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De novo designed synthetic mimics of antimicrobial peptides

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Antimicrobial peptides are small cationic amphiphiles that play an important role in the innate immune system. Given their broad specificity, they appear to be ideal therapeutic agents. As a result, over the last decade, there has been considerable interest in developing them as intravenously administered antibiotics. However, it has proven difficult to accomplish this goal with peptide-based structures. Although it has been possible to solve some relatively simple problems such as susceptibility to proteolysis, more severe problems have included the expense of the materials, toxicity, limited efficacy, and limited tissue distribution. In an effort to overcome these problems, we developed small synthetic oligomers designed to adopt amphiphilic conformations and exhibit potent antimicrobial activity while being nontoxic to host cells. One class of these synthetic mimics of antimicrobial peptides (SMAMPs) is being developed as intravenous antibiotics.

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Introduction

Antimicrobial peptides

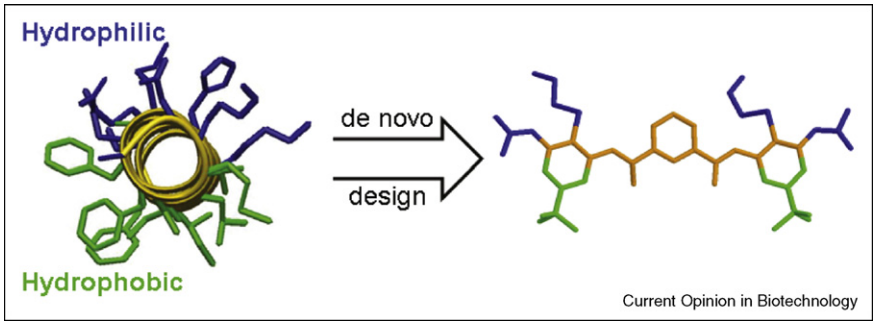
Antimicrobial peptides (AMPs), isolated from organisms across the phylogenetic spectrum, are an important part of the innate immune system [1]. They are typically small (12–80 amino acids) cationic amphiphiles that provide protection against a wide variety of pathogenic organisms, are the first line of defense against microbial infection in many species and are particularly important for infection control in insects and plants, which lack an adaptive immune response based on T-cells and antibodies. Many excellent reviews are available [1–4]. In mammals, the peptides are produced and secreted in skin, mucosal surfaces, and neutrophils, and act locally in response to infection. There are two types of AMPs comprising ribosomally and nonribosomally synthesized peptides. Over 700 AMPs have been identified and are generally α-helical (magainin and cecropin) or disulfide-rich β-sheets (bactenecin and defensin). Despite the large diversity observed in AMPs, they generally adopt highly amphiphilic topologies in which the hydrophilic and hydrophobic sidechains segregate into distinctly opposing regions or faces of the molecule — referred to as facially amphiphilic. Figure 1 highlights this facially amphiphilic structure for Magainin 2. Numerous studies with linear and cyclic peptides have strongly supported the hypothesis that their physicochemical properties, rather than any precise sequence, are responsible for their activities. It is generally believed that this amphiphilic topology is essential for insertion into and disruption of the cytoplasmic membrane leading to bacteria death [2].

Several models, such as the *toroidal pore*, *carpet*-like, and *barrel stave*, have been proposed to explain the complex structures formed between lipids and various AMPs during their interactions with membrane lipids (see references for details of these mechanisms) [1,5–7]. None of the proposed mechanisms are receptor based, consistent with the findings that all D-peptides are generally as active as all L-peptides [8,9]. In addition, certain AMPs have been proposed to indirectly modulate antimicrobial activity by interacting with other components of the innate immune system (reviewed in [10,11]). It is unclear which of these events are primarily responsible for bacterial killing and multiple target models have been proposed with the exact mechanism being dependent on the peptide, concentration, and bacterium in question [12–14]. Bacteria have also been shown to respond to AMPs [15,16] and even evolve novel resistance to their toxic affects [17,18]. Nevertheless, AMPs have remained an effective weapon against bacterial infection over evolutionary time indicating that their mechanism of action thwarts bacterial responses, which lead to overall resistance.

Mimetics of the AMPs

Given their broad specificity, amphiphilic AMPs appear to be ideal therapeutic agents. Over the last decade, there has been considerable interest in developing AMPs as intravenously administered antibiotics. However, despite extensive efforts in the pharmaceutical and biotechnology industry, it has proven difficult to accomplish this goal [2]. As a first step in this direction, various workers designed AMPs by idealizing the amphiphilic α-helical

Figure 1



(left) Magainin 2 shown with cationic groups in blue and nonpolar groups in green. The peptide backbone is shown as a yellow ribbon. (left) *De novo* designed SMAMP which captures the facially amphiphilic architecture and critical physicochemical properties needed to establish robust antimicrobial activity.

arrangement of sidechains [19–21] observed in the natural structures, leading to a large number of potent and selective antimicrobial compounds [3]. Also, cyclic peptides [22], turn-forming, and hairpin-forming peptides [19–21,23,24] have been extensively studied, but are beyond the scope of this review (see [25] for a recent review).

Peptidomimetic approaches that mimic the peptide primary structure have been pursued using amide bond isosteres or modifications of the peptide backbone by chain extension or heteroatom incorporation [26]. Several groups designed β-peptides (peptides built from β-amino acids with an additional methylene group in their backbone relative to α-amino acids), which maintained this amphiphilic architecture [27–29]. A systematic analysis of β-peptide structure and activity revealed that facial amphiphilicity as well as a precise ratio of charged to hydrophobic residues was essential for potent and selective activity, similar to what has been described for the antimicrobial α-peptides [30,31]. Both diastereomeric (D-amino acid substitutions) peptide sequences and α/β-peptides that did not adopt globally amphiphilic helices showed antimicrobial activity suggesting a well-defined amphiphilic secondary structure was not essential [29,32]. Although these peptides may be unable to adopt regular α-helix structures, their potent antibacterial activity may well require the presence of highly flexible backbones that permit the formation of irregular but globally amphiphilic conformations upon interaction with membranes. Helical, cationic, facially amphiphilic peptoid mimics (poly-*N*-substituted glycine) of magainin-2 have also been produced that show selective and potent antibacterial activity against Gram-positive and Gram-negative bacteria [33]. Significantly, the β-peptides and peptoids are resistant to proteases, providing important advantages over α-peptides. However, β-peptides and peptoids are relatively difficult and expensive to synthesize in large quantities.

Shai and coworkers have explored the use of short basic peptides with an N-terminal fatty acyl chain as potential antimicrobials [34,35] (for a review see [35]). Linear oligomers containing alternating acyl chains and cationic amino acid (lysine) residues have been shown to be active against Gram-negative bacteria. The addition of an acyl group to the N-terminus enhanced potency, leading to a molecule active *in vivo* in a mouse peritonitis model [36,37]. Recently, this group described a series of palmitoylated dipeptides, tripeptides, and tetrapeptides [38,39] with varying specificities for a variety of bacteria and fungi. These lipopeptides assemble into nanostructures that might be related to their biological activities.

Nonpeptidic compounds that are facially amphiphilic have also been produced from a steroid scaffold [40,41]. These compounds, termed ceragenins, have antibacterial activities similar to AMPs, but unsatisfactory safety profiles have limited their use to topical and medical devise applications [42]. Polymeric molecules have attracted significant attention recently as progress toward biomimetic macromolecules continues. Several polymers have been synthesized that likely adopt amphiphilic structures in the membrane environment and further confirm that well-defined amphiphilic secondary structures are not essential. These include phenylene ethynylene [43–45], short polymethacrylate [46,47], β-lactam [48], and polynorbornene backbones [49–52] that are active against Gram-positive and Gram-negative bacteria and have lower hemolytic activities against human erythrocytes. Such compounds are not preferred for systemic therapeutic use but their ease and economical cost of synthesis make them highly suitable for many material applications to create antimicrobial surfaces and products [53,54].

Design of arylamide and heteroarylamide antimicrobial peptide mimetics

The goal of our synthetic approach was to capture the structural and biological properties of AMPs within the framework of inexpensive oligomers [55]. Although it has been possible to solve some relatively simple problems

such as susceptibility to proteolysis, more severe problems have included the expense of the materials, toxicity, limited efficacy, and limited tissue distribution. We reasoned that small synthetic oligomers that adopt amphiphilic secondary structures while exhibiting potent and selective antimicrobial activity would be less expensive to produce, they might have better tissue distribution, and that it would be much easier to fine-tune their structures and activities to minimize toxicity. Therefore, an active program to *de novo* design synthetic mimics of antimicrobial peptides (SMAMPs) from inexpensive synthetic oligomers was undertaken (see Fig. 1). This effort has led to the identification of a class of SMAMPs that possess efficacy, safety, and pharmaceutical qualities suitable for development as intravenous antibiotics.

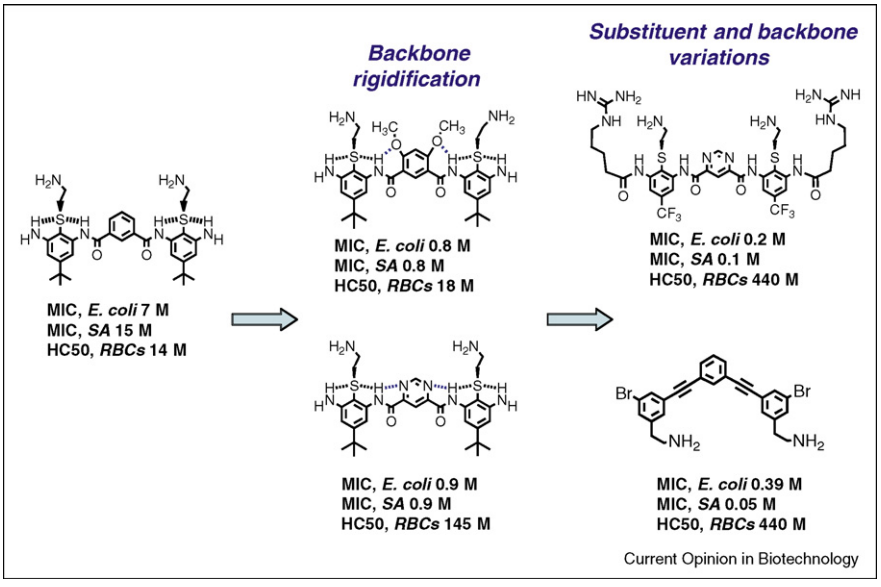
In general, *de novo* design of oligomers was accomplished in several steps. We first defined a three-dimensional framework, or backbone, using molecular dynamics and quantum force field calculations to assure it was able to assume a semi-rigid conformation [55–60]. Next, side groups were added computationally to maximize diversity and maintain drug-like properties. The best combinations of functional groups were then computationally selected to produce a cationic, amphiphilic structure [59]. Representative compounds were synthesized from this selected library to evaluate their activities. In our initial studies, we prepared compounds related to the core structure, which contained alternating 1,3-phenylene diamine units connected by a isophthalic acid [55].

Once an active series had been established, the activity was optimized by increasing the rigidity of the backbone through hydrogen-bonding and/or by introducing new substituents as shown with the arylamides in Figure 2. Hydrogen bonding in the backbone does not appear to be essential as the phenylene ethynylene was highly active [61–63].

Mechanism of membrane activity

Understanding the interactions between SMAMPs and lipid bilayers is a complicated, multicomponent problem yet scientifically important. Beyond understanding the structure–function correlation of SMAMPs, learning how these unique molecules interact with lipid membranes will contribute to our fundamental understanding of molecular-membrane self-assemblies which are critically important for numerous biological processes. Bacterial membranes and mammalian cell membranes are very distinct with respect to their phospholipid compositions [53]. It was shown that the exact lipid type influenced the leakage of calcein-loaded vesicles and that specifically the presence of 'bacterial' type lipids was essential. For example, the phenylene ethynylene SMAMP (Figure 2) exhibited ~75% dye leakage from PE/PG vesicles versus only 10–15% leakage against PC/PS and PC/PG vesicles, despite the presence of the same negatively charged lipid mol% [64]. Very similar results were obtained with SMAMPS in the arylamide series where leakage activity was again dependent on the volume fraction of negative intrinsic curvature PE. This dependence on negative intrinsic curvature lipids is consistent with the observed biological activity of this SMAMP because bacteria contain much higher volume fractions of negative intrinsic curvature lipid than mammalian cells. Synchrotron small-angle X-ray scattering (SAXS) studies further confirmed these findings and showed how this SMAMP interacts with membranes of four different

Figure 2



Representative SMAMPs highlighting design principles leading to potent *in vivo* activity.

lipid compositions [63]. This SMAMP induced an inverted hexagonal phase (H_{II}) in PE/PG vesicles, but only when the PE content was above a minimum threshold concentration of 64%. It was concluded that the ability of the SMAMP to restructure membranes critically depends on the concentration of negative intrinsic curvature PE present in the membrane. These results strongly support the hypothesis that exact lipid type may be essential for the SMAMPs ability to distinguish microorganisms from mammalian cells.

Antimicrobial activities of lead oligomers

Structure–activity relationships in the arylamide series identified lead compounds that were optimized for Gram-positive activity. Table 1 illustrates results from a susceptibility screen with two of these lead compounds, PMX30016 and PMX10129, against Gram-positive and Gram-negative human clinical isolates. Both compounds exhibited broad coverage against the Gram-positive pathogens with *S. aureus* and coagulase-negative Staphyloccal species showing the lowest MICs (0.5 μg/ml). *S. pneumoniae* and *S. viridians* were the least susceptible with MICs of 8 and 8–16 μg/ml, respectively. For the Gram-negative pathogens, PMX30016 had more extensive coverage than PMX10129 but overall coverage was less than for the Gram-positive organisms.

These SMAMPs are very stable in plasma and MICs are not affected by the addition of pooled human plasma at 40–60% concentrations. Full antimicrobial activity is retained under physiological salt concentrations. The arylamides are bactericidal and reductions in viable bacteria of greater than 3 logs (2–4× MIC concentrations) occur in 30 min to 6 hours upon exposure, depending on the compound and organism. The time-kill kinetics for the oligomers are concentration-dependent and appear to be somewhat slower than those for many of the AMPs which are reported to be ≤30 min. The reasons for this are unclear but may be related to the smaller size of the SMAMPs (MWs = 520–960 Da) since polymeric mimetics with MWs > 2000 Da show time-kill kinetics of less than 30 min, despite having higher MICs (6–25 μg/ml).

Cytotoxicity of PMX30016, PMX10129, and PMX30006, a progenitor of PMX30016, was evaluated using mammalian cell lines (Table 2). Although antimicrobial activities between the three SMAMPs are similar, PMX30016 and PMX10129 are significantly less cytotoxic versus

Table 1

Clinical isolate screen versus Gram-positive and Gram-negative pathogens.

	MIC range (μg/ml) 2 isolates/organism				
	PMX30016	PMX10129	Linezolid	Vancomycin	Ceftazidime
Gram-positive organisms					
Entero. faecalis	2	2–8	1–2	1	>64
Entero. faecium (VRE)	1	1	1–2	>128	>64
Staph. aureus (MRSA)	0.5	1	1–2	0.5–1	32
Staph. epidermidis	0.5	0.5	0.5–1	2	16–32
Staph. saprophyticus	0.5	0.5	1–2	1–2	32–>64
Staph. spp. (coagulase)	0.5	0.5–2	1	1–2	16–32
Strept. agalactiae	2–4	1–4	1	0.5	0.5
Strept. pneumoniae	8	8	1	0.5	0.25
Strept. pyogenes	1–2	1–2	1	0.5	0.12
Strept. viridians	8–16	16	1	0.5–1	0.5–4
Gram-negative organisms					
Citrobacter freundi	4	16	>16	>128	0.25–2
Citrobacter koseri	2–4	8	>16	>128	0.12–0.25
Enterobacter cloacae	2	8–16	>16	>128	0.25
Escherichia coli	2	4–8	>16	>128	0.06
Klebsiella oxytoca	2–4	16	>16	>128	0.06–0.12
Klebsiella pneumoniae	2–4	16	>16	>128	0.06–0.12
Morganella morganii	>64	>64	>16	>128	2–16
Proteus mirabilis	64	>64	>16	>128	0.03–0.06
Proteus vulgaris	8–64	>64	>16	>128	0.12
Providencia stuartii	4–16	32–>64	>16	>128	0.12–64
Acinetobacter spp.	2–16	8–16	>16	128–>128	2–64
Pseudomonas aeruginosa	8	16	>16	>128	1–8
Serratia marcescens	16–32	32–>64	>16	>128	0.12–0.25
Stenotroph. maltophilia	4–64	16–>64	>16	32–128	4–8
Haemophilus influenzae	8	>64	16–>16	128	0.06–0.12

The *in vitro* susceptibility assays were performed in accordance with defined CLSI guidelines for the specific organisms tested. PMX30016 and PMX10129 are members of the arylamide series.

Table 2

Selectivity indices between MRSA and mammalian cells.

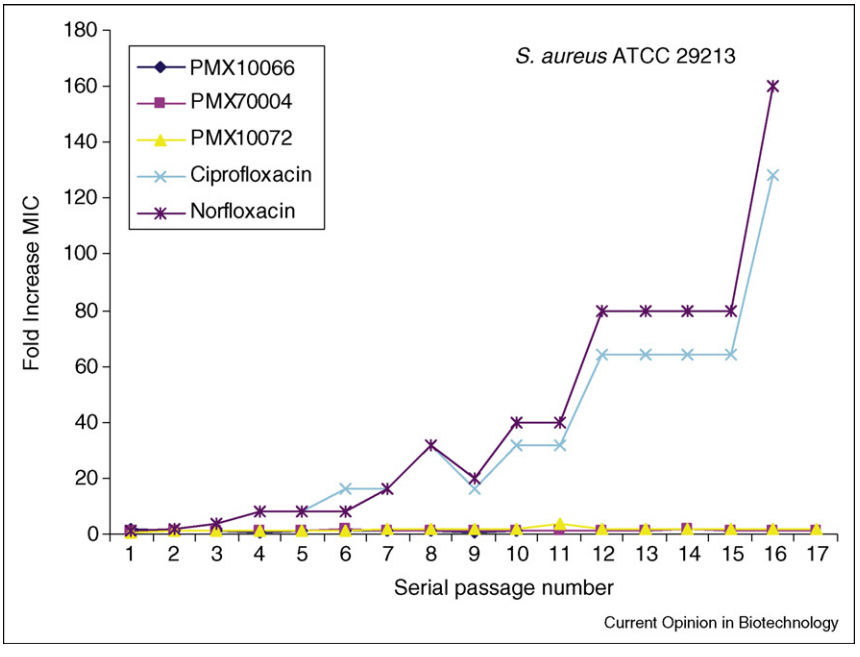
PMX compound	MIC (μg/ml)	Cytotoxicity (EC_{50}, μg/ml)		Selectivity (EC_{50}/MIC)	
	MRSA	3T3	HepG2	3T3	HepG2
30006	0.5	21	28	42	57
30016	0.5	113	341	226	682
10129	1.0	>2000	>2000	>2000	>2000
Melittin	2	4	1	2	0.5

Cytotoxicity was evaluated in a colorimetric assay using a transformed human liver cell line (HepG2 cells) and an embryonic mouse cell line (NIH/3T3 cells). This assay measures the bioreduction of a novel tetrazolium compound to a soluble formazan product by viable cells. Cells were incubated for one hour in the presence of compound in serum-free medium before viability determinations. Melittin is a nonselective antimicrobial peptide that has little selectivity for bacteria. PMX30006, PMX30016, and PMX10129 are members of the arylamide series.

mammalian cells and have superior selectivity indices (ratios of EC_{50} values for cytotoxicity to MIC for MRSA). Factors that have been associated with enhanced selectivity for bacterial cells over mammalian cells are the number of positive charges, identity of the positively charged endgroup, rigidity across the backbone, hydrophobicity, and stiffness of the sidechains.

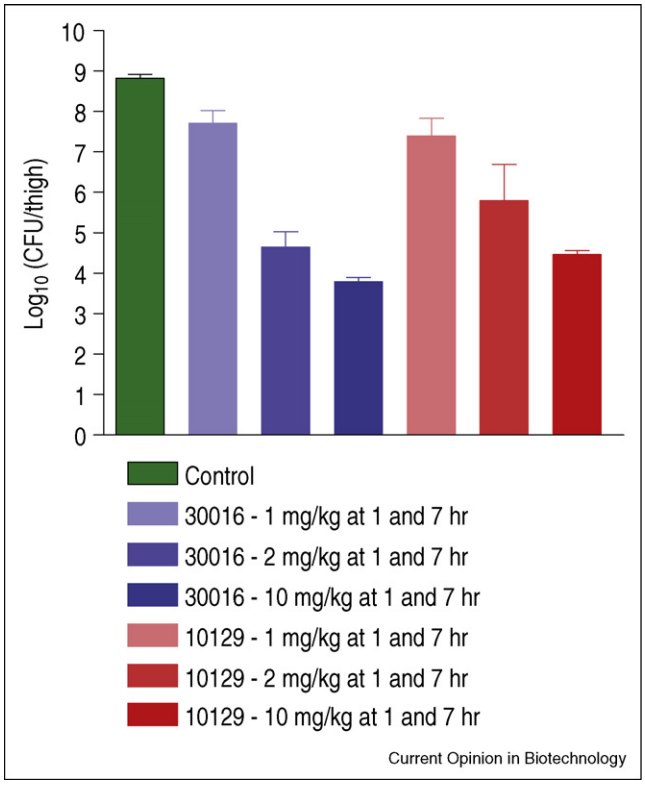
To investigate the potential for bacteria to develop resistance against the antimicrobial oligomers, serial passage experiments in the presence of sublethal SMAMP concentrations were performed with *S. aureus*, MRSA, and *P. aeruginosa*. Figure 3 shows results for SMAMPs PMX10066, PMX10072, and PMX70004 up to 17 passages with *S. aureus* ATCC 29213. MIC values were determined at each passage and the culture at 0.5× the MIC was selected for further passage. As a control, parallel cultures were also exposed to 0.5× MIC concentrations of ciprofloxacin or norfloxacin, two broad spectrum fluoroquinolones for which resistance has been reported in this experimental format [65]. There is no change in the MICs for PMX70004, PMX10072, or PMX10066 over the entire 17-passage time course. Conversely, an increase in the MIC is readily observed by passage seven for both ciprofloxacin and norfloxacin. These data are very similar to results from resistance

Figure 3



The development of bacterial resistance with three different classes of SMAMPs and two fluoroquinolones against *S. aureus* ATCC 29213. Serial passage and MICs were performed in microtiter panels containing antimicrobials, each over a range of doubling dilution concentrations. After the 20-hour incubation period, the entire content of the well with the highest concentration of compound permitting visible growth was taken, diluted to the correct inoculum and reinoculated in a fresh panel with compound dilutions. PMX 10066 and 10072 are members of the arylamide series while PMX70004 is a member of the phenylene ethynylene series.

Figure 4



In vivo efficacy in the mouse thigh burden model. Neutropenic mice were inoculated in their thigh muscles with *S. aureus* ATCC 13709 and at one and seven hours after infection the mice were administered compound by IV injection (n = 4/group). At 25 hours after infection, the thigh muscles were harvested and viable bacteria quantitated. Control mice were untreated and received bacterial inoculum only.

studies with the AMPs and together demonstrate the difficulties bacteria have in building resistance to this mechanism of cell killing.

Animal efficacy studies

PMX30016 and PMX10129 have been evaluated for *in vivo* efficacy versus *S. aureus* in a mouse thigh burden model (SW Choi, WF DeGrado, Design of potent non-toxic nonpeptide-based antimicrobial agents. *Proc Natl Acad Sci U S A*, unpublished data). Robust efficacy is observed with both PMX30016 and PMX10129. Efficacies for PMX30016 exceed 5 $\log_{10}$ and 4 $\log_{10}$ reductions in viable bacterial cell counts (colony forming units, cfu) with total doses of 10 mg/kg (5 mg/kg/dose) and 4 mg/kg (2 mg/kg/dose), respectively (see Figure 4). PMX10129 is slightly less efficacious in this dosing format. Comparable efficacy was achieved with PMX30016 against methicillin-resistant *S. aureus* (MRSA ATCC 33591) in the mouse thigh burden model done under similar conditions (not shown). The activity of the lead SMAMPs in the mouse thigh burden when administered by IV injection distinguishes their efficacies from many of the AMPs that lack efficacy when administered parentally in different compartments from the infection [32,36,66]. This robust efficacy, likely due to their smaller size and abiotic structures which enhance bioavailability and stability *in vivo*, illustrates the power of this SMAMP approach.

Table 3

Maximum tolerated doses in mice and rats.

Dosage	MTD (mg/kg)	
	Mouse	Rat
PMX30006		
IV bolus	25	20
PMX30016		
IV bolus	20	15
IV infusion — one hour	N.D.	20–30
PMX10129		
IV bolus	15	10
IV infusion — one hour	N.D.	20–30
IV infusion — four hours	N.D.	45

Mice or rats where administered a single dose of compound and clinical signs were recorded over a four-day to seven-day period following compound administration. Gross necropsy was also performed at the conclusion of the study. Reported values represent the highest dose where no adverse signs were recorded.

Animal safety studies

Single dose toxicity studies were conducted to define the maximum tolerated dose (MTD) where no adverse signs are evident over a four-day to seven-day period following SMAMP administration (Table 3). Compounds were administered either by IV bolus injection in the tail vein of mice and rats or by IV infusion via femoral vein catheter in rats. For PMX10129, the MTD in rats is 10 mg/kg by IV bolus injection, 20–30 mg/kg by one-hour IV infusion and 45 mg/kg by four-hour IV infusion. For PMX30016, the MTD is 15 mg/kg by IV bolus injection and 20–30 mg/kg by one-hour IV infusion. The sequentially higher MTDs observed for these SMAMPs when administered by IV bolus or IV infusions indicate that the MTD-related adverse events are primarily dependent on maximum concentrations attained in the blood (C_{max}) rather than the total dose of drug (AUC).

Conclusions

A number of peptidomimetic approaches have identified potent and selective compounds that demonstrate broad antimicrobial activities *in vitro* and low cytotoxic activities against mammalian cells and erythrocytes. However, robust *in vivo* antimicrobial activity has been a greater challenge due likely, at least in part, to poor bioavailability and systemic toxicity. This has limited their therapeutic uses to indications where they can be administered locally. PacGen Pharmaceuticals is currently in Phase II testing with a histatin peptidomimetic for oral candidiasis using a mouthrinse formulation. Ceragenix is developing

the ceragenins to reduce nasal colonization of *S. aureus* and lower bacterial contamination of catheter surfaces. Improved bioavailability for two peptidiomimetics has been reported. Optimized acyl-lysine oligomers show good blood levels and efficacy in a mouse peritonitis model when administered intraperitoneally [37] and cyclic D,L-peptides are active in mouse peritonitis and thigh burden infection models when administered intravenously, although improved therapeutic indices appear necessary for further development [22].

Using *de novo* design principles, it has been possible to develop SMAMPs with potent *in vivo* activity via IV administration. The synthetic flexibility of the small oligomeric scaffolds allowed rapid structure optimization and elimination of unwanted side reactions. In a short time, leads were taken from the design laboratory to the clinic. At this early stage, it may be too soon to predict the potential of this approach but the success to date would suggest many important opportunities lie ahead for the design of synthetic oligomers with protein-like activity.

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