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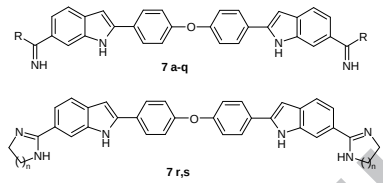

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ABSTRACT

A series of 4,4'-bis-[2-(6-*N*-substituted-amidino)indolyl] diphenyl ether have been synthesized and tested for their in-vitro antibacterial activity including a range of Gram-positive and Gram-negative pathogens and cytotoxicity. Most of these compounds have mainly shown anti-Gram positive bacteria activities especially against drug resistant bacterial strains MRSA、MRSE and VRE. The anti-MRSA and anti-MRSE activities of compound 7a and 7j were more potent than that of the lead compound 2, levofloxacin and vancomycin. Interestingly, 7j had greatly improved anti negative bacterial activity, especially for the producing NDM-1 *Klebsiella pneumonia* strain and less toxic than that of the lead compound 2.

The increasing emergence of multi-drug-resistant (MDR) bacteria has become a significant challenge in the clinic. Infections caused by drug-resistant Gram-positive bacteria, particularly MRSA (methicillin-resistant *Staphylococcus aureus*) and VRE (vancomycin-resistant *Enterococcus faecium*) are on the rise in the community and hospital.[1,2] Additionally, MDR Gram-negative bacteria such as extended-spectrum beta-lactamases (ESBL)-producing *Enterobacteriaceae* and Carbapenemase-producing *Klebsiella pneumonia* (KPC) are now resistant to most antibiotics and have emerged as an important therapeutic challenge.[3] One important strategy to address these resistance issues is the development of new classes of antibiotic drugs with activity against resistant pathogens.

During the course of our efforts to develop novel antimicrobial agents, we have discovered a new class of bis-benzimidazole bisamidine derivatives **1** (Fig. 1) which displayed potent anti-MRSA and anti-VRE activities.[4] These agents are structurally unrelated to any clinically used antibiotic. Optimization of the benzimidazole ring of dicationic bis-benzimidazole derivatives **1** resulted in the discovery of the lead bisamidine compound 4,4'-bis-[2-(6-N-substitution-amidino)indolyl] diphenyl ether **2** (Fig. 1) which has shown significant antibacterial activity including MRSA, methicillin-resistant Staphylococcus epidermidis (MRSE) and VRE.[4] Another important research group on bisamidine compounds is mainly focused on the structure of single heterocycle between two indole cycles. These compounds are found to be potent antifungal agents and some of them showed powerful antibacterial activities.[5]. Furthermore, the indole nucleus is a widespread substructure found in molecules which exhibit significant antimicrobial activities.[6]

To assist the development of novel antibiotics that are active against resistant bacteria, as well as to extend the SAR analysis for this class of compounds, we have kept the core structure of lead compound **2** and replaced the iso-propyl group on the *N*-position with different size chains and rings in order to evaluate the biological effect induced by steric bulk of the substituents in this position. Meanwhile, compared with dicationic bis-benzimidazole derivatives **1**,[4] it was observed that whether or not the same bisamidine substitutions showed similar effects on the antibacterial activity in these two series.

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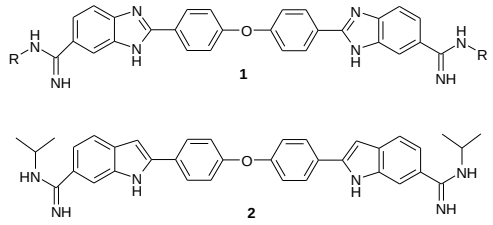
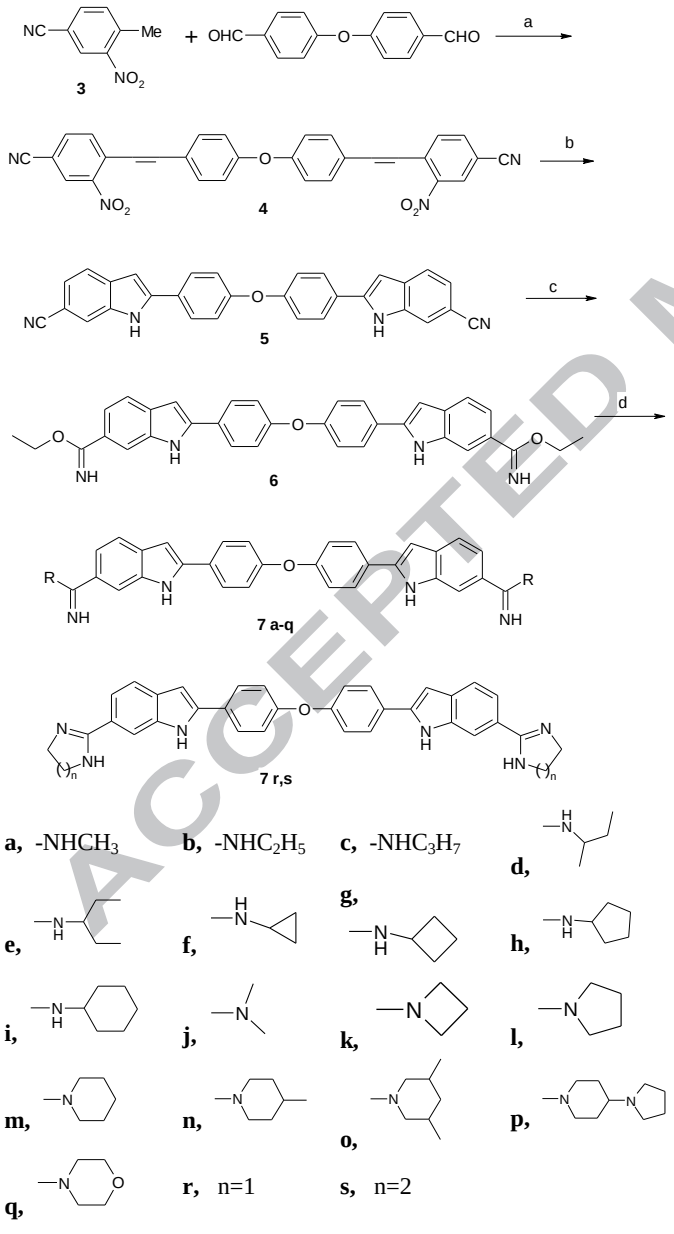


Figure 1. Benzimidazole and indole amidine compounds.

Scheme 1 depicts a convenient route for the synthesis of compounds **7a-s**. 4,4'-Bis-[2-(6-cyanoindolyl)] diphenyl ether **4** was prepared from 4-methyl-3-nitrobenzonitrile **3** by condensation with 4-(4-formylphenoxy) benzaldehyde and followed by heating with neat triethylphosphite. The dicyano compound **5** was converted to the key intermediate by using the Pinner method as previously reported.[7] The desirable target compound **7a-s** was prepared from **6** by reaction with the corresponding amines.



Scheme 1. Reagents and conditions: (a) 4-(4-formylphenoxy)benzaldehyde, piperidine, 100 ℃, 48%; (b) P(OEt)3, 160℃, 30%; (c) HCl (gas), EtOH; (d) the corresponding amines, EtOH, reflux, 20-70% in two steps.

The synthesized compounds were tested for their in-vitro antibacterial activity against a panel of 36 reference bacterial strains belonging to 16 different species representative of common human pathogens including MRSA, VRE and resistant *Enterobacteriaceae* and *Klebsiella pneumonia* strains. Using the agar dilution method[8] for the qualitative determination, the results of antibacterial screening of newly prepared compounds **7a-s** were expressed as the MIC values, comparative with the starting **2**, levofloxacin and vancomycin are summarized in Table 1. The information for other strains can be found in the supplementary data. By now, some evidences showed the potent bactericidal activity of the bisamidines is likely the result of the inhibition of DNA synthesis.[5] So the cytotoxicity (CC_{50}) of each analog against HEK 293T cell line[9] was investigated in Table 1.

The data generated from this study showed that most compounds displayed mainly good activity against Gram-positive strains and showed low or none activity against Gram-negative strains. It is interesting to note that **7e** with *iso*-pentylamino and **7j** with dimethylamino substitution exhibited improved activity against Gram-negative strains. We note that the observed activity against resistant Gram-positive bacteria is similar to that of sensitive Gram-positive bacteria. Most of them showed greater activity against MRSA, MRSE and VRE than levofloxacin and vancomycin. So we focused on the resistant bacteria such as MRSA, MRSE and VRE for discussion.

Based on our results (see Table 1), it was apparent that the carbon chain length and bulk has great influence on anti-bacteria activity. For compounds **7a-c**, as longer length of *N*-substitution from methyl to propyl, the compounds showed less and less anti-MRSA activity with MIC values from 0.5 to 8 μ g/ml. The trends against MRSE and VRE were similar. Compound **7a** showed more potent activity than levofloxacin with 16-32 increase in antibacterial activity and more active than vancomycin with 4-8 fold for MRSA and MRSE, especially with 128-256 fold greater activity for VRE strains: *faecalis 09-9, faecium ATCC 700221*. From the structural perspective, the carbon chain length seemed to have significant impact on anti-MRSA, MRSE, VRE activities. The shorter the carbon chain length on *N*-substitution, the more potent the activity of the compound. The compounds **2**, **7d** and **7e** with branched chain exhibited potent anti-MRSA and anti-VRE activities. However, as the branched chain becomes larger, the activity becomes weaker. **7d** and **7e** were more active than **7c** possessing the straight chain propyl group.

Replacement of *N*-isopropyl in **2** with cyclopropyl, cyclobutyl, cyclopentyl and cyclohexyl in **7f-i** reduced the activity. Furthermore, compounds **7g** and **7h** having the cyclobutyl and cyclopentyl group showed only weak activities. Interestingly, Compound **7i** with cyclohexyl had a slight increase of antibacterial activity relative to **7g** and **7h**. This may be because *N*-cyclohexyl group in **7i** is more flexible than the smaller more rigid rings.

Based on the structure-activity relationship, we found that the more branched and the shorter chain exhibited the more powerful activity. So, **7j** with the dimethylamino group was synthesized. Consistent with the expected result, **7j** showed the four times better activity against MRSA and MRSE than the lead compound **2**. And interestingly, the antibacterial activity against Gram-negative bacteria of **7j** had greatly improved, it was 8-fold more potent than lead compound **2** and more than 16 times potent for referenced drug levofloxacin for producing NDM-1 (New Delhi Metallo-beta-lactamase-1) *Klebsiella pneumonia* strain. At the same time, **7b-7j** showed relatively low cytotoxicity against HEK 293T cells.

We introduced an *N*-atom in different rings to synthesize compounds **7k-q,** comparing to **7j** with the dimethylamino group. **7k** with a four member ring was more potent than levofloxacin by 16-64 fold against MRSA, MRSE and VRE and more potent than vancomycin with 4-8 fold more activity against MRSA and MRSE. With increasing ring size, the compounds showed a large decline in activity. Likewise, the activity of compounds **7n**, **7o** with methyl additions to the six member ring of **7m** was inferior to that of **7m**. This result suggests that the more bulky groups are not beneficial to the antibacterial activity. For the compounds **7p** and **7q,** introduction of heteroatom increased the antibacterial activity relative to **7m** and **7n**. However, all of those compounds **7k-q** were less potent relative to **7j** with the dimethylamino group. Except **7k** with a tetramethylene imine ring, **7l-7k** all showed relatively high cytotoxicity against HEK 293T cells with $CC_{50} < 10$ μ g/ml.

In order to further understand the structure-activity relationship of the amidine position, we synthesized cyclic amidines **7r, 7s**. They showed promising activity. **7s** with six membered ring displayed more potent activity and much less cytotoxicity than **7r** with five membered ring.

Interestingly, compared with dicationic bis-benzimidazole derivatives **1**, dicationic bis-indole derivatives were much more sensitive to the change of *N*-substitution. When isopropyl was replaced by cyclopentyl, the piperidinyl, 4-Methylpiperidnyl and the 3,5-dimethylpiperidinyl group, compounds **7h** and **7m-n**, showed decreased the activity greatly by 16-64 fold while dicationic bis-benzimidazole analogs **1h**, **1m** and **1n** displayed only slightly less antibacterial potency up-to 2 folds [3]. So it is more meaningful to find the suitable substitution on *N*-position in dicationic bis-indolyl analogs **7**. The only difference of the two nitrogen atoms in these two series makes a great impact on the SAR. It is clear that a number of additional analogues need to be synthesized for a more in depth understanding the SAR of these dicationic amidine compounds.

For anti-Gram-negative bacteria activity, most novel derivatives showed less potent activity than lead compound **2**. However, **7d**, **7e** and **7j** had comparable or better activity, especially against producing NDM – 1 *Klebsiella pneumonia* strain. Their common structure feature is that the branched chain on an amidine *N*-atom is not cyclic. And more attractively, their cytotoxicity is less that the lead compound.

In conclusion, we have designed and synthesized a series of diphenyl ethers diamidines with bis-indole rings that have remarkable antibacterial properties especially against antibiotic-resistant bacteria including MRSA, MRSE and VRE. Obviously, the most active compound is **7j** with dimethylamino group (MIC = 0.25 μ g/mL for MRSA and MRSE, MIC = 2 μ g/mL for VRE) 4-fold better than the leading compound **2** and much more effective than the standards levofloxacin and vancomycin. At the same time, **7j** had excellent performance against gram negative bacteria and less toxic than the leading compound **2**. Our results represent a valuable starting point for the preparation a new series of bisamidine derivatives with bis-indole rings with the aim of improving their antibacterial activity and reducing toxicity. While the mechanism of action of these compounds isn't very clear, efforts to determine the origin of their antibacterial activity are underway.

Acknowledgments

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Supplementary data

Supplementary data (the activity data of all compounds for 36 strains) associated with this article can be found, in the online version, at *http://dx.doi.org/XXXX/j.bmcl*.

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9. **Determination of mammalian cytotoxcity.** Human embryonic kidney 293t cells were seeded onto a 96-well plate at a concentration of 1×10^5 Vero cells per mL and a volume of 200 μL per well. Following 24 h incubation at 37 °C, a confluent cell monolayer was confirmed. Test compounds were diluted with DMSO reach the different concentrations. Negative control dilution of DMSO at 1 % was also included. 2 μL/well of each diluted compound or DMSO was added to the plates in triplicate. After incubation at 37 °C with 5% CO^2 for 48h , cell supernatants were replaced with fresh medium containing MTS reagent (110 μL medium containing 10 μL MTS reagent per well). The trays were further incubated for 2h to allow MTS production. The absorbance was determined with an ELISA reader at a test wavelength of 490 nm. Data were calculated as the percentage of inhibition using the following formula: inhibition % = [100 – (At/As) × 100] %. At and As refer to the absorbance of the test substances and the solvent control, respectively. CC_{50} values, defined as the concentration of 50% cellular cytotoxicity (CC_{50}) of test compounds.

Table 1.

Antibiotic activity (MIC μ g/ml) and cytotoxicity (CC_{50}) of **7a-s** against Gram-positive and Gram-negative stains

Strain	Enzyme[a] production	Drug[b] resistance	**7a**	**7b**	**7c**	**7d**	**7e**	**7f**	**7g**	**7h**	**7i**	**7j**	**7k**
S. aureus ATCC29213	+	MSSA	1	2	16	2	2	4	64	128	4	0.25	2
S. Aureus	+	MRSA	0.5	1	16	1	2	4	64	64	2	0.25	0.5

09-13													
S. epidermidis ATCC12228	-	MSSE	0.25	1	8	0.5	0.5	1	8	8	1	0.125	0.125
S. Epidermidis 09-3	-	MRSE	0.5	1	2	1	1	2	16	16	2	0.25	0.5
E. faecalis ATCC29212	-	VSE	1	2	16	2	4	4	64	128	4	0.5	2
E. faecalis 09-9	-	VRE	1	1	16	8	8	8	64	128	8	2	8
E. faecium 09-10	-	VSE	1	16	16	8	4	8	64	128	8	2	8
E. faecium ATCC700221	-	VRE	0.5	2	2	1	2	4	32	128	2	2	2
E. coli ATCC25922	-	ESBLs (-)	128	64	>128	16	16	128	>128	>128	64	4	16
E. coli 09-1	+	ESBLs (+)	128	64	>128	32	16	128	>128	>128	128	8	64
K. pneumonia 7	-	ESBLs (-)	>128	128	>128	64	16	>128	>128	>128	128	8	64
K. pneumonia 09-25	+	ESBLs (+)	>128	64	>128	32	16	>128	>128	>128	128	8	64
K. pneumonia BAA-2146	+	NDM-1 (+)	>128	128	>128	32	16	>128	>128	>128	128	8	128
cytotoxicity CC_{50}[d]	/	/	12.2	36.3	26.2	40.4	25.4	36.9	32.6	28.9	59.2	21.6	21.6

Strain	Enzyme production	Drug resistance	**7l**	**7m**	**7n**	**7o**	**7p**	**7q**	**7r**	**7s**	**2**	**Lflox**[c]	**VCM**[c]
S. aureus ATCC29213	+	MSSA	2	16	16	64	8	2	16	2	1	0.125	0.5
S. Aureus 09-13	+	MRSA	1	16	16	16	8	2	2	1	1	32	2
S. epidermidis ATCC12228	-	MSSE	0.5	8	4	16	8	2	1	0.25	1	0.25	0.5
S. Epidermidis 09-3	-	MRSE	1	8	8	16	8	2	1	0.5	1	8	4
E. faecalis ATCC29212	-	VSE	8	32	64	128	8	8	16	4	1	0.5	1
E. faecalis 09-9	-	VRE	32	32	128	64	16	16	8	8	2	128	>128
E. faecium 09-10	-	VSE	8	16	128	128	8	4	8	16	2	64	1
E. faecium ATCC700221	-	VRE	2	32	16	128	8	2	2	1	1	64	128
E. coli ATCC25922	-	ESBLs (-)	16	>128	>128	>128	32	32	>128	64	16	0.06	>128
E. coli 09-1	+	ESBLs (+)	64	>128	>128	>128	64	128	>128	64	32	16	>128
K. pneumonia 7	-	ESBLs (-)	64	>128	>128	>128	128	>128	>128	128	64	1	>128
K. pneumonia 09-25	+	ESBLs (+)	64	>128	>128	>128	128	>128	>128	>128	64	4	>128
K. pneumonia BAA-2146	+	NDM-1 (+)	128	>128	>128	>128	128	>128	>128	128	64	>128	>128
cytotoxicity CC_{50}[d]	/	/	3.2	2.5	3.2	2.6	1.1	4.9	1.7	43.1	7.3	/	/

[a] Enzyme production: Beta-lactamase produced.

[b] MSSA, methicillin sensitive staphylococcus aureus. MRSA, methicillin resistant staphylococcus aureus. MSSE, methicillin sensitive staphylococcus aureus. MRSE, methicillin resistant staphylococcus aureus. VSE, Vancomycin sensitive Enterococcus faecalis. VRE, Vancomycin resistant Enterococcus faecalis. ESBL, extended-spectrum beta-lactamase. NDM-1, New Delhi Metallo-beta-lactamase-1.

[c] Lflox, Levofloxacin. VCM, vancomycin.

[d] CC_{50}, cytotoxic concentration value against human HEK 293T cell line.