50} \geqslant 50$ μg/ml in 72-h MTT test). Compound **3d** with two S atoms in aliphatic linker was more toxic in both tests, with $IC_{50} = 50$ μg/ml, and 10 μg/ml in 48- and 72-h MTT test, respectively. As A549 cell line is relatively resistant to xenobiotic toxicity due to the elevated level of survival signaling, we decided to test all compounds on more sensitive HeLa cell line. However, similar toxicity pattern was also observed for HeLa cells with analogs **3a**–**3c** exhibiting low toxicity (IC_{50} >100 μg/ml in 48- and 72-h MTT tests), and compound **3d** exhibiting higher toxicity (IC_{50} in both tests below 50 μg/ml).

The marked antimicrobial properties of the most active analogs **1d**, **1i**–**1j**, **2a**–**2b**, **3a** and **3b** against Gram-negative and Gram-positive strains suggest that other effects than those related only to the differences in composition of the bacterial cell wall may be implicated in the antimicrobial activity of pentamidine analogs. Nevertheless, for new derivatives **3a**–**3d** the greater influence on Gram-positive strains is remarkable.

In conclusion, we evaluated pentamidine analogs **1d**, **1f**, **1i**, **1j**, **2a**, **2b**, **3a**, **3b** with a broad activity spectrum which inhibited the growth of all tested strains. Among them the derivatives **1d**, **1j**, **2a**, **2b**, **3a** and **3b** showed strong activity against yeast strains. The analog **1j** served as the lead compound for the synthesis of new pentamidine analogs **3a**–**3d** which were also evaluated against MRSA strains. 1,5-Bis[4-(*N*-cyclohexylamidino)phenoxy)]-3-thiapentane dihydrochloride **3b** showed very promising MIC values (0.012–0.23 μmol/ml) against MRSA strains as compared with pentamidine, linezolid and vancomycin. We hypothesize that *N*,*N*′-dialkylated bis-amidines with the S atom in the middle of the aliphatic linker are the promising template molecules for future studies, especially towards anti-fungal and anti-MRSA medicines.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.04.075.

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