Current Topics in Medicinal Chemistry, 2017, *17*, 576-589

REVIEW ARTICLE

Mimics of Host Defense Proteins; Strategies for Translation to Therapeutic Applications

Richard W. Scott[*,1] and Gregory N. Tew[2]

[1]*Fox Chase Chemical Diversity Center, Pennsylvania Biotechnology Center, Doylestown, PA, USA;* [2]*Polymer Science and Engineering, Veterinary and Animal Science, Cell and Molecular Biology, University of Massachusetts, Amherst MA, USA*

Abstract: New infection treatments are urgently needed to combat the rising threat of multi-drug resistant bacteria. Despite early clinical set-backs attention has re-focused on host defense proteins (HDPs), as potential sources for new and effective antimicrobial treatments. HDPs appear to act at multiple targets and their repertoire includes disruptive membrane and intracellular activities against numerous types of pathogens as well as immune modulatory functions in the host. Importantly, these novel activities are associated with a low potential for emergence of resistance and little cross-resistance with other antimicrobial agents. Based on these properties, HDPs appear to be ideal candidates for new antibiotics; however, their development has been plagued by the many therapeutic limitations associated with natural peptidic agents. This review focuses on HDP mimetic approaches aimed to improve metabolic stability, pharmacokinetics, safety and manufacturing processes. Early efforts with β-peptide or peptoid analogs focused on recreating stable facially amphiphilic structures but demonstrated that antimicrobial activity was modulated by more, complex structural properties. Several approaches have used lipidation to increase the hydrophobicity and membrane activity. One lead compound, LTX-109, has entered clinical study as a topical agent to treat impetigo and nasal decolonization. In a more significant departure from the amino acid like peptidomimetics, considerable effort has been directed at developing amphiphilic compounds that recapitulate the structural and biological properties of HDPs on small abiotic scaffolds. The lead compound from this approach, brilacidin, has completed two phase 2 studies as an intravenous agent for skin infections.

ARTICLE HISTORY

Received: May 22, 2015
Revised: October 29, 2015
Accepted: November 30, 2015

DOI: 10.2174/1568026616666161607 13130452

Keywords: Antibiotic, Antimicrobial peptides, Antiseptic, Host defense proteins, *In vivo*, LPS, LTA, Membrane, Mimetics, Mimics, Pharmacokinetics, Resistance, Therapeutic, TNF.

1. INTRODUCTION

Infections caused by multi drug-resistant (MDR) bacteria are increasing at an alarming rate and have become a major medical threat in the US and worldwide. Even though most of these infections happen in the community, the majority of attributable deaths, coming from infections, happen in healthcare settings such as hospitals and nursing homes. A recent CDC report estimates that over 2 million illnesses per year in the US are caused by MDR bacteria and result in greater than 23,000 deaths [1]. The risk of death is 2-fold higher in patients infected with resistant strains [2] and associated annual costs of drug-resistant over drug-susceptible infections range from $20 to $35 billion [1, 3]. Further contributing to the global antibiotic resistance health crisis is the limited development of new antibiotics. Within known classes of antibiotics, the rate of important new compounds has declined; however, more problematic, is that few distinct classes of antibacterials with novel sites of action, have been developed since the 1980s [4]. Recent studies show that to reverse the trend of increasing resistance it is necessary not just to reduce antibiotic use but to employ antibiotics with fundamentally different modes of action [5].

To identify new agents that can impact this growing emergence of drug resistant bacteria, substantial attention has been re-directed at antimicrobial peptides (AMPs), also termed host defense proteins (HDPs) because of their roles in the innate immune system. AMPs have been isolated from organisms across the phylogenetic spectrum and are typically small (12-80 amino acids) cationic amphiphiles that provide protection against a wide variety of pathogenic organisms including bacteria, fungi and parasites [6, 7]. In mammals, the peptides are produced and secreted in skin, mucosal surfaces and neutrophils to act locally in response to infection. Currently the Antimicrobial Peptide Database con-

*Address correspondence to this author at Fox Chase Chemical Diversity Center, 3805 Old Easton Road, Doylestown, PA 18902, USA; Tel/Fax: 215-589-6416/215-589-6335; email: rscott@fc-cdci.com

1873-5294/17 $58.00+.00

tains 2,517 members that are predominantly either α-helical (cathelicidin LL-37, magainin) or disulfide-rich β-sheets (alpha- and beta-defensins).

Despite a large diversity in primary amino acid sequence, AMPs, typically adopt facially amphiphilic (FA) topologies [8] with hydrophilic and hydrophobic side chains segregating to opposing regions or faces of the molecule. 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 membrane leading to microbe death [9, 10]. Membrane association may also permit entry into the bacterial cell and disruption of essential metabolic processes necessary for viability [11, 12]. The underlying principal for activity is that cationic charge of the AMP provides an electrostatic interaction with the anionic surface of bacterial membranes and the hydrophobic surface mediates entry into the membrane [13, 14]. Although, the details of this process and the essential steps in the process remain to be understood in greater detail.

Another distinguishing feature is the relative enrichment of negative intrinsic curvature lipids such as phosphatidylethanolamine in Gram-negative and cardiolipin in Gram-positive bacteria, which help facilitate formation of transmembrane pores [10, 15-18]. Importantly, the novel mechanisms of action, including at the membrane, have been attributed to their low risk for resistance development and comparable activities against both drug-sensitive and drug-resistant strains. Additional activities, outside of their direct antimicrobial action, have also been described for AMPs including immune modulatory functions such as suppression of proinflammatory cytokines [19] and stimulation of B- and T- cell responses which serves to link the innate and adaptive immune systems [20, 21]. These multi-faceted roles evident in the AMP family are essential for maintenance of proper barrier function in the host and provide a coordinated and effective response to microbial infection. Their efficacy can be so potent that for some species, like moths, they are the only immune system [14].

Given their broad activity and low resistance potential, amphiphilic HDPs appear to be ideal therapeutic agents. However, significant pharmaceutical issues, including metabolic instability, poor tissue distribution, systemic toxicity, as well as difficulty and expense of manufacturing, have severely hampered clinical progress. Due to these limitations, therapeutic development of the peptides has been limited largely to topical or local administrations. In initial attempts to address these issues, several peptidomimetic approaches have been taken to improve safety by increasing selectivity for target pathogens over mammalian cells and minimize proteolytic susceptibility.

β-PEPTIDES

An early strategy to improve proteolytic stability was through incorporation of β-amino acids in which the amino group of alanine is attached to the β- rather than α-carbon (Fig. **1**) [22]. β-peptides have longer backbones than their α-amino acid counterparts but are able to attain stable, folded structures similar to secondary structures of proteins. A variety of β-helical structures can be formed in solution but the L+2 helix defined by a 14-membered hydrogen bonded (14-helix) ring is one notable stable helical structure, presenting a 3-residue repeat motif. Through incorporation of hydrophobic sidechains, modulation of chain length, and amino acid composition, amphiphilic β-peptides were designed that displayed negligible protease susceptibility and had antibacterial IC50s *vs. E. coli* of approximately 2 μg/ml but were only at most 2 fold selective in hemolytic assays [23] (Fig. **1**; 1 and 2). As found with many AMPs and AMP mimetics, achievement of selectivity is challenged by the direct correlation of hydrophobicity with both antimicrobial and hemolytic activities. However, proper tuning by adjusting the helical stability and content of hydrophobic subunits produced more selective agents [24-27]. Incorporation of cyclohexyl (3) or cyclopentyl (4) constraints in the β-peptide backbone stabilize 14- or 12-helix conformations, respectively, and minimal inhibitory concentrations (MICs) *vs.* Gram-positive bacteria ranged from 0.8 to 3.1 μg/ml while EC_{50}s for hemolytic activity were approximately 100 to 400 μM. Cyclization of 8 residue α/aza-β hybrid peptides has also produced analogs preferentially active against Gram-positive bacteria with low hemolytic activity and reduced susceptibility to proteolysis [28].

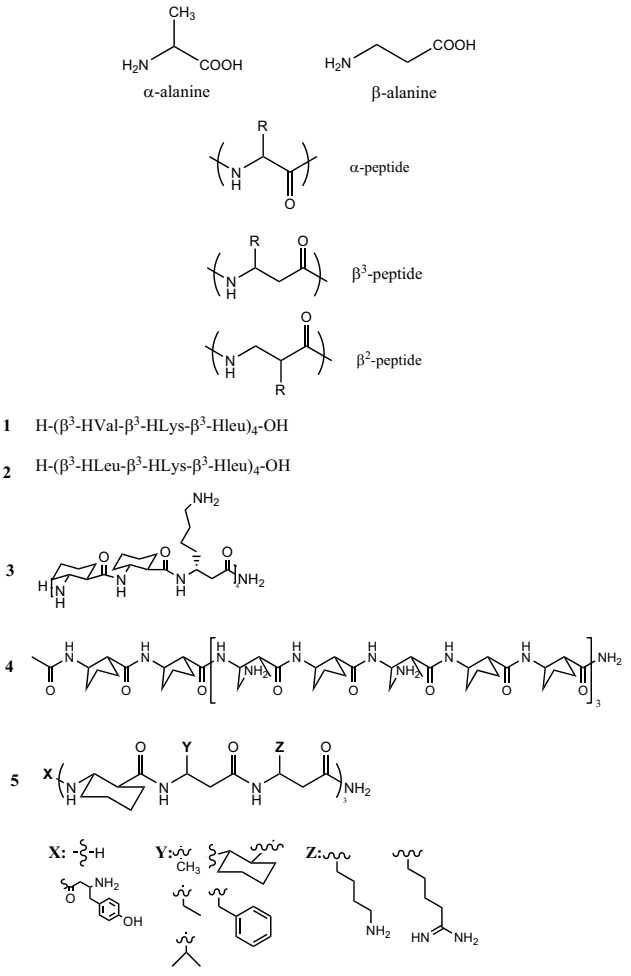


Fig. (1). Structures of β-peptides.

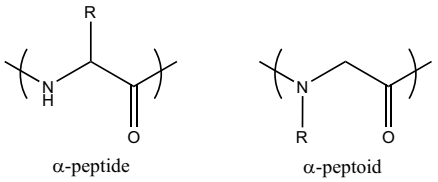
Global helical amphiphilicity was considered important for antimicrobial activity but results from hybrid peptides containing both α- and β-amino acids showed a scrambled α/β-peptide could be more potent and less hemolytic than helical hybrid peptides indicating that helical propensity was not a prerequisite for anti-bacterial activity [29, 30] and that structure-activity relationships (SARs) are more complex than the simple amphiphilicity argument. Antifungal activity has also been reported for β-peptides [31-33]. Several selective peptides with MICs of 8 μg/ml *vs*. *Candida albicans* were identified that exhibited < 2.5% hemolysis at their MIC [33]. In this series of β-peptides (5), helicity was linked to activity. Using synthetic strategies similar to those reported above, incorporation of cyclic ACHC (aminocyclohexane carboxylic acid) sidechains stabilized 14-helix conformations and the more stable β-peptides exhibited better anti-*Candida* activity than less stable peptides with similar hydrophobicity. Therefore, the role helicity plays on antimicrobial activity is likely dependent on the scaffold structure, their ability to attain an amphiphilic structure in a membrane environment, and possibly the target pathogen.

Further homolongation of the backbone such that three atoms separate the nitrogen from the carbonyl group produces γ-peptides that have well-defined backbone conformations capable of forming stable, proteolytically-resistant helical structures with 9-, 12- or 14-helix conformations [34]. N,N'-linked oligourea γ-peptide analogs have moderate antibacterial and low hemolytic activities [35].

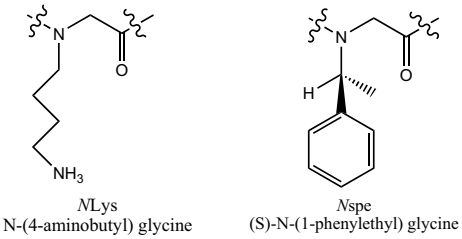
PEPTOIDS

Peptoids (oligo-N-substituted glycines) are mimics of α-peptides with their sidechains linked to the backbone amide nitrogen rather than to the α-carbon [36]. This change simplifies synthesis and renders the peptoid resistant to proteolytic cleavage [37, 38]. Although peptoids lack intra-chain H-bonding capabilities, the incorporation of bulky sidechains periodically along the backbone produces helical structures that when made FA display broad antimicrobial activities against Gram-positive and Gram-negative pathogens with low cytotoxicity in hemolytic assays [39-42]. A 12 subunit peptoid analog (**6**; Fig. **2**) with a predicted three-fold helical periodicity was found to have comparable antimicrobial activities to the natural 22 amino acid antimicrobial peptide pexiganen versus *Escherichia coli* and *Bacillus subtilis* (MICs = 3.1 and 1.6 μg/ml, respectively) and similar hemolytic activities resulting in selectivity indices of 20 to 24 [40]. Moderate anti-bacterial activities were observed with lysine:peptoid hybrids, such as LP5 (**7**), that contains 5 lysine residues linked to a peptoid core [43]. Mechanism of action studies indicated that LP5 acts both intracellularly by inhibiting DNA replication and, with higher concentrations, at the membrane.

Potent activity with shorter lysine:peptoid hybrids (**8**) has been reported with MICs versus Gram-positive and Gram-negative bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* and vancomycin-resistant *Enterococcus*, in the 2 to 6 μg/ml range with selectivity indices of 10 to >20 fold for 50% hemolysis concentrations (HC_{50}s)/MICs [44]. Bactericidal activity was correlated to membrane depolarization with *S. aureus* and



6 H-NLys-NSpe-NSpe-NLys-NSpe-L-Pro-(NLys-NSpe-NSpe)$_2$-NH$_2$



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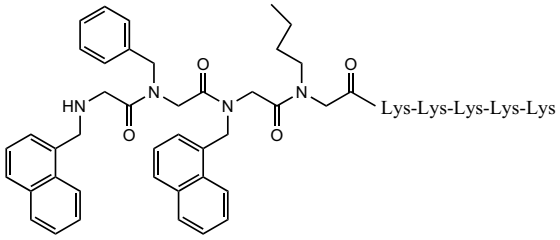

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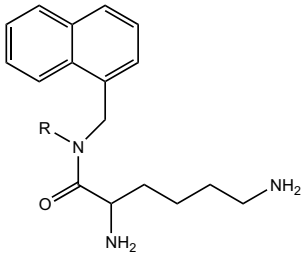


9 DOTA-(NLys-NSpe-NSpe)4-NH2

10 DOTA-(NLys-NSpe-NSpe)$_3$-NLys-NSpe-NH$_2$

Fig. (2). Structures of peptoids.

inner and outer membrane permeabilization with *S. aureus* and *E. coli*, respectively. A recent publication describes improved pharmacokinetic properties over peptides [45] using short peptoids (11–12mers) linked N-terminally to a ^{64}Cu-DOTA (Tri-*tert*-butyl 1,4,7,10, tetraazacyclododecane-1,4,7,10-tetraacetate). The peptoid conjugates (**9, 10**) showed higher tissue accumulation, slower elimination and higher *in vivo* stability relative to comparator peptides. Although not orally bioavailable, the peptoid conjugates also demonstrated greater stability in the gastrointestinal tract. Such improvements, including oral bioavailability, are critically important steps in expanding the utility of HDP mimics for clinical use.

Hybrid peptoid structures have been examined and show promising activities. Oligomers comprised of alternating cationic natural amino acids and hydrophobic N-substituted

glycines (α-peptoids) or N-substituted β -alanines (β-peptoids) are proteolytically stable and active against Gram-negative bacteria [46, 47] (11, 12; Fig. **3**). In a study employing lysine as the cationic moiety [46], α -peptoid hybrids were more active *vs*. *E. coli* than their homotypic α-peptoid counterparts and significantly more active and less hemolytic than the all L- or D- natural amino acid analogs.

Similar trends were also observed for β-peptoid hybrids. The most active analogs contained a hydrophobic α - or β-peptoid and cationic natural amino acid (**13, 14**). Since α - and β -peptoid oligomers possess greater conformational diversity due their achirality and lack of hydrogen bonding, structural flexibility in this scaffold design appears to be more important for favorable anti-bacterial and hemolytic properties than attainment of rigid and amphiphilic secondary structures. All of the hybrids, bearing either α - or β-peptoid subunits were poorly active against another *Enterobacteriacae* family member, *Klebsiella pneumoniae*, and Gram-positive *S. aureus.* In all examples, the cationic moiety was lysine and incorporation of homoarginine as the cationic residue would be expected to broaden the spectrum of antimicrobial activity but possibly at the expense of increased cytotoxicity [48].

In fact, similar scaffolds containing both lysine (Lys) and homoarginine residues (**15**) were more broadly active against Gram-negative bacteria including MDR strains of *E. coli*, *Acinetobacter baumannii*, and Gram-positive bacteria [47]. To improve cytotoxicity of the arginine (Arg)-bearing hybrids, effects of chain length and side chain α-chirality were investigated with both α - and β-peptoid hybrids. In agreement with previous studies [48, 49], cytotoxicity, as measured in metabolic assays using a diverse panel of mammalian cell lines and anti-bacterial activity, was associated directly with oligomer chain length [47]. As the chain length of α- or β-peptoids containing either achiral or α -chiral sidechains increased from 10 to 12 to 14 residues there was a progressive increase in cytotoxicity and anti-bacterial activity versus *A. baumannii* and to a lesser extent, *E. coli*, MRSA and vancomycin-resistant *Enterococcus faecium* (VRE). As expected, oligomers with achiral sidechains were less cytotoxic

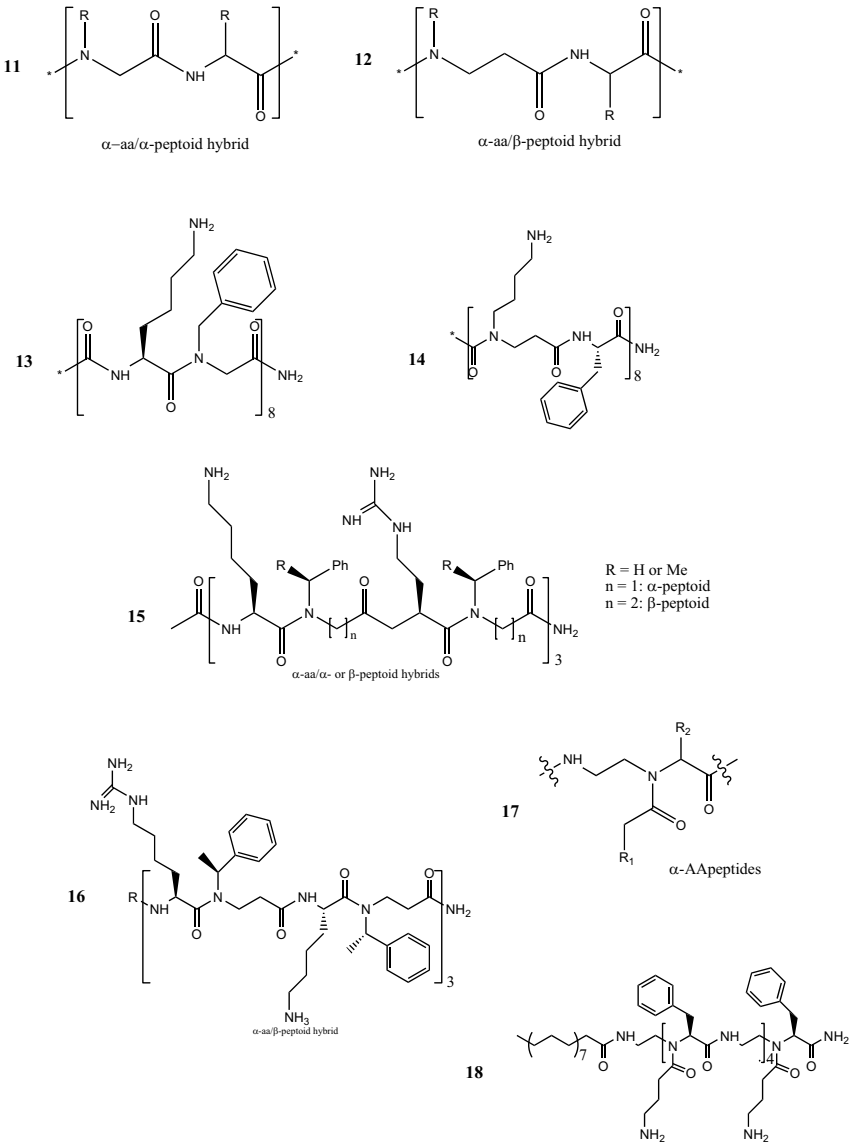


Fig. (3). Structures of α-peptide/peptoid hybrids.

than their α -chiral sidechain analogs indicating conformational rigidity hampers selectivity within this series.

However, contrary to results with all Lys-hybrids described above [46], sidechain chirality had little influence on activity against *E. coli* and oligomers with α-chiral sidechains were significantly more active against *A. baumannii*, MRSA and VRE. Taken together, results from this series of studies with α- and β-peptoid hybrids indicate that antibacterial activity is modulated by different structural properties depending on the targeted pathogen [46, 47]. The presence of guanidino sidechains drives activity against *E. coli* and minimizes influences of scaffold flexibility and chain-length seen with other less robust Lys-hybrids, whereas susceptibilities of MRSA, VRE and *A. baumannii* are dependent not only on the presence of guanidino sidechains but longer oligomer chain length and more rigid scaffolds as well. Improvement in Gram-positive activity was accomplished by modifications at the N-terminus [50]. Similar to results obtained with other AMP-scaffolds [51, 52], addition of hydrophobic end-groups onto a N-acetylated Lys, Arg β-peptoid hybrid oligomer (**16**) led to 4 - 16 fold improvements in MICs versus MRSA and VRE (MICs = 1 μg/ml) without affecting activity against *E. coli*. Although these modifications were associated with increased cytotoxicity, several analogs containing cyclohexylacetic acid or pentafluorophenylacetic acid showed 50 to 150 fold and 400 to 600 fold selectivities in cytotoxicity and hemolysis assays, respectively [50]. Therefore, by tuning various structural properties, including charge group, hydrophobicity, scaffold length, and scaffold rigidity, the design of potent, broadly active antimicrobial agents with low cytotoxicity is possible.

LIPO-PEPTIDOMIMETICS

A number of approaches have used lipidation to increase the hydrophobicity of cationic subunits and improve associations with microbial membranes [53, 54]. Precedence for this strategy centers on several lipidated peptides that are highly effective antibiotics. Daptomycin and colistin/Polymyxin B contain an acyl chain of 6 to 9 carbons in length that is essential for their antimicrobial activities. Alkylation of peptoid oligomers with acyl chain lengths of 10 or 13 carbons preserved antimicrobial activities and improved selectivity indices in hemolysis assays 2 to > 3-fold [55]. Lipidation of novel α -AA peptides (**17**) containing N-substituted-N-acylated amino acid residues of various length (3 to 5 subunits) with a C-16 hydrophobic tail produced a set of oligomers with MICs of 4 to 8 μg/ml *vs.* Gram-positive (*S. aureus, Staphylococcus epidermidis, E. faecalis*) and Gram-negative (E. coli, *K. pneumoniae, P. aeruginosa*) bacteria and low hemolytic activity [56, 57]. One of the more potent lipidated α-AA peptide oligomers (**18**) was membrane-active in propidium iodide uptake assays [57].

OAKS

An alternate lipo-peptidomimetic strategy employs acyl chains of varying lengths separating two cationic amino acids. Oligomers of acylated lysine (OAKs) possess both charged and hydrophobic domains but lack a defined secondary structure due to rotational freedom of the acyl carbon atoms [58-60]. This lack of dependence on a stabilized secondary structure distinguishes the OAKs from many AMPs and other peptidomimetics. There are several excellent reviews on the antimicrobial spectrum, mechanism of action and SARs of these acylated cationic oligomers [61, 62]. Typical designs include an N-terminal acyl chain (usually 12 carbon atoms in length) linked to repeating acyl-Lys (α: **19**; Fig. **4**) or Lys-acyl-Lys (β: **20**) subunits of varying carbon lengths. Early oligomers containing α or β acyl chains of 4 (C_4) or 8 (C_8) carbon atoms in length were found to be selectively active against Gram-negative bacteria whereas longer C_{12} subunits self-aggregated in solution and were poorly active and hemolytic [60]. Introduction of tandem lysine motifs between long (C_{12}) acyl chains prevented aggregation and enabled the production of short non-hemolytic oligomers that were selectively active against Gram-positive species [60, 63].

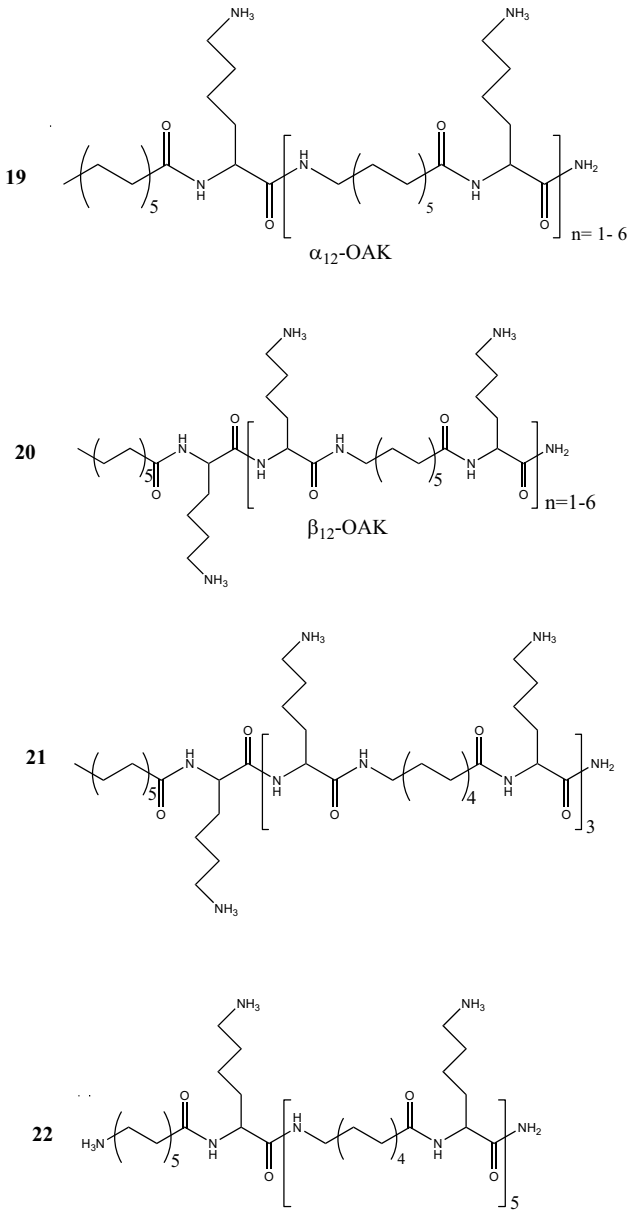


Fig. (4). Structures of acyl-lysyl oligomers.

OAKs containing C_{10} β-subunit acyl chains also possessed low hemolytic and aggregation properties; however, the preferred oligomer in this class (**21**) was potently bactericidal against both Gram-positive and –negative bacteria with MICs in the 1.6 to 6.2 μM range [64]. Furthermore, **21** was efficacious *in vivo* versus *S. aureus* with a single 2 mg/kg IV dose in a mouse tissue burden model and efficacy was comparable to vancomycin administered at 20 mg/kg. Mechanism of action studies indicate that plasma membrane interactions of this OAK may be more robust with Gram-negative bacteria but that membrane depolarization and ATP leakage is evident with both. Subsequently, a modified C_{10} hexamer (**22**) was shown to be broadly active against Gram-negative bacteria including *E. coli, P. aeruginosa, K. pneumoniae* and *Salmonella* spp. with an MIC_{90} of 6.2 μM [65]. Interestingly, in addition to overall charge and hydrophobicity, charge distribution along the oligomer backbone was found to be an important determinant for activity. Drug interaction studies also indicated strong synergy with erythromycin versus *E. coli*, attributed at least in part to loss of membrane potential induced by the OAK hexamer, which in turn causes loss of protein motive force and inactivation of drug efflux pumps [65]. Potentiation of antibiotic activity by membrane active agents including HDP mimetics may be a highly useful treatment strategy for future drug combination therapies to combat MDR microbial infections. Indeed, robust potentiation of antibiotic action versus *P. aeruginosa* has been observed with several membrane-active small mimics of HDPs (see below) at sub-MIC concentrations in checkerboard MIC and time-kill studies (unpublished results).

LTX-109

The concept of adding hydrophobic bulk to cationic subunits has also been applied to the outcome of a pharmacophore scan of lactoferrin to identify the minimal requirements of cationic peptides for antibacterial activity. The resulting compound, LTX-109, is a tripeptide (Arg-Tbt-Arg-NH-EtPh) containing a central modified tryptophan moiety linked to two arginine residues and a C-terminal ethylphenyl group [66] (**23**; Fig. **5**). Although small in size, the peptide has eight stereoisomers and the four least hydrophobic isomers, as measured by reverse phase HPLC retention times, displayed significantly less antibacterial and hemolytic activities [67]. Molecular dynamic simulations suggest the more active isomers have amphiphilic structures which are not evident with the less active isomers. LTX-109 displays a broad range of activity against a panel of drug-resistant strains of *S. aureus* with a very tight MIC range of 2 – 4 μg/ml [68]. Robust bactericidal activity *vs.* methicillin-resistant (MRSA), vancomycin-intermediate resistant (VISA) and vancomycin-resistant (VRSA) staphylococcal strains was observed in time-kill assays where > $3\log_{10}$ reductions in viable cfus were obtained within 0.5 hrs to 6 hours following exposure.

Anti-fungal activity has also been reported [69]. MIC values were 8 μg/ml *vs. Saccharomyces cerevisiae* and killing kinetics were comparable to amphotericin B at 5x the MIC. Membrane activity as the underlying mechanism of activity was supported by membrane permeabilization assays measuring efflux and influx of solutes. A screen of a haploid

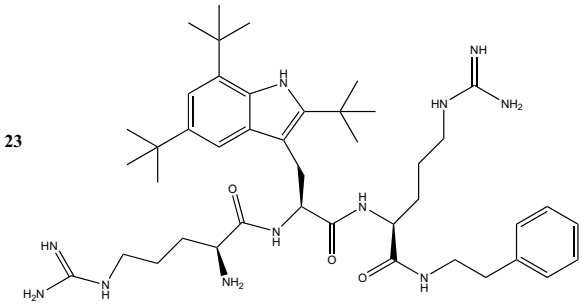


Fig. (5). Structure of LTX-109.

S. cerevisiae gene deletion library implicated a direct or indirect interaction of LTX-109 with sphingolipds in eliciting its killing activity [69].

LTX-109 has entered clinical development for two topical indications, impetigo and nasal decolonization. The Phase 2 study for impetigo was a randomized, double-blind, placebo controlled study to evaluate two doses of LTX-109 versus vehicle [70]. Test agent was administered topically, three times daily for 5 days. The primary endpoint was clinical success at one or more of the visits during the 12 day period of treatment and follow-up. A high proportion of clinical success and bacteriological response in the placebo-treated group complicates interpretation but a trend for clinical success was observed at Day 6 and Day 12 in the two treatment groups receiving 1% or 2% LTX-109 [70]. Both doses of LTX-109 were well-tolerated as only one adverse event, possibly related to the study drug was reported in a total population of 206 patients. Systemic exposure following topical skin administration was negligible. Results for a Phase 1 study for nasal decolonization using a 3 day treatment of 1%, 2% or 5% LTX-109 compared to vehicle showed a significant reduction in *S. aureus* burden at 2 and 3 days of treatment [71]. Recolonization was commonly observed in all treatment groups and it was suggested that more frequent or extended dosing regimens could be tested to improve long-lasting effects. Adverse events that were reported with higher frequencies in the 2% and 5% LTX-109 treatment groups than in the 1% LTX-109 or vehicle groups included runny nose, itching, burning, pain and minimal epithelial lesions in the nasal cavity and septum [71]. All adverse events were predominantly mild in severity and resolved quickly after cessation of study drug administration. The possibly of reformulating LTX-109 was suggested to help control the nasal congestion associated with LTX-109 by preventing access of the drug to the upper nose.

SMALL MIMICS OF HOST DEFENSE PROTEINS

In a more significant departure from amino acid like peptidomimetics, considerable effort has been directed at developing small, nonpeptidic, abiotic scaffolds with amphiphilic structures for therapeutic uses, so called SMAMPs (Synthetic Mimics of Antimicrobial Peptides) or smHDPs (small mimics of Host Defense Proteins). This has included short oligomeric structures as well as easily-prepared, polymerizable monomers generating samples with molecular weight heterogeneity [22, 72-75]. The ability to capture AMP-like biological activity in simple polymers significantly expanded the design space for smHDPs; however, it is not the focus

here and has been recently reviewed [76]. Instead, discussion is focused on oligomeric smHPDs since this approach has been given significantly more medical chemistry effort and generated numerous clinical leads including Brilacidin, which has completed two phase 2 clinical trials.

The overarching program goal was to recapitulate the biological (and structural) properties of HDPs into oligomeric backbones without trying to duplicate the dimensional structures of the peptides but rather by creating small FA compounds [77]. These smHDPs would be expected to have better pharmacokinetic and tissue distribution properties due to their size and improved stability; they should also be less expensive to produce with selection of appropriate building blocks. Furthermore, synthetic chemistry, expanded beyond amino acids, provides considerably more phase space to fine-tune their structures for enhanced antimicrobial activities and minimized toxicities. Numerous amphiphilic oligomer families including meta-phenylene ethynylene and triaryl backbones with differing ring linkages have been studied [78-83]. A recent review provides an excellent description of SARs that govern their antimicrobial and cytotoxic activities [76]. It is generally straightforward to design highly potent compounds by selecting the appropriate cationic group, typically amine or guanidine, and hydrophobicity. However, the challenge is in building selectivity while maintaining activity against target pathogens to provide adequate safety margins for therapeutic development. One important principal in developing smHDPs is that proper balance between several structural features including the nature and number of cationic charges, charge distribution, hydrophobicity and amphiphilicity is required for optimization. Furthermore, the relative influence each of these parameters contributes to the potency and selectivity of antimicrobial activity differs among different scaffolds. This presents challenges to the development of robust, global SAR principles which could guide the field; however, it is not especially surprising given the complex landscape related to mechanisms of action (multiple targets, membrane complexity, pathogen, etc).

Mechanism of action studies utilizing progenitor arylamide smHDPs **24** and **25** (Fig. **6**), active against *E. coli*, support membrane action as an important aspect of their antimicrobial activity [84]. Large changes in membrane morphology and outer membrane permeability were found following

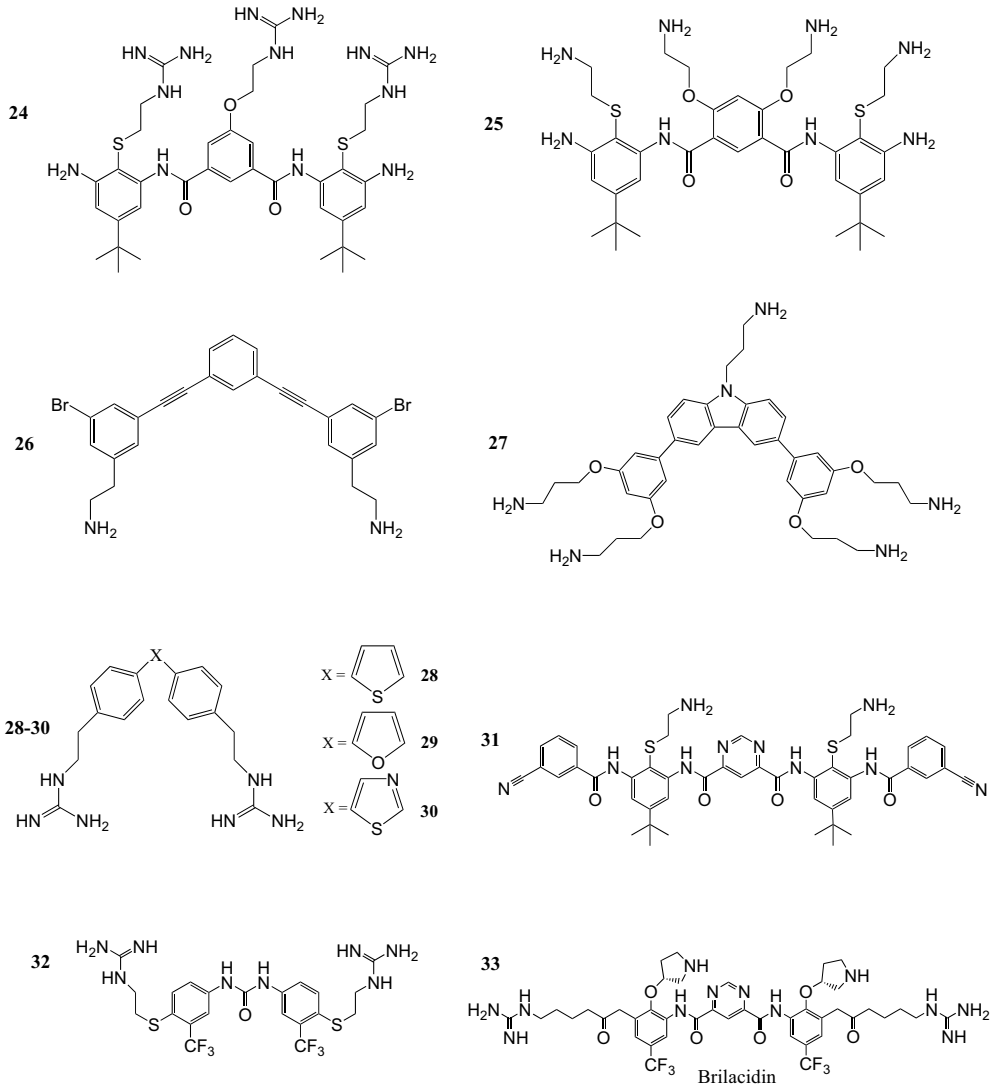


Fig. (6). Structures of smHDPs.

exposure to killing concentrations of the smHDPs. More limited permeabilization of the inner membrane was also associated with inhibition of protein processing and secretion into the periplasmic space. Induction of response regulons that mediate membrane and oxidative stress was also observed in transcriptional profiling experiments using sublethal concentrations of compound and there was partial overlap with regulons induced by the membrane-active peptide Polymyxin B [84]. Together these results substantiate the goal of recapitulating the function of AMPs on small amphiphilic scaffolds.

Spectrum of Activity Versus Microbial Pathogens

Although highly detailed knowledge on the mechanism of target interactions are not known for the smHDPs and their peptide counterparts, differences in membrane structure and composition do appear to provide the opportunity to identify pathogen-selective mimetics capable of distinguishing bacterial, fungal, parasitic and mammalian cell types. This selectivity became evident when discovery programs were expanded from anti-bacterial to anti-fungal and anti-parasitic indications. A number of structurally distinct smHDPs were found to possess potent activity against *C. albicans* and non-albican Candida species [85, 86] showing MICs from 0.5 to 8 μg/ml. Selectivity for Candida over mammalian cells, as measured by the ratio of EC_{50}s in metabolic assays with 3 mammalian cell lines versus Candida MICs, ranges from 4 to 10-fold for the least selective meta-phenylene ethynylene (**26**) to >100 fold for a diphenyl dibenzopyrole (**27**), a diphenyl thiophene (**28**), a diphenyl furan (**29**) and a diphenyl thiazole (**30**). Interestingly, these latter compounds also displayed very poor activity against a panel of Gram-positive and Gram-negative bacteria whereas mPE is broadly active against bacteria [80, 87]. This selectivity appears to be dependent on the correct ratio of charge, provided by a limited number of guanidine groups (2) or greater numbers of amine groups (5), to hydrophobicity, which in these examples is provided by the scaffold [86]. As opposed to the arylamides, where restricted torsional freedom was important for potent anti-bacterial activity [81, 82], a rigidified scaffold does not appear to be necessary for anti-Candida activity. The highly selective smHDPs, **27** and **28**, were also shown to be robustly active as topical agents in both immune-competent and immune-deficient mouse models of oral candidiasis where single administrations to well-established (4 day) Candida infections on the tongue reduced the fungal burden by up to 3 logs with efficacy comparable or superior to that of the widely used topical antifungal agent, Nystatin [86].

A library of smHDPs was also screened for compounds that kill *Plasmodium falciparum*, the infectious parasite that causes the most lethal form of malaria [88]. The selected compound, **31**, was active against chloroquine-sensitive and –resistant parasites with IC_{50}s in infected human erythrocytes of 75 to 100 nM. Furthermore, **31** had very low cytotoxicity against mammalian cells (EC_{50}s > 400 μM) and was inactive against both Gram-positive and Gram-negative bacteria (MICs > 50 μg/ml). Compound **31** is a biamino triarylamide with a pyrimidine center ring which has been shown to restrict rotational freedom through intramolecular H-bonding between the ring nitrogens and amide bond linkers [81]. The unusually high potency of **31** is most likely due to its unique mechanism of action where rather than targeting the plasmodium cytoplasmic membrane, it targets the membrane of the digestive vacuole. Shortly after exposure, **31** was found to concentrate specifically in the plasmodium digestive vacuole leading to rapid lysis of the vacuole and parasitic death [88]. An alternate compound, **32** (a biguanidyl biarylurea), had comparable activity versus *P. falciparum* to **31** but was significantly less selective against both mammalian cells and bacteria. Like **31**, **32** appears to kill *P. falciparum* by a digestive vacuole-dependent mechanism [88]. Results from the screen indicate that an overall lower cationic charge, as found on **31**, is suitable for activity against *P. falciparum* and contributes to strong selectivity for the parasite over other microbial pathogens. The promise of the smHDPs as antimalarials was validated in mouse models of *Plasmodium* infection using *P. yoelli* 17XNL and *P. bergei* ANKA parasites. Parasitemia was completely eliminated in both models by IV administration of **31** over 4 days after infection and all treated mice infected with *P. bergei* ANKA survived through 20 days. The challenge for smHDPs as useful antimalarials is oral bioavailability which is typically poor for this class of compounds. However, the relatively low cationic charge needed for anti-parasitic activity provides an opportunity for optimizing the smHDPs for oral delivery. In fact, progress along this line within the program has identified leads with increased oral bioavailability from ~6%, which is impractical, to ~40% (a reasonable threshold for therapeutic consideration).

Brilacidin

One of the more clinically advanced smHDPs is brilacidin (PMX30063, **33**); now being developed for Acute Bacterial Skin and Skin Structure Infections (ABSSSI) as an intravenous agent (Cellceutix, Inc., Beverly MA). Brilacidin is an arylamide (MW = 936.9; CAS 1224095-98-0) that bears two guanidino and two pyrrolidine cationic end-groups and two trifluoromethyl hydrophobic sidechains. Brilacidin and close analogs are highly active against staphylococcal and other Gram-positive bacteria with MIC_{90} values ≤ 1 ug/ml against methicillin-sensitive and –resistant *S. aureus* and coagulase-negative *S. aureus* [89]. Mechanistic studies with *S. aureus* revealed a dose-dependent membrane depolarization by brilacidin comparable to the effect elicited by daptomycin [90]. Transcriptome interrogation by deep sequencing showed up-regulation of regulons responsive to cell wall and membrane stress and protein misfolding with kinetics similar to effects of brilacidin on bacterial growth [90]. Similarities to the transcriptome responses to daptomycin and the AMP LL37 were observed and found to be largely related to genes under control of the 2 component systems GraSR, VraSR and NsaR, which are typically upregulated by cell membrane and cell wall damaging agents. Although these regulons are commonly associated with development of resistance to the stimulating antibiotic, serial passage studies with brilacidin showed no significant changes in susceptibility over 20 passages. Furthermore, resistance to daptomycin was not associated with decreased susceptibility to brilacidin [89]. Therefore, while such cellular responses can lead to resistance to other antibiotics, their

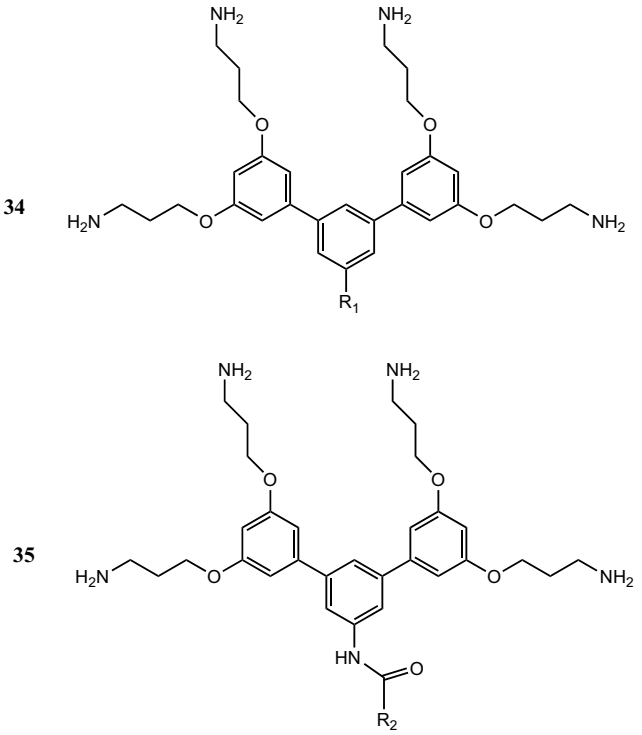
induction does not appear to readily cause resistance to brilacidin.

Low cytotoxicity of brilacidin and its analogs versus mammalian cells in metabolic activity and hemolysis assays, and efficacy in mouse tissue infection models following IV administration were important qualifications for entering clinical studies as a systemic IV antibiotic. Brilacidin has completed several Phase 1 and two Phase 2 clinical studies for ABSSSI. Brilacidin displayed predictable pharmacokinetic properties with a half-life of approximately 17 hours following IV administration [91]. In the first Phase 2A ABSSSI trial, brilacidin was administered IV once daily for 5 days at three dosages with approximately 50 patients per dosage arm (www.clinicaltrials.gov; NCT01211470). The active comparator was daptomycin administered IV once daily for 7 days according to label instructions. Clinical response rates in all analysis populations were high across all three dose groups and similar to daptomycin [92]. The most common side effect was paraesthesia and hypoaesthesia (numbness and tingling) that resolved quickly after treatment cessation. Infrequent increases in blood pressure and heart rate that were rapidly reversible were also observed and appeared to be dose-related. A Phase 2B trail in ABSSSI for dose optimization has been completed (www.clinicaltrials.gov; NCT02052388) and results were reported at the 2015 25th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) conference in Copenhagen, Denmark [93]. The trial included 2 single dose arms of brilacidin (0.6 and 0.8 mg/kg IV) with the positive control comparator, daptomycin administered IV once daily for 7 days. Single dose brilacidin was safe and well-tolerated. Efficacy was high in both regimens at the early Food and Drug Administration (FDA) timepoint (day 2 -3) indicating an immediate response and high at later timepoints (day 7-8 and 10-14) indicating a sustained clinical response. Efficacy was also similar to 7 days of treatment with daptomycin. There were no treatment-related serious adverse events (SAEs) or cardiovascular SAEs and blood pressure events were low in the 0.6 mg/kg single-dose regimen, similar to or better than daptomycin, an antibiotic with no reported blood pressure effects. Numbness and tingling was reported in both single dose regimens but were mild and transient. A phase 3 trial is currently being planned.

Given the broad activity profiles of these smHDPs with anti-bacterial, -fungal, and –parasitic action, including agents with activity across this spectrum, or agents with potent but select antimicrobial activity in one category, the future looks incredibility bright for their continued development. Additionally, rapid improvement in oral bioavailability despite the limited research effort due to the program size, suggests smHDP designs hold considerable promise. Two additional areas of serious need and opportunity include improved Gram-negative activity (especially *in vivo*) and understanding (as well as exploiting) their immune-modulatory activity.

Structure – Activity Relationships

In an effort to better understand some of the subtleties between FA structure and antibacterial activity, a new series of smHDPs were prepared in which one set was 'uniformly FA' while the other set had the amphiphilicity disrupted (DA, disrupted amphiphilicity) by the insertion of a polar amide linker into the hydrophobic region (**34, 35**; Fig. **7**). The SAR revealed that amphiphilic topology is critical for the broad spectrum activity of these smHDPs, especially against Gram-negative bacteria.



R_1 and R_2 = phenyl, 4-methylphenyl, 4-t-butylphenyl, 3,5-ditrifluoromethylphenyl and indole

Fig. (7). FA *vs.* DA smHDPs.

All the FA compounds with pendant side groups showed improved antibacterial activity against *S. aureus* as compared to a smHDP which was much less hydrophobic and therefore not active. The substitution of the pendant phenyl group with a methyl-phenyl group resulted in a 16-fold increase in activity against *S. aureus* with an MIC of 0.78 μg/ml. The incorporation of a pendant indole ring yielded the highest selectivity in the FA series against *S. aureus*, with selectivity values of ~400 for *S. aureus* and ~100 for *E. coli*, along with very low toxicity (HC_{50} = 634 μg/ml).

This may be due to the unique membrane affinity of the indole ring as observed with tryptophan [94]. These FA smHDPs had Gram-negative MIC values in the 1-10 μg/mL range. In contrast, insertion of the amide group essentially obliterated antibacterial activity (>50 μg/mL). Even when the pendent group in the DA series was made more hydrophobic to 'match' the overall HPLC retention time of FA smHDPs, they remained impotent. Efforts to better understand the relationships between structure and molecular amphiphilicity explored the use of the integy moment (IM), a vector pointing from the center of mass of the molecule to the center of the hydrophilic region [95]. A higher IM value is indicative of a higher concentration of the hydrophilic regions towards one face of the molecule allowing IM to be

a convenient, quantifiable parameter for a molecule's amphiphilicity. The IM values indicate a threshold value of amphiphilicity (0.24) is required to achieve activity against Gram-negative bacteria within this backbone.

While smHDPs likely kill bacteria via multiple mechanisms, membrane interaction seems to be critical for many reasons [96]. This increased importance of the FA topology in killing Gram-negative bacteria is consistent with the need to generate negative Gaussian curvature [97]. Negative Gaussian curvature, a requirement for pore formation in the bacterial membranes requires both positive and negative mean curvature components [98]. The smHDPs contribute both at the same time [96]; Gram-negative lipid membranes are typically richer in the negative curvature lipid phosphatidylethanolamine (PE - around 80%), compared to the Gram-positive lipid membranes, which contain large amounts of cardiolipin (CL). Thus, it may be a requirement that disruption of Gram-negative bacteria requires more efficient insertion of hydrophobic components. So, for example, although two smHDPs may have similar HPLC retention times, or overall hydrophobicities, the hydrophobicity of the DA series is disrupted. The high energy penalty of 2 kcal/mol required for the insertion of a polar amide moiety into the hydrophobic core may limit the penetration of the DA smHDPs [99] which reduces the total volume of the inserted hydrophobic groups resulting in less positive curvature generation and therefore less negative Gaussian curvature which is required for a broad range of barrier disruption events. Another challenge with respect to Gram-negative potency is the need to disrupt or transverse the inner plasma membrane. This means that smHDPs must get passed the outer membrane first and this likely reduces the 'effective' concentration of smHDPs at the inner membrane [100].

Changing the nature of the 'linker' and type of pendant group generated another series of smHDPs (**36**; Fig. **8**). The amide containing oligomers showed some Gram-positive activity, especially against *S. aureus* but essentially no Gram-negative potency, as expected. Either replacement generated improved potency especially against Gram-negative pathogens with MICs of 0.5-3.0 μg/mL.

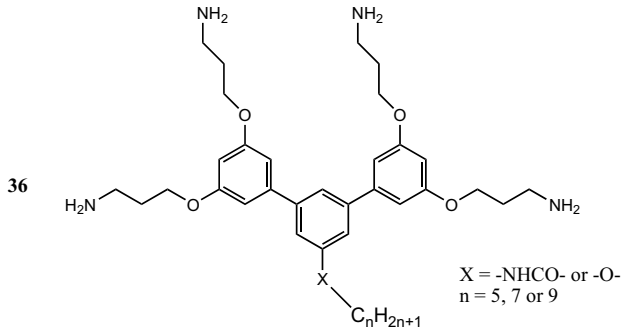


Fig. (8). Amide *vs.* ether containing smHDPs.

Immune-modulatory activities

The ability of HDPs to participate in additional roles of the immune response has been recognized more recently. For example, LL-37 and defensins affect innate immune cell functions, including the induction and modulation of chemokine and cytokine production, direct chemo-attraction of immune cells, angiogenesis promotion and wound healing. Towards this end, a number of immunomodulatory molecules, including smHDPs and innate defense regulators (IDRs) that do not show direct antimicrobial response, are being developed into therapeutic candidates and adjuvants [101]. The combination of direct antimicrobial activity and controlled immunomodulation in an antimicrobial system would present a novel strategy to treat infections with multiple mechanisms of action against pathogens, which is expected to minimize pathogen-antimicrobial responses.

Exploring the ability of smHDPs to directly engage the immune system showed that smHDP **37** (Fig. **9**) was able to induce cytokines, tumor necrosis factor (TNF), interleukin-6 (IL-6), and interleukin-10 (IL-10) in murine macrophages [RAW 264.7 or bone marrow derived macrophages (BMDMs)]. Natural peptides, like human neutrophil α-defensins, have been shown to stimulate TNF production in monocytes [102]. In addition to modulating pro- and anti-inflammatory cytokine production, this smHDP also induced significantly higher levels of murine KC (chemokine CXCL1, a neutrophil chemo-attractant) compared to the dimethylsulfoxide (DMSO) control in BMDMs (~300 *vs*. ~160 pg/mL). Increased KC expression has been associated with neutrophil influx during inflammatory conditions [103]. The protective activity of a synthetic cationic peptide against bacterial infection was also associated with the induction of chemokines such as CXCL1 [104]. Thus, the ability of this non-toxic, non-peptidic, antimicrobial smHDP to modulate both cytokine and chemokine production is encouraging for the design of synthetic molecules with multiple biological functions.

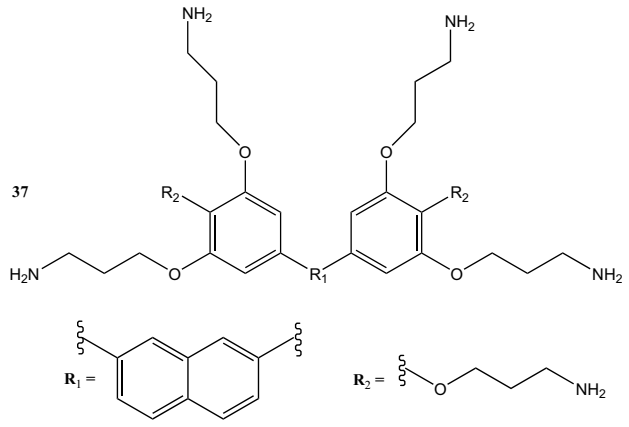


Fig. (9). Immuno-modulatory smHDP.

The TNF production was smHDP concentration dependent but its relationship with lipopolysaccharide (LPS) was unknown so the immunomodulatory activity was studied in the presence of LPS. LPS-induced production of both TNF and IL-6 increased in the presence of the smHDP. However, LPS-induced anti-inflammatory cytokine (IL-10) production was inhibited in the presence of the smHDP to background levels. This unique activity is different from the peptides like LL-37 and IDR peptides, which do not directly stimulate TNF production but suppressed LPS-induced TNF produc-

tion by up-regulation of IL-10 and, in the case of LL-37 also binding to LPS [105, 107]. The addition of rIL-10 (recombinant protein) did not significantly affect the self-agonistic effect [108, 109]. However, with LPS stimulation, the presence of rIL-10 resulted in the elimination of the smHDPs' enhanced TNF production suggesting that the decrease in IL-10 production was at least partially responsible for increased TNF production. Therefore, this smHDP seems to orchestrate the balance of the pro- and anti- inflammatory cytokine responses in macrophages. While many fundamental questions still remain, these unique immunomodulatory properties can be used to trigger immune responses in a very specific way. For example, MLA, a TLR4 (toll-like receptor 4) agonist is already an effective adjuvant for hepatitis B and influenza [101].

The complex role HDPs and smHDPs will play in clearing infections will require much more study but smHDPs can be expected to modulate the immune system in various ways. This may include sequestering anionic endotoxins. For example, in another set of studies a group of smHDPs reduced lipoteichoic acid (LTA)-induced TNF production by >65% at the transcriptional level [110]. The same inhibitory effect was observed for the proinflammtory cytokine IL-6. Using whole bacteria generated even more robust responses. The production of IL-10, in response to *S. aureus*, was also suppressed in the presence of the smHDPs, indicating that the observed inhibitory effect in TNF and IL-6 production induced by these oligomers does not involve the induction of IL-10 and support specific interactions between the smHDP and LTA. These *in vitro* observes were also observed during *in vivo* experiments. Using the standard tissue burden model, but with fully competent animals showed that an average bacterial burden of 10^4 c.f.us/gram was detected in the vehicle-treated control mice while treated animals showed no bacterial burdens. At the same time, the sera from treated, infected mice contained significantly lower levels of TNF and IL-6 compared to control infected mice. This demonstrates that a nonpeptidic smHDP effectively eliminates the proinflammatory activity associated with *S. aureus* infection while simultaneously clearing the microorganism in the animal.

CONCLUSION

Host defense proteins appear to offer new infection strategies for the critical problem of multi-drug resistant bacteria. Although peptides remain a potential source of new therapies, challenges associated with their metabolic stability, pharmacokinetics, safety and manufacturing processes have limited therapeutic success to date. Alternatively, smHDPs have quickly demonstrated remarkable potency, encouraging safety, *in vivo* activity, oral bioavailability, and wide antimicrobial profiles. Importantly, these novel activities are associated with a low potential for emergence of resistance and little cross-resistance with other antimicrobial agents. Global SARs remain challenging due to the complex landscape and multiple targets of interaction, yet steady progress is being made. One lead compound, LTX-109, has entered clinical study as a topical agent to treat impetigo and nasal decolonization and brilacidin has completed two phase 2 studies as an intravenous agent for skin infections.

CONFLICT OF INTEREST

Richard W. Scott is a consultant for and equity stock holder in Cellceutix, Inc. (Beverly, MA), a company that is developing a lead host defense protein mimic, brilacidin, for clinical use.

Gregory N. Tew has no conflict of interest to report.

ACKNOWLEDGEMENTS

Declared none.

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