Branched Peptides: Acridine and Boronic Acid Derivatives as Antimicrobial Agents

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S Supporting Information

ABSTRACT: The emergence of microbial resistance presents a challenge in the development of next generation therapeutics. Herein, we report the discovery of branched peptides decorated with acridine and boronic acid moieties with potent antimicrobial activity. The results revealed minimal inhibitory concentrations (MICs) as low as 1 $\mu\text{g/mL}$ against *Staphylococcus aureus*, *Candida albicans*, and *Escherichia coli*. These peptides were nonhemolytic, and significantly inhibited growth of *C. albicans* in suspension and biofilm formation. Structure–activity relationship studies suggest the acridine functional group as a driving force for the potent inhibition observed against bacteria.

KEYWORDS: Branched peptides, Acridine, Boronic Acid, MIC, Antimicrobials, Biofilms

One forefront global health crisis is the emergence of antibiotic resistance. Most prominent among antibiotic-resistant bacteria include methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE), which are constituents of ESKAPE pathogens.^{1,2} The U.S. Centers for Disease Control and Prevention (CDC) estimates that antibiotic resistance is responsible for more than 2 million infections and 23,000 deaths each year in the United States.³ Further, the cost of antibiotic-resistance is estimated at \$20 billion per year. These staggering numbers provide the overwhelming impetus for developing the next generation therapeutics against these microbes.

One class of emerging antibiotics that displays broad antimicrobial activity is antimicrobial peptides (AMPs).⁴ AMPs are typically six to 60 amino acids in length, contain cationic and hydrophobic residues, demonstrate amphipathicity, and can affect microbial viability through several mechanisms including inhibition of formation of transmembrane pores or interference with cell-wall, nucleic-acid, and protein syntheses.⁵ An advantage of AMPs is that they are components of the innate immune system of microorganisms.⁶ Their mechanism of action is postulated to decrease the possibility of the emergence of antibiotic-resistant bacteria.⁴ However, there is mounting evidence that resistance to AMPs can develop through mechanisms that involve remodeling of the bacterial cell wall.^{7,8} Nonetheless, several AMPs are currently undergoing phase 2 and 3 clinical trials.⁸

AMPs have gained momentum as complementary alternatives to conventional antibiotic agents. Synthetic analogues (β peptides,^{9,10} peptoids,^{11–13} oligoureas,^{14–16} dendrimers,^{17–19}

and sulfono- γ -AApeptides²⁰) that mimic the structure and function of AMPs have been developed to increase proteolytic stability and decrease host toxicity. These AMPs capitalize on the electrostatic interaction with negatively charged bacterial cell membranes composed of phospholipids such as phosphatidyl ethanolamine, cardiolipin, and phosphatidyl glycerol.

We previously developed a branched peptide (BP) library that revealed 14 hits containing boronic acid and/or acridine moieties when screened against HIV-1 Rev Response Element (RRE) RNA.^{21,22} The BPs were synthesized via standard solid-phase peptide synthesis protocol using Fmoc-Lys(Fmoc)-OH as the branching amino acid as previously reported.^{21,22} The presence of cationic (lysine) and hydrophobic (leucine, acridine) residues has the potential to display amphipathic character commonly seen with AMPs, and their cationic properties could aid in selectively targeting the more negatively charged membranes such as LPS and teichoic acids found in bacteria.²³ Further, bacteria also have an overexpression of polysaccharides on the cell surface,²⁴ where boronic acid could be used to target and selectively exert antimicrobial effects. Herein, we report the identification and characterization of a family of novel antimicrobial BPs containing boronic acid and acridine. This class of BPs potently inhibited the microbial growth of *C. albicans*, *S. aureus*, and *E. coli* with MICs of 1–2 $\mu\text{g/mL}$. Further, these BPs were capable of inhibiting biofilm formation and

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Table 1. Minimal Inhibitory Concentrations of BPs against a Panel of Microbes ($\mu\text{g/mL}$)

peptide	sequence ^a	<i>S. aureus</i>	<i>E. coli</i>	<i>M. smegmatis</i>	<i>C. albicans</i>	<i>A. niger</i>
A1	(KKK) ₂ *LK _{BBA} K _{BBA} Y	32	8	32	4	62.5
A2	(KKK) ₂ *LK _{BBA} LY	32	16	32	4	62.5
A3	(KKK) ₂ *K _{ACR} LKY	16	16	16	1	62.5
A4	(KKK) ₂ *KLK _{ACR} Y	16	8	32	2	62.5
A5	(KKK) ₂ *K _{ACR} K _{ACR} KY	1	8	16	4	62.5
A6	(K _{ACR} KK) ₂ *KLK _{BBA} Y	1	4	16	8	32
A7	(KK _{ACR} K) ₂ *KLK _{BBA} Y	8	2	32	2	32
A8	(KK _{BBA} K) ₂ *LLKY	62.5	16	62.5	16	125
A9	(KKK _{BBA}) ₂ *KLK _{BBA} Y	62.5	16	62.5	8	125
A10	(KLK) ₂ *LKK _{BBA} Y	62.5	16	32	4	125
A11	(KLK) ₂ *KKK _{BBA} Y	32	16	32	2	125
A12	(KKL) ₂ *LKK _{BBA} Y	62.5	16	32	4	125
A13	(LLK) ₂ *KK _{BBA} KY	62.5	16	125	8	250
A14	(KKL) ₂ *KK _{BBA} K _{BBA} Y	62.5	16	62.5	8	125
CA3	(KKK) ₂ *K _{BZ} LKY	62.5	32	32	2	125
CA4	(KKK) ₂ *KLK _{BZ} Y	62.5	8	32	1	125
CA5	(KKK) ₂ *K _{BZ} K _{BZ} KY	62.5	16	32	1	250
CA6	(K _{BZ} KK) ₂ *KLK _{BBA} Y	62.5	8	62.5	8	125
CA7	(KK _{BZ} K) ₂ *KLK _{BBA} Y	32	32	62.5	4	125

^a*= Lysine branching unit.

growth against *C. albicans* and were nontoxic to mammalian cells at 10 times the MIC.

We initiated our investigations by screening BPs against a panel of microbes including Gram-positive bacteria (*S. aureus*), Gram-negative bacteria (*E. coli*), mycobacteria (*Mycobacterium smegmatis*), yeast (*C. albicans*), and fungi (*Aspergillus niger*) (Table 1). Some of the BPs demonstrated potent growth inhibition as low as 1 $\mu\text{g/mL}$. While *A. niger* and *M. smegmatis* appeared least sensitive to BPs, *S. aureus* was inhibited by acridine and benzoylboronic acid functionalized BPs A5 and A6 with MIC of 1 $\mu\text{g/mL}$. Against a recent clinical isolate of MRSA (strain 43300), A5 and A6 resulted in MICs of 62.5 and 2 $\mu\text{g/mL}$, respectively. These results demonstrate the ability of A6 to efficiently inhibit growth of either antibiotic-resistant or -sensitive strains of *S. aureus*. While there has been reported resistance in various strains of MRSA against acriflavine and proflavine, there appears to be no reported clinical resistance shown against aminacrine derivatives,²⁵ making acridinyl lysine a potentially useful pharmacophore against resistant strains of *S. aureus*.

Isomeric BPs A6 and A7, which contain similar amino acid composition but with residue K_{ACR} placed at different positions, inhibited *E. coli* with MICs of 2–4 $\mu\text{g/mL}$. In contrast, BPs A1–A5, A7, and A10–A12 inhibited *C. albicans* with MICs of 1–4 $\mu\text{g/mL}$, suggesting that the BP scaffold might be generally toxic to this yeast strain. In general, isomeric sequences (A2/A11, A3/A4, A8/A14, and A10/12) displayed similar MICs against the panel of microbes tested with the exception of A6 and A7. Along with A7, both A3 and A4 also severely inhibited the growth of *C. albicans* with MICs of 1–2 $\mu\text{g/mL}$. We were particularly excited to discover BPs capable of inhibiting the growth of *C. albicans*. Rise in fungal infections specifically involving *C. albicans* are

associated with those infected with HIV/AIDS or cancer, where depression of immune functions has led to decreased efficacy of therapies designed to treat both mucosal and systematic fungal infections.²⁶ In fact, species of *Candida* are involved in the majority of nosocomial fungal infections.²⁷ Unfortunately, resistance to all the common classes of antifungal agents used to treat *C. albicans*, including polyene, flucytosine, azole, and echinocandin derivatives, has emerged.^{26,28} This resistance in conjunction with the few antifungal drugs available for treatment of fungal infections, in general, underscores the importance of novel drug discovery toward targeting *Candida*.

As the presence of acridine in K_{ACR} of the BPs correlated with strong antimicrobial activity, control (CA) peptides of the five sequences containing acridine were synthesized, and their MICs were measured against the same panel of microbes (Table 1). Benzoyl lysine (K_{BZ}) was chosen as a substitute for acridinyl lysine in order to maintain similar structural properties of the amino acid, including length of the alkyl chain and aromatic functional group but with the removal of the intercalating abilities of acridine. As shown in Table 1, substitution of K_{BZ} for K_{ACR} in sequences A5 and A6 resulted in a 63-fold reduction in *S. aureus* activity (i.e., CA5 and CA6), and this substitution in A7 also resulted in a decrease in *E. coli* inhibitory activity (CA7). Overall, removal of the acridine moiety markedly reduced inhibition of *S. aureus* activity for all BPs tested. These results are not surprising as aminoacridine derivatives have been demonstrated to bind to bacterial nucleic acids and exert their effects through intercalation or stabilization of topoisomerase II-DNA cleavage complexes.^{29,30} It also appears that the moderate inhibition of *A. niger* is related to the presence of acridine moiety, as its removal led to loss of *A. niger* activity. Most significantly, removal of acridine in the BPs did not affect the strong antifungal

activity against *C. albicans*. Further, lytic activity of A3, A4, A7, and their corresponding control sequences had no effect on membrane integrity at MIC (Table 2). These results indicate that

Table 2. Percent Lysis Measurements of BPs against Yeast^a

compound	<i>S. cerevisiae</i>	<i>C. albicans</i>
A3	0.95 ± 0.05	1.0 ± 0.2
CA3	0.7 ± 0.1	6.0 ± 0.05
A4	1.4 ± 0.2	1.0 ± 0.1
CA4	1.0 ± 0.2	<1
A7	1.8 ± 0.2	4.0 ± 1.0
CA7	0.4 ± 0.05	18 ± 3
lyticase	100	100

^aPercent release of maltase after 60 min of exposure of compounds at their MICs. Lyticase served as the positive control. Average of two replicates.

the mechanism of action of these compounds against yeast most likely do not involve intercalation of acridines into nucleic acids or membrane disruption.

Candida spp. forms biofilms, and infections are often associated with *Candida* biofilms in patient tissue and medical devices.³¹ Substantial resistance to antimicrobial therapy of biofilms stems from the inability of drugs to penetrate beyond the exopolysaccharide matrix as well as the slower growth of mature biofilms, heterogeneity within the biofilm, development of resistant phenotypes, and quorum sensing of colonies within the biofilm.^{31–33} Therefore, we measured the effect of select BPs (A3, A4, and A7) for their ability to inhibit *Candida* growth in suspension and in biofilms (Table 3). Gratifyingly, both A4 and

Table 3. MICs (μg/mL) against *C. albicans*

compd	suspension	biofilm formation
A3	1	62.5
A4	1	1
A7	2	1
amphotericin B	0.25	0.25
miconazole	1.0	4

A7 inhibited biofilm formation with MICs comparable to those measured in suspension as well as control amphotericin B. Further, both peptides were more effective than miconazole in inhibiting biofilm formation (Table 3). The hemolytic activity of the BPs was then measured to assess the safety relative to their efficacies as antimicrobial agents (Table 4). All BPs revealed

Table 4. Hemolytic Activity of BPs^a

compd	EC _{10%} hemolysis (μg/mL)	safety factor (EC _{10%} /MIC)
A3	175 ± 5	>100
A4	>250	>100
A7	>250	>100
Triton X-100	10–12	N/A

^aAverage of two replicates.

negligible hemolytic effects on red blood cells. While A3 has an EC_{10%} value of 175 μg/mL, this EC_{10%} value is still well over the MIC of A3 against *C. albicans* (1 μg/mL). Indeed, all BPs showed a safety factor of over 100-fold. Additionally, we determined the toxicity of BPs A3–A7 in a U87 mammalian cell line.²² At concentrations 10-fold over MIC, none of the BPs were toxic in

an MTT assay over 24 h (Figure 1). Collectively, these results demonstrate the selectivity of BPs toward microbes over mammalian cells.

Figure 1. MTT assay with U87 CXCR4 cell line at >10-fold MIC of BPs (10 μM) over 24 h.

In summary, a series of BPs screened against a panel of microbials revealed compounds that inhibited the microbial growth of *S. aureus*, *E. coli*, and *C. albicans* with MICs as low as 1–2 μg/mL. The acridinyl lysine moiety in the BPs was responsible for the potent inhibitory effects observed against the bacteria *S. aureus* and *E. coli*. These BPs also inhibited the growth and formation of biofilms in *C. albicans* at MICs similar to or lower than currently employed antifungal drugs. Current efforts in our laboratories are targeted toward BPs as a general platform for antimicrobial development as well as the elucidation of mechanism of action.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmmedchemlett.7b00119.

Detailed experimental procedures and characterization of branched peptides (PDF)

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The authors declare no competing financial interest.

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