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Antimicrobial activity of an abiotic host defense peptide mimic

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Abstract

Bacterial drug resistance is emerging as one of the most significant challenges to human health. Antimicrobial peptides (AMPs), which are produced by many tissues and cell types of invertebrates, insects, and humans, as part of their innate immune system, have attracted considerable interest as alternative antibiotics. Interest in novel mimics of AMPs has increased greatly over the last few years. This report details a new AMP mimic, based on phenylene ethynylene, with improved antimicrobial activity and selectivity. Screening against a large set of bacterial and other organisms demonstrates broad spectrum antimicrobial activity including activity against antibiotic resistant bacterial like methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE) as well as activity against yeast (*Candida albicans*) and fungus (*Stachybotrys chartarum*). Bacterial resistance development studies using *Staphylococcus aureus* show a rapid increase in MIC for conventional antibiotics, ciprofloxacin and norfloxacin. In sharp contrast, no change in MIC was observed for the AMP mimic. Cytotoxicity experiments show that the AMP mimic acts preferentially on microbes as opposed to mammalian red blood cells, 3T3 fibroblasts, and HEPG2 cells. In vivo experiments determined the maximum tolerated dose (MTD) to be 10 mg/kg suggesting a therapeutic window is available. These studies indicate that nonpeptidic amphiphilic AMP mimics could be developed as potential new treatments for antibiotic-resistant bacterial infections.

Keywords: Magainin; Phenylene ethynylene; Foldamer; Antimicrobial; Antibiotic-resistant

1. Introduction

Bacterial resistance and its rapid increase is a major concern of global public health. Resistance development is an even bigger problem since the bacterial resistance is often not restricted to the specific antibiotic prescribed, but generally extends to other compounds of the same class. Alarmingly, methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *Staphylococcus epidermidis*, vancomycin-resistant *enterococci* (VRE), ampicillin-resistant *Escherichia coli*, and even vancomycin-resistant *Staphylococcus aureus* (VRSA) have emerged as common nosocomial infections [1–3]. Antimicrobial peptides (AMPs) operate via non-specific mechanisms, targeting the bacterial cell membranes and other macromolecules, unlike conventional antibiotics, and as a result have shown a low potential for the emergence of bacterial resistant development [4]. This unique feature has resulted in substantial research efforts directed toward the potential of AMPs and their mimics as novel antimicrobial therapies. This special issue covers much of the exciting current research in this area.

Native AMPs with broad-spectrum antimicrobial properties such as magainin, defensin, melittin, and thionins are small host-defense peptides (typically, 20–40 residues) found in a wide range of species, including plants, frogs, worms, and humans [5,6]. They vary in size, length, sequence, and structure from the α-helical (magainin and cecropin) to the β-sheet (bactenecin and defensin) peptides which represent two major classes of AMPs. Despite this large diversity, they adopt highly amphiphilic topologies in which the hydrophilic and hydrophobic side chains segregate into distinctly opposing regions or faces of the molecule. This is referred to as facially amphiphilic. This unique amphiphilic topology and polycationic nature leads to membrane insertion, eventually depolarizes the membrane potential, disrupts membrane integrity, and ultimately kills the microbe [7–9].

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Developing new antimicrobial agents that are cell selective is a major challenge, especially when the target is the cell membrane. However, AMPs have already evolved excellent cell discrepancy so that learning to capture, or mimic, these essential properties in non-native structures will lead to fundamental understanding of this delicate and complicated process. Even more importantly, these discoveries offer the potential to develop novel agents to combat bacterial infections. This would have a major positive impact on global health care. Over the last decade, new classes of peptidic and nonpeptidic antimicrobials with structures similar to that of cationic and facially amphiphilic AMPs have been extensively investigated. There have been a number of studies reporting non-native antibiotics that follow the mechanism of natural AMP, such as α-amino acids [10–12], β-amino acids [13–16], peptoids [17], aromatic oligomers [18–20], and synthetic polymers [21–26].

We recently designed a series of polymeric phenylene ethynylenes with both good activity and selectivity [26]. This report demonstrated that abiotic structures, and even polymeric materials, could be designed with antibacterial activity that is non-hemolytic. Polymeric systems with average molecular weight of 1600 Da had an MIC of 50 μg/mL and HC_{50} of 540 μg/mL compared to the potent magainin analog, MSI-78 [27–31], which had an MIC of 12.5 μg/mL and HC_{50} of 120 μg/mL. Although the polymeric analog was not quite as potent as MSI-78, it was just a selective based on a comparison of the HC_{50} and MIC values. This notion is now extended to discrete, small oligomers with increased potency, better selectivity, broad spectrum activity, and significantly decreased bacterial resistance compared to ciprofloxacin and norfloxacin. These results demonstrate that the phenylene ethynylene oligomer has excellent biological activity and maybe a promising candidate for potential treatment of antibiotic resistant bacterial infections.

2. Results and discussion

2.1. Antibacterial activity

Based on our previous study of polymeric phenylene ethynylene [26], a series of discrete smaller molecular weight (MW) oligomers were prepared. This includes the three structures shown in Fig. 1 that contain three (**1**), five (**2**), and seven (**3**) aromatic rings, respectively. These oligomers have

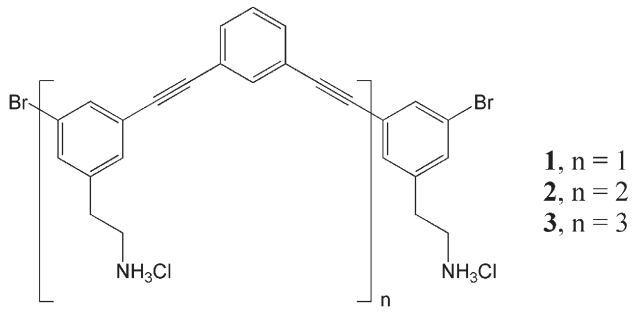

Fig. 1. Chemical structure of discrete, facially amphiphilic phenylene ethynylene antimicrobial AMP mimics.

Table 1
Antibacterial activities of **1**

Oligomer	MIC (μg/mL)				
	MW	*S. aureus*	*E. coli*	*E. faecalis*	*K. pneumoniae*
1	575	0.2	0.1	0.2	1.56
2	875	3.13	25	12.5	>100
3	1155	12.5	50	25	>100
MSI-78	2478	12	12	–	–

MW ranging between 595 and 1155 Da. Antibacterial screening against a small battery of both gram-positive and gram-negative bacteria shows clearly that the short three ring oligomer, **1**, is the most potent (see Table 1). The MIC of **1** is below 0.2 μg/mL for three of the four bacteria and reaches a maximum of 1.5 μg/mL for *K. pneumonia*. This is significantly more potent than the magainin derivative MSI-78. In contrast, the MIC of **2** and **3** was above 10 μg/mL, comparable to MSI-78, with only one exception. These results agree with similar findings on arylamide and arylurea oligomers recently reported in which the three aromatic ring structure represents a maximum in potency and selectivity [19,20]. The potency of **1** motivated further characterization of its antibacterial activity.

Table 2 shows the antibacterial activity of **1** against a larger panel of bacteria. In each case, two isolates per strain were used to uncover any unexpected selectivity between isolates. The MIC of **1** is generally between 0.5 and 4 μg/mL which compares favorably with Linezolid, a member of the

Table 2
MIC of 1 against a large panel of bacteria

Organism description	Isolate number	MIC[a]	MIC[a]
		1	Linezolid
Enterococcus faecium	1,162,827	8	2
Enterococcus faecium	1,162,828	8	2
Enterococcus faecium	1,162,826	0.5	2
Enterococcus faecium	1,162,818	0.5	4
Enterococcus faecium	1,162,819	0.5	8
Enterococcus faecium	1,162,820	0.5	2
Enterococcus faecalis	1,162,825	1	2
Enterococcus faecalis	1,162,836	1	2
Haemophilus influenzae	1,162,808	4	>8
Haemophilus influenzae	1,162,810	4	8
Stenotrophomonas (Xanthomonas) maltophilia	1,162,814	1	>8
Stenotrophomonas (Xanthomonas) maltophilia	1,162,815	1	>8
Staphylococcus epidermidis	1,162,840	0.5	1
Staphylococcus epidermidis	1,162,846	0.5	1
Staphylococcus haemolyticus	1,162,842	0.5	1
Staphylococcus haemolyticus	1,162,841	0.5	1
Staphylococcus hominis	1,162,844	0.5	1
Staphylococcus hominis	1,162,838	1	1
Staphylococcus saprophyticus	1,162,798	0.5	2
Staphylococcus saprophyticus	1,162,834	0.25	1
Streptococcus pyogenes	1,162,822	4	1
Streptococcus pyogenes	1,162,823	2	1
Streptococcus pneumoniae	1,162,833	2	1
Streptococcus pneumoniae	1,162,832	2	1

[a] μg/mL.

oxazolidinones antibiotics. As expected, the activity of **1** is generally independent of the isolate with one exception, *E. faecium*. A larger screening of this organism showed two distinct MIC values of 8.0 and 0.5 μg/mL. Similar variation is observed in the control antibiotic with MIC values ranging from 2 to 8 μg/mL. In all cases shown in Table 2, the MIC of **1** is comparable to the control.

2.2. Activity against antibiotic-resistant bacteria

AMP mimic **1** was screened against a series of antibiotic-resistant bacteria and the results are shown in Table 3. Potent activity of **1** was demonstrated against antibiotic resistant *S. aureus, E. faecium, E. faecalis,* and *S. pneumoniae*. This includes potent activity against MRSA and VRE. Activity was also measured against *P. aeruginosa* and showed variation of the MIC of 4, 16 and 32 μg/mL against three isolates. The MIC of AMPs is known to increase against *P. aeruginosa* and so these higher MIC values for **1** are not unexpected; even the control showed an increase in MIC. The ability of **1** to remain potently active against a large series of antibiotic resistant bacteria is extremely encouraging.

To further evaluate the prospect of **1** against antibiotic resistance, experiments were performed to measure the susceptibility of *S. aureus* to develop resistance against two conventional antibiotics, ciprofloxacin and norfloxacin which are members of the fluoroquinolones, as well as **1**. These experiments were performed by a serial passage of *S. aureus* to sub-lethal concentrations of the three antibiotics, followed by determination of the MIC values every 24 h period [32]. Fig. 2 shows that no significant change in the MIC is observed for **1** over the entire time course of the experiment in sharp contrast to the conventional antibiotics. In as little as six exposures, the MIC for ciprofloxacin and norfloxacin both increased. By the 16th passage, the MIC has increased from 0.195 to >12 μg/ml for ciprofloxacin and from 0.78 to >60 μg/ml for norfloxacin. These data demonstrate that resistance development for **1** is more difficult compared to these fluoroquinolones and suggests that the antibacterial mechanism is likely different. Previous results demonstrated that **1** acts on the cytoplasmic membrane of *S. aureus* using the membrane potential-sensitive dye, 3, 5′-dipropylthiacarbocyanine $DiSC_3(5)$ [33]. In addition, experiments performed on model liposomes demonstrated the ability of **1** to preferentially cause leakage of 10 mol% phosphatidylglycerol-phosphatidylethanolamine vesicles compared to 10 mol % phosphatyl-L-serine-phosphatidylcholine vesicles [33]. These membrane studies along with the resistance data presented above suggests that direct action on the bacterial membrane is a likely mechanism of action [24,33,34]. The broad activity against antibiotic resistant bacteria and lack of increase in MIC compared to ciprofloxacin is encouraging evidence for the development of AMP mimics as potential new therapies for antibiotic-resistant bacterial infections.

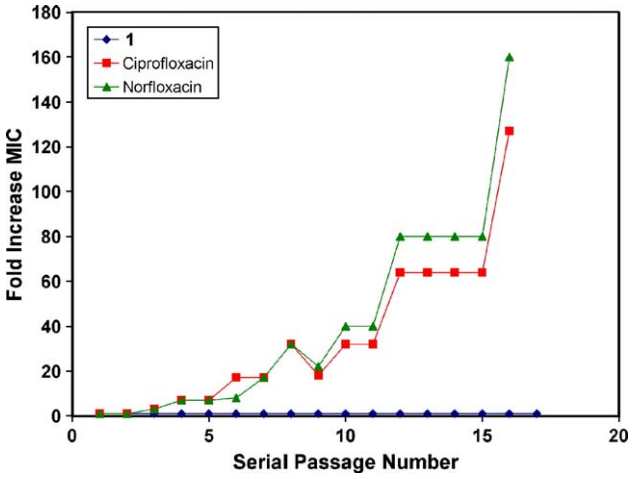

Fig. 2. Susceptibility of *S. aureus* to develop resistance against two conventional antibiotics, ciprofloxacin and norfloxacin, as well as **1**.

Table 3
Antimicrobial Activity of 1 against antibiotic resistant strains

Organism description	Isolate number	Antibiotic resistance	MIC [a]	MIC [a]
			1	Linezolid
Staphylococcus aureus	1,162,847	OX-R	0.5	2
Staphylococcus aureus	1,162,848	OX-R	0.5	2
Staphylococcus aureus	1,162,849	OX-R	0.5	2
Enterococcus faecium	1,162,818	VAN-R	0.5	4
Enterococcus faecium	1,162,819	VAN-R	0.5	8
Enterococcus faecium	1,162,820	VAN-R	0.5	2
Enterococcus faecalis	1,162,824	VAN-R	1	1
Enterococcus faecalis	1,162,837	VAN-R	1	2
Enterococcus faecalis	1,162,835	VAN-R	1	1
Pseudomonas aeruginosa	1,162,811	MDR	32	>8
Pseudomonas aeruginosa	1,162,812	MDR	4	>8
Pseudomonas aeruginosa	1,162,813	MDR	16	>8
Streptococcus pneumoniae	1,162,829	PEN-R	2	0.5
Streptococcus pneumoniae	1,162,830	PEN-R	2	0.5
Streptococcus pneumoniae	1,162,831	PEN-R	1	1

[a] μg/mL.

Table 4
Antimicrobial Activity of **1**

Strain	MIC/MFC (μg/mL)		
	1	Amphotericin B	Fluconazole
A. viscosus	0.8	–	
S. mutans	0.1 [a]	–	–
H. marina	0.1	–	–
P. Haloplanktis	1.0	–	–
Aspergillus fumigatus ATCC 204304	32/32	1/4	>32/>32
Aspergillus niger	4/16	0.5/2	>32/>32
Aureobasidium pullulans ATCC 11942	1/1	0.06/0.25	>32/>32
Chaetomium globosum ATCC 13835	0.5/8	0.06/1	0.25/0.25
Penicillium funiculosum ATCC 10446	1/2	0.06/8	4/>32
Trichoderma virens ATCC 13213	32/32	2/8	>128/>128
Trichophyton rubrum	2/2	0.5/0.5	32/32
Trichophyton mentagrophytes	2/4	0.5/1	32/>32
Stachybotrys chartarum ATCC 11695	2/2	2/2	>32/>32
Candida albicans ATCC 10231	1/2	0.25/1	0.5/1
Cryptococcus neoformans ATCC 36556	1/1	0.25/0.25	32/32

[a] MIC determined in a biofilm.

2.3. Antimicrobial activity and cytotoxicity

The excellent activity of **1** against the large panel of bacteria, including antibiotic resistant strains, and limited resistance induction encouraged us to screen for wider antimicrobial activity. Table 4 shows the MIC values of **1** against a range of microbes including marine bacteria, oral healthcare pathogens, yeast, and fungi. The activity against bacteria remains in the <1 μg/mL range including against *S. mutans* in biofilms. The MIC against yeast and fungus increases slightly to 1–2 μg/mL for most except *C. globosum* which is 0.5 μg/mL and *A. niger* which is 4.0 μg/mL. Two fungi, *A. fumigatus* and *T. virens*, show higher MIC values of 32 μg/mL; however, these show corresponding MIC increases for the two controls, amphotericin B and fluconazole. These results demonstrate that **1** is not only active against bacteria but also against yeast and fungus.

Cell selectivity is one of the most difficult challenges in antibiotic development, especially if the target of action is the cytoplasmic membrane [4]. In order to determine the ability of **1** to differentiate between microorganisms and mammalian cells, the EC_{50} for three different cell types was determined. Human red blood cell (RBC) lysis, or hemolysis, is the most common assay of selectivity for AMPs and their mimics. In addition to this assay, 3T3 fibroblasts, and HEPG2 liver cells, were also assayed for cytotoxicity. Table 5 compares the cytotoxicity of these three cell lines and selectivity of **1**. The selectivity value is a conservative estimate since it compares MIC, or >90% reduction, to EC_{50}, or 50% reduction. The AMP mimic, **1**, is approximately six times more cytotoxic to 3T3 fibroblasts and HEPG2 cells than RBCs. When comparing selectivity between microbial cells and mammalian cells, good selectivity is observed. For *S. aureus*, the selectivity is over 400 for RBCs as well as 60 and 70 for 3T3 fibroblasts and HEPG2 cells, respectively. Compared to melittin and MSI-78, **1** is significantly less cytotoxic. Selectivity for all MIC values can be determined. For example, selectivity compared to MRSA and VRE (MIC 0.5 μg/mL) is 176, 24, and 28 for RBCs, 3T3s, and HEPG2s, respectively. Using *C. albicans* and *S. chartarum* as examples of yeast and fungi, respectively, the selectivity is 88 and 44 for RBCs and 12 and 6 for 3T3 fibroblasts.

The difference in cytotoxicity between RBCs and 3T3 fibroblasts suggested in vivo studies would be critical to evaluate the therapeutic potential of **1**. Before determining the MTD for **1**, assays were performed to determine the MIC in the presence of serum and whole blood. In the presence of 40% mouse serum, the MIC was observed to increase from approximately 1 to 16 μg/mL. In contrast, experiments performed in whole human blood showed no change in the MIC and no change in the time needed to reach the MIC. Using mouse models, the MTD for **1** was determined to be 10 mg/kg. This value suggests a therapeutic window exists for **1**.

Table 5
Cytotoxicity of **1**

Compound	MIC (μg/mL)	Cytotoxity (EC_{50} μg/mL)			Selectivity (EC_{50}/MIC)		
	S. aureus	RBCs	3T3	HepG2	RBCs	3T3	HepG2
1	0.21	88	12	14	440	60	70
Melittin	2*	2	4	1	1	2	0.5

3. Conclusions

The development of novel antibacterial agents is critically important. Designing abiotic oligomers with distinct polar and non-polar facers, similar to AMPs, is a powerful approach to creating broadly active antimicrobial agents. A unique phenylene ethynylene oligomer is reported here which shows activity against bacterial, yeast, and fungus. Because it is a non-natural backbone without amide or ester functionality, it will not undergo proteolytic degradation from enzymes, like natural AMPs. This oligomer is active against antibiotic-resistant bacteria and shows a lower propensity toward developing resistance in *S. aureus* than ciprofloxacin. Integrating the physiochemical studies with in vivo activity is a significant challenge; however, a deeper understanding of the molecular mechanism will provide critical clues to this problem. Bringing a variety of analytical tools to this problem is sure to provide new, fundamental insight. The broad spectrum activity, potency, and selectivity of **1** strongly suggest this structure is a promising AMP mimic.

4. Experimental section

4.1. Antimicrobial activity

This procedure is a modification of the standard broth microdilution assay recommended by the National Committee for Clinical Laboratory Standards (NCCLS) which has been developed for determining in vitro antimicrobial activities of cationic agents by Hancock et al. [32] Bacteria were grown in Mueller–Hinton broth (MH broth) at 37 °C overnight, and the bacterial growth was measured by turbidity as optical density at $\lambda = 600$ (OD_{600}) using an Eppendorf BioPhotometer. After the regrowth of bacteria ($OD_{600} = 0.5–0.6$), the suspension is diluted to approximately 5×10^5 CFU/ml and inoculated into a polypropylene (Costar) 96-well round bottom plate (90 μl volumes). Solution containing DMSO without compound was prepared as a negative control. Compound stock solutions are prepared in DMSO and serial two-fold dilutions of compound are made in 0.01% acetic acid and 0.2% bovine serum albumin directly in the wells of the polypropylene plate at 10 μL/well. DMSO concentrations should not exceed 1% in the assay. One set of control wells includes broth-only samples with dilution buffer for testing sterility and providing blank values for the assay readings. Vehicle-control wells containing the bacterial suspension with DMSO (no compound) are also included. The assay plate was incubated at 37 °C for 18 h. The minimum inhibitory concentration was measured by observing the presence of cell growth, defined by NCCLS as a ≥2 mm button or definite turbidity.

4.2. Resistance study

In brief, the first MIC determination of **1** and two antibiotics (ciprofloxacin and norfloxacin) against *S. aureus* was

performed as described above. Bacteria samples from duplicate wells at the concentration of one-half MIC were removed and used to prepare the bacterial dilution (OD_{600} = 0.001) for the next experiment. These bacteria solutions were then replated on new 96 well plates with fresh dilutions of compound and the two controls. After incubation at 37 °C overnight, the change of MIC values was determined. This experiment was repeated each day for 16 passages.

4.3. Cytotoxicity assays

4.3.1. Hemolytic activity

Compound stock solutions and control solutions were prepared by the same procedure in the antimicrobial activity assay. Fresh human red blood cells (RBCs) were obtained by centrifuging a whole blood (3000 rpm, 10 min) and removing the plasma and the white blood cells. The RBCs (1 mL) was diluted with 9 mL of TBS buffer (10 mM Tris buffer, pH = 7.0, 150 mM NaCl) and this suspension was further diluted by a factor of 40 to give a RBC stock suspension (0.25% blood cells). This RBC stock (120 μL), TBS buffer (15 μL) and the compound stock solutions (15 μL) (or control solutions) were added to a 200-μL centrifugation tube and incubated at 37 °C for 1 h. The tube was centrifuged at 4000 rpm for 5 min. Supernatant (30 μL) was diluted with TBS buffer (100 μL), and OD_{414} of the solution was measured as hemoglobin concentration. Melittin was used as a positive control, and the most concentrated sample (100 μg/mL) was used as a reference for 100% hemolysis. A control solution containing serially decreasing amounts of DMSO without compound was used as a reference for 0% hemolysis.

4.3.2. MTS cytotoxicity assay

This protocol is based on one included with the CellTiter 96® AQ_{ueous} Assay Reagents (Promega, Madison, WI). The assay measures the reduction of MTS to Formazan by dehydrogenase enzymes found in metabolically active cells. 0.1 mL of cells were pipetted into a 96-well polystyrene TC-treated plate at 10^4 cells/well. This plate was incubated at 37 °C overnight in a CO_2 incubator for 18 h. The cells were washed once with 0.1 mL of assay media, or tissue culture media without the serum. Then, 0.09 mL assay media was added to each well and serial dilutions of drug were pipetted in 0.01 mL volumes; final well volume is 0.1 mL. The plate was then incubated for 1 h and the cells were washed with media containing serum. The MTS and PMS solutions were thawed in a 37 °C water bath for 15 min before use. A fresh 0.1 mL of tissue culture media and 0.02 mL of MTS-PMS solution (2 mL MTS + 0.1 mL PMS) was added to each well and shaken gently by hand for 20 s. The plate was then incubated from 2.5 (3T3 cells) to 4 (HepG2 cells) h. Following the final incubation, the plate is gently shaken again for 20 s and read at 490 nm in a microplate reader.

Acknowledgments

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