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Host Cell Interactions are a Significant Barrier to the Clinical Utility of Peptide Antibiotics

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Abstract

Despite longstanding promise and many known examples, antimicrobial peptides (AMPs) have failed, thus far, to impact human medicine. Based on the physical chemistry and mechanism of action of AMPs, we hypothesized that host cell interactions could contribute to loss of activity *in vivo* where host cells are highly concentrated. To test this idea, we characterized AMP activity in the presence of human red blood cells (RBC). Indeed, we show that most of a representative set of natural and synthetic AMPs tested are significantly inhibited by preincubation with host cells, and would be effectively inactive at physiological cell density. We studied an example broad-spectrum AMP, ARVA (RRGWALRLVLAY), in a direct, label-free binding assay. We show that weak binding to host cells, coupled with their high concentration is sufficient to account for loss of useful activity, for at least some AMPs, because $>1 \times 10^8$ peptides must be bound to each bacterial cell to achieve sterilization. The effect of host cell preincubation on AMP activity is comparable to that of serum protein binding. Feasible changes in host cell binding could lead to AMPs that do not lose activity through interaction with host cells. We suggest that the intentional identification of AMPs that are active in the presence of concentrated host cells can be achieved with a paradigm shift in the way AMPs are discovered.

Introduction

Since their discovery in the 1980s as effectors of innate immunity in plants and animals, antimicrobial peptides (AMPs) have generated interest for their therapeutic potential^{1,2}. With epidemic levels of antibiotic resistance established or emerging across an increasingly broad spectrum of human pathogens^{3,4}, AMPs are especially attractive⁵ due to their unique mechanism of action: disruption of bacterial membrane integrity^{6–8}. By targeting membranes, AMPs are active against many strains of Gram negative and Gram positive bacteria¹, including drug-resistant strains^{9,10}, and simultaneously minimize the emergence of resistance compared to conventional antibiotics^{2,7,11}. Interest in AMPs has also given rise to promising mimics, such as polymers¹² and other synthetic molecules¹³, that also bind to and disrupt microbial membranes.

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Supporting Information Available: This material is available free of charge *via* the Internet.

AMPs and other membrane permeabilizing antibiotics are unique because they target the whole cytoplasmic membrane rather than a specific site in a specific biomolecule. Membrane binding is best described in physical chemical terms as partitioning. Although some mechanistic details are still unknown, there are two important elements of AMP activity. First, activity requires a significant accumulation of an AMP on bacterial membranes^{8,14,15}, driven by both electrostatic and hydrophobic interactions. These interactions are influenced by the mostly anionic bacterial lipid composition^{2,6}, spatial lipid distribution¹⁶, membrane curvature¹⁷, and peptide secondary structure^{2,18}. AMPs also accumulate on other anionic structures such as the cell wall, LPS and DNA¹⁹. Second, microbicidal activity requires that bound peptides cause physical disruption of the membrane barrier, driven by amphipathicity, or interfacial activity⁷.

Standard, *in vitro* assays^{20,21} for antimicrobial activity against diverse strains of bacteria, including those that are resistant to conventional antibiotics, have demonstrated that many AMPs have potent, broad-spectrum activity. Yet, such assays do not recapitulate the complex environment in which systemically administered antibiotics must perform *in vivo*, perhaps explaining why AMPs have had little clinical impact to date^{2,5}. Factors that are known to inhibit the activity or usefulness of AMPs (and other peptide drugs) *in vivo* include proteolytic degradation, binding to serum proteins, host cell toxicity, and clearance by glomerular filtration²². AMPs may also be challenged *in vivo* by their very nature as effectors of membrane disruption⁷ which depends on selective membrane partitioning. While their affinity for bacteria is high^{8,14}, weak host cell binding nonetheless occurs²³. As illustrated in Figure 1, we hypothesize that weak interactions and slow exchange between AMPs and host cells, which are highly concentrated *in vivo*, could overwhelm the selective binding to bacteria and, alone or in combination with other known impediments, reduce or eliminate the activity of some AMPs *in vivo*.

The effect of direct host cell interactions on AMP activity, and how such interactions might be related to host cell toxicity, have not been well explored. Here, we show directly that human red blood cells (RBC) strongly inhibit the activity of many, but not all, of a set of 12 representative natural and synthetic AMPs. Even some naturally-occurring AMPs are shown to be susceptible to host cell inhibition, presumably because few have evolved to be systemic anti-infectives, especially in vertebrates. Host cell interactions likely contribute to the impediments to the development of clinically-useful systemic AMPs (Figure 1) especially since pre-clinical, *in vitro* characterization of AMPs is rarely done in the presence of host cells.

RESULTS AND DISCUSSION

To test the hypothesis that host cells inhibit AMP activity, we measured the effect of washed, serum free human RBCs in two assays. First, we determined the effect of preincubation of peptide with RBCs on the minimum inhibitory concentration (MIC) of a representative collection of natural and synthetic AMPs by broth dilution: an all-or-none sterilization assay²⁰. Second, we measured the effect of RBCs using radial diffusion²⁰ on a subset of natural and synthetic, broad-spectrum AMPs. Finally, we measured the binding of peptide to both RBCs and bacteria to explain the observed loss of activity in our experiments.

The effect of host cells in broth dilution

We studied 12 cationic AMPs of diverse structure by broth dilution. See Figure 2. The five natural AMPs range from 13 to 37 residues, and are derived from humans, cows, insects and frogs: a broad survey of the animal kingdom. The seven synthetic peptides range from 9 to 26 amino acids and include peptides with α -helical and with β -sheet secondary structure. These include ARVA²⁴, a 12-residue AMP in both L- and D-amino acid form, WLBU2, a peptide that was previously reported to retain activity in the presence of RBCs²⁵, and MSI-78, or pexiganan, a peptide that is the subject of ongoing human clinical trials, including a phase 3 trial for diabetic foot ulcers⁵. As controls, we tested the conventional antibiotics, streptomycin and vancomycin, against susceptible organisms.

Selectivity is a critical factor in determining the clinical potential of candidate antibiotics¹⁸. The selectivity, or therapeutic index, of an AMP is a measure of its antimicrobial activity, relative to its host cell toxicity. Of the many AMPs known, only a small fraction have little or no toxicity to host cells. Here, we hypothesize that host cell interactions may be correlated with host cell toxicity because peptides must bind, at least partially, to host cells to cause lysis, although they could also bind without causing lysis. To test this idea, we studied peptides that cover the entire range of possible selectivities, which we represent in Figure 2 by showing their propensity to lyse human RBCs at a high peptide concentration of 100 μ M. We included the natural AMPs cecropin A and magainin II, both of which are highly selective, as shown by their near zero propensity to lyse dilute human RBCs at high peptide concentration (Figure 2 and Supplementary Figure 1). At the other extreme, we included melittin, a lytic bee venom toxin, and Melp5, a synthetically evolved gain-of-function analog of melittin²⁶. These peptides are completely non-selective; both potentially bactericidal, and highly hemolytic.

Escherichia coli and *Staphylococcus aureus* were used as model Gram-negative and Gram-positive human pathogens, respectively. Peptides were preincubated with human RBCs for ~30 minutes at 0, 1×10^7 , 1×10^8 , and 1×10^9 cells per milliliter prior to the addition of bacterial cultures in growth media. In the absence of RBCs, most of these AMPs, as well as the conventional antibiotics, have low micromolar MIC values (Figure 2). Eight of the peptides were similarly active against both bacteria, underscoring their appeal as therapeutics. After preincubation with 1×10^9 RBC/ml (20% of the concentration in blood) the MIC values for most of the AMPs, including all of the broad spectrum peptides, increased dramatically (Figure 2). By extrapolation, these AMPs will be effectively inactive (MIC $> 50 \mu$ M) at the human physiological concentration of 5×10^9 cells/ml. The conventional antibiotics were not strongly affected by RBCs. In Supplementary Figure 2 we show that the inhibitory effect of serum on MIC in broth dilution is similar to the effect of RBCs at the same relative concentration.

The synthetic peptide WLBU2 lost activity in the presence of RBCs as readily as the other broad-spectrum peptides (Figure 2), in our studies with *E. coli* and *S. aureus*, contrasting with a previous study²⁵ reporting that WLBU2 retained activity in whole blood against *Pseudomonas aeruginosa*. The activity of the human peptide LL-37 was not strongly affected by RBCs, but its activity in this sterilization assay is known to be poor against *E. coli* and undetectable against *S. aureus*²⁷. Cecropin A, a helical AMP originally derived from the

moth *Hyalophora cecropia*²⁸, was the only peptide that was both highly active and not strongly inhibited by RBCs. However, cecropin A was only active against *E. coli*, and not against *S. aureus*, as reported by others²⁹. Similarly, magainin II, from the skin of the frog *Xenopus laevis*³⁰, was active only against *E. coli*. However magainin II, unlike cecropin A, was strongly affected by preincubation with RBCs.

Surprisingly, we observed no strong correlation between selectivity and host cell inhibition. For example, we observe no RBC effect with cecropin A which is highly selective, but a strong RBC inhibition with magainin II, which is also highly selective. We also observe a similar RBC effect with MSI-78, a peptide with moderately high selectivity that has been studied in clinical trials. Further, melittin, which is the least selective peptide by far, is not the peptide that is most strongly affected by host cells. This lack of correlation probably results from the fact that host cell binding does not necessarily mean that lysis will take place.

The effect of host cells in radial diffusion

Four representative, broad-spectrum AMPs were subjected to a closer examination by radial diffusion assays. We selected the L- and D-amino acid variants of ARVA, a cationic, 12-residue peptide previously identified in a peptide library screen³¹. Like most AMPs, ARVA kills bacteria by rapidly permeabilizing the bacterial cytoplasmic membrane^{24,31}. ARVA-L and ARVA-D have very similar activities against many strains of bacteria²⁴ and also have fair selectivity (Figure 2 and Supplementary Figure 1)³¹. By comparing protease-resistant ARVA-D to protease-sensitive ARVA-L, we test for the effect of proteolytic degradation. We also studied two naturally occurring, broad-spectrum AMPs. We examined indolicidin, a 13-residue AMP found in bovine neutrophil granules that acts against phagocytosed microbes³² and has poor selectivity. There is some disagreement in the literature as to whether indolicidin acts *on* bacterial membranes or translocates through membranes to act intracellularly. There is strong evidence for multiple mechanisms^{19,33}. Finally, we examined melittin, a broadly membrane lytic, 26-residue, amphipathic, α -helical peptide toxin from the venom of the European Honey Bee (*Apis mellifera*)³⁴. Melittin is not an AMP by evolutionary design, but rather a toxin, and is entirely non-selective.

With these four peptides, we performed radial diffusion assays^{20,35} against *E. coli* and *S. aureus* in the presence of increasing concentrations of human RBCs. In radial diffusion, a bacteria-inoculated plain agarose layer is overlaid with a nutrient rich agarose layer that enables the growth of an opaque lawn of bacteria (Figure 3A). Antibiotics are added to small wells in the inoculated agarose. The radius of inhibition around a well reflects the inherent minimum inhibitory concentration of the antibiotic and its ability to diffuse into the agarose (Supplementary Table 1). In our modified assays, peptide, fixed at a concentration of 20 μ M, is preincubated with increasing concentrations of RBCs for 30 minutes at 37°C, then delivered to wells in the inoculated agarose. For comparison, we also measure the zones of inhibition caused by serially diluted peptide alone on the same plate as in the example in Figure 3A.

Figure 3B and 3C, show that in the presence of increasing RBCs the activity of the AMPs decreases exponentially with 50% loss of activity at $2-5 \times 10^8$ cells/ml (4–10% of whole

blood). These AMPs will be effectively inactive in 5×10^9 RBC/ml, in agreement with broth dilution assays in Figure 2. The conventional antibiotics, streptomycin and vancomycin, were not affected significantly by preincubation with RBCs. Importantly, the normalized effect of RBCs on AMP potencies against *E. coli* and *S. aureus* are similar despite species-specific differences in MIC supporting the idea that the reduction in activity is explained mostly by the interaction of AMPs with RBCs.

For comparison, we also show that AMP activity decreases in human serum (Figure 3A and Supplementary Figure 3). While the effect is significant, it is smaller than the effect of RBCs at the same concentration, relative to whole blood. We note that ARVA-D, which is resistant to proteolytic degradation, is affected by serum like the others, indicating that serum protein *binding* is a major contributor to the observed effect. Unlike RBC inhibition, serum inhibition reaches an apparent plateau where the peptide is still active, as can be seen in Figure 3A, row 4 and in Supplementary Figure 3. The conventional antibiotics are hardly affected by serum. When serum and RBCs are both present, the four AMPs are strongly inhibited and the conventional antibiotics are not. We conclude that host cell inhibition can be as large as or larger than serum protein inhibition in radial diffusion, as well as in broth dilution (see above).

Direct measurement of cell binding

Having shown that preincubation with host cells dramatically decreases AMP potency in two assays, we sought to explore the physical basis for this effect. We used a label-free method to directly measure cell binding of protease sensitive ARVA-L and protease resistant ARVA-D to *E. coli*, *S. aureus*, and human RBCs. Peptide solutions were mixed with bacterial or RBC suspensions in PBS at room temperature for 30 min, after which the cells were removed by centrifugation. Fractional binding of peptide in Figure 4 was determined by measuring the peptide remaining in the supernate, using HPLC¹⁵ (Supplementary Figure 4) and comparing it to a peptide solution without cells, but otherwise treated identically. When plotted on a linear scale, binding follows hyperbola-like behavior. While we cannot rule out some metabolism or degradation of L-amino acid peptides under these conditions, we note the short time scale, the similarity between the results for ARVA-L and ARVA-D in most cases, and the continued presence of the intact ARVA-L in HPLC chromatographs (Supplementary Figure 4).

Binding of L- and D-ARVA to bacteria is very strong, and appears to occur in a saturable manner; decreasing peptide concentration from 20 μ M to 5 μ M significantly shifts the midpoint of the binding curve to lower cell counts without changing the shape of the curve. Such behavior is not surprising since the total mass of bacteria in these experiments is similar to the total mass of peptide. The binding of ARVA-L and ARVA-D to RBCs is much weaker than binding to bacteria. In this case, the mass of cells is much greater than the mass of peptide and saturable behavior is neither expected nor observed. The midpoint of RBC binding of ARVA occurs at $\sim 5 \times 10^7$ cells per ml, equivalent to 1% of the physiological concentration in human blood. After accounting for the differences in membrane surface areas, the apparent dissociation constants of ARVA for bacteria are more than two orders of magnitude smaller than for RBCs (Supplementary Table 2).

The direct measurement of ARVA-D binding to bacterial cells in Figure 4, coupled with MIC measurements made under the same conditions, enabled us to determine the minimum number of bound peptides required to sterilize bacteria. We varied the initial *E. coli* count and measured surviving colony forming units (CFU) in the presence of 20 μ M ARVA-D (Figure 5). By extrapolation, sterilization occurs up to 4×10^7 CFU/ml. Using this value and the binding curve in Figure 4, we calculate that there are no surviving CFUs when there are 1.5×10^8 peptides per cell. A survival rate of about 1% occurs when there are $\sim 0.5 \times 10^8$ peptides per cell. We do not know, at the current time, how representative ARVA-D is of the universe of known AMPs, but we note that it is a typical, synthetic, linear AMP with respect to spectrum of activity, amino acid composition, MIC in broth dilution, MIC in radial diffusion, and selectivity.

Antimicrobial peptides as drugs

More than three decades of research have shown that early expectations for the immediate, systemic use of antimicrobial peptides in the clinic were overly optimistic. While *in vitro* measures of efficacy highlight many sequences with potent, broad-spectrum antimicrobial activity, translational studies and *in vivo* experiments have yielded mixed results, including some reports of the effective use of AMPs in animal models^{25,36–42}. Perhaps more revealing is the absence of clinical trials initiated for the treatment of human subjects via systemic administration of AMPs^{2,5,43}. The success rate for antimicrobial peptides in clinical trials for any indication is null at present, as FDA/EMA approval has not been granted for any AMP studied in clinical trials. However, there are a few promising examples under development, including topical MSI-78 (pexiganan) for infected diabetic foot ulcers^{5,18,44}. In sharp contrast to AMPs, multiple peptide drugs have had significant impacts in the clinic for treatment of HIV infection, hormone therapy, cancer and more^{45,46}. Developing a clearer understanding of all the obstacles to the clinical utility of AMPs will be vital if this class of molecule is to have a wide-ranging impact in human medicine.

Accumulation of AMPs on bacteria is necessary for activity

The mechanism of action of most AMPs requires their accumulation on bacterial membranes, and likely also LPS, cell wall, DNA, and cytosolic proteins that carry negative charges or expose hydrophobic domains. Membrane accumulation is followed by disruption of membrane integrity. A critical number of bound peptides are required for bactericidal activity^{14,47} and our experiments showed that the number is very large; for ARVA-D, a typical, synthetic, broad-spectrum AMP, $\sim 1.5 \times 10^8$ peptides must accumulate on each bacterial cell to achieve sterilization. This is equivalent to an effective local “concentration” of 40–80 mM bound peptide over the whole cell volume, in line with predictions made elsewhere^{8,14,15}, and probably saturates the cell with bound peptide. This is many-fold higher than the concentration of cellular lipids or of any critical metabolite. Cell killing by massive peptide accumulation on bacteria probably explains why resistance to AMPs and mimics does not arise as easily as to chemical antibiotics^{1,13}.

One group of researchers¹⁵ used HPLC to measure binding of *Rhesus* theta defensin to *E. coli* at peptide concentrations slightly above the MIC. They measured a binding capacity of about 1 μ g/ml in the presence of 1×10^6 bacteria/ml. This is equivalent to $\sim 3 \times 10^8$ peptides

bound per bacterial cell, in excellent agreement with our measurement of 1.5×10^8 molecules required to kill *E. coli*. Other researchers¹⁴ have reported that sterilization of *E. coli* by a different AMP occurs when 1.1×10^7 peptides are bound to the membrane of each cell. Our somewhat larger value may reflect inherent differences between peptides, but likely also reflects the fact that we are measuring *all* peptides bound to the cell, including those bound to non-membrane anionic macromolecules such as lipopolysaccharide, cell wall components, and DNA (Supplementary Figure 5). This idea is supported by the fact that membrane binding of ARVA-D can only physically account for a minor portion of total peptide because peptide mass $\approx$ total bacterial membrane mass in the experimental system. The requirement for significant accumulation of peptide on bacteria does not prohibit clinical utility, but it emphasizes why it is critical to reduce the inhibition caused by host cell interactions and other factors.

The selectivity of AMPs

Multiple kinetic factors likely affect potential host cell inhibition, as we illustrate in Figure 1. Thus, antimicrobial peptide activity can be thought of as a race between the rate of exchange of peptide between cell types, the incubation time required to kill bacteria, and the rate of growth of bacteria in rich media. *In vivo*, the kinetic network race also includes serum protein-bacteria-host cell exchange of peptides, serum proteolysis, glomerular filtration, as well as other innate immune responses that affect bacterial growth.

In this work we used moderate bacterial cell counts of $\sim 10^5/\text{ml}$ and high host cell counts which partly mimic an *in vivo* situation such as might be encountered in the bloodstream during a major disseminated infection (where even 10 to 1000 CFU/ml in blood indicate a serious infection)⁴⁸. We also preincubated peptides with host cells to mimic the administration of a concentrated peptide AMP by injection (because orally available peptide drugs are not yet a reality). Preincubation is appropriate to mimic a hypothetical injected peptide antibiotic because it would have to be transported by diffusion and/or circulation before achieving systemic distribution and thus would encounter many host cells before encountering the majority of pathogenic bacteria. Topical application of AMP drugs might more reasonably be simulated by experiments without preincubation. Preincubation will increase the effect of host cell interactions if equilibration in either or both cell types is slow. To test this effect, we compared the MIC values for ARVA-L and ARVA-D with and without preincubation with host cells. The host cell effect is much smaller when there is no preincubation (Supplementary Figure 6), however, 5–30 minutes of preincubation with host cells, at low concentrations without mixing, is sufficient to strongly inhibit these AMPs. We conclude that kinetic effects are significant and hypothesize that slow off-rates from cells may be the rate-limiting steps.

Binding of ARVA to bacterial cells is always much stronger than binding to host cells. This is likely true for most other AMPs, also. Thus, preferential accumulation of peptide on bacteria is predicted to always occur, and local peptide concentration on bacteria is expected to always be higher than local concentration on host cells, as has been observed experimentally for other AMPs^{49,50}. Yet, these observations are still consistent with our conclusions. Despite preferential binding to bacteria, this work shows that pre-contact with

host cells and weak binding *in vivo* can dramatically reduce AMP activity because i) a very large number of bound peptides are required to kill bacteria, and ii) host cells are very abundant. Based on our experiments using modified *in vitro* assays of AMP potency, any reduction in net accumulation due to weak host cell interactions (in addition to reductions caused by proteolytic degradation, serum protein binding and filtration) will decrease the effectiveness of an AMP against bacteria *in vivo*.

It is perhaps not surprising that natural AMPs are susceptible to these various impediments as few have evolved for systemic activity. Instead, many natural AMPs are employed locally. For example, some α -defensins from humans, ν -defensins from primates, bovine indolicidin, and other AMPs, are encapsulated in the granules of neutrophils and deployed against microbes only within phagosomes^{1,32}. This may help to explain the very poor selectivity of indolicidin (Figure 2). Other mammalian AMPs act locally after transient secretion; for example some defensins are secreted from intestinal Paneth cells into intestinal crypts when a bacterial pathogen is present⁵¹. Other well-known AMPs (e.g. the anuran magainins and dermaseptins) are found extracorporeally in the skin secretions³⁰. Some insect AMPs may be exceptions, as they are constitutively present and active in hemolymph. It is thus interesting to note that the only insect AMP we studied, cecropin A, was the most selective in our set, and was the only effective AMP we studied that was not affected by preincubation with host cells. In mammals, peptides are presumably too costly an approach for continuous, systemic innate immunity against occasional bacterial threats. However, in the clinic, no cost is too high to save the lives of patients infected with drug-resistant bacteria.

Outlook

The need for novel antimicrobials to combat the rising toll of drug-resistant bacterial infections is becoming increasingly more urgent. Efficacious AMPs could be useful as topical antibiotics to prevent initial drug-resistant bacterial colonization of, for example, trauma wounds, burns, surgical incisions, diabetic extremity ulcers, pressure ulcers and more. But ultimately, new classes of antibiotics with systemic activity are needed; a characteristic that has yet to emerge from the myriad AMP sequences that have been discovered and studied. We show here that preincubation with host cells can inhibit many natural and synthetic AMPs and that weak binding to host cells at physiologically relevant concentrations is sufficient to account for loss of activity for at least some AMPs. In the supplemental material we present a model indicating that this particular barrier can be bypassed with small, feasible changes in binding strength to host cells and/or bacteria (Supplementary Figure 7).

Proteolytic degradation, serum protein binding, lack of selectivity due to background cytotoxicity, and clearance by glomerular filtration have all previously been identified as impediments to the systemic activity of AMPs². Here we have shown that interaction with host cells, coupled with their high concentration, *in vivo*, is also sufficient to inhibit AMPs, especially when taken in combination with the other impediments.

Host cell interactions, serum protein binding, proteolytic degradation, and selectivity (i.e. lack of host cell toxicity) rarely, if ever, have been simultaneously optimized in AMP development. In part, this may be because AMP researchers, including us³¹, usually take a

“peptide first” approach, in which novel AMPs are discovered, selected, engineered, or refined in simple media, then tested against panels of microbes in standard antimicrobial assays, then tested for cytotoxicity in separate assays, and finally tested for activity under physiologically relevant conditions: in serum, less often in blood, and *in vivo*. The results and methods presented here suggest a shortened path to the identification of clinically-useful AMPs. First, the impediments caused by host cell interactions and serum protein interactions can be taken into account and tested for if all *in vitro* steps in the development, discovery or engineering of potential AMP drugs, from the initial design and discovery to preclinical characterization, are carried out in the presence of concentrated host cells and serum. As we have shown here, this assay modification can readily be accomplished with little loss in efficiency. A likely benefit of this approach is that AMPs that are active after preincubation with concentrated host cells will probably also have high selectivity (low toxicity). Second, because cells and/or serum will be present at all times, realistic proteolytic degradation is almost certain to occur if the AMP is susceptible. The simplest solution to this impediment is for all work to be done with protease resistant peptides, such as folded, disulfide crosslinked or cyclized AMPs, D-amino acid peptides, or peptide analogs. Third, peptide selectivity should be assessed under the most stringent possible conditions to identify peptides with the smallest effect on host cells. Since we are suggesting here that RBCs be present at all times, parallel hemolysis and bactericidal assays are readily accomplished. Taken together, these small changes in the standard approaches to AMP discovery or selection could lead to the more rapid discovery of AMPs with the potential for clinical utility as systemic antibiotics.

Materials and Methods

Peptides and Antibiotics

Peptides were obtained from Bio-Synthesis Inc. and had purities >95% by HPLC. Vancomycin and streptomycin were obtained from Acros Organics. Unless otherwise stated, all solutions were prepared by dissolving lyophilized peptide powders in 0.025% (v/v) acetic acid. Peptide sequences are melittin: GIGAVLKVLTTGLPALISWIKRKRQQ-NH₂; Cecropin A: KWKLFKKIEKVGQN-IRDGIIKAGPAVAVVGQATQIAK; Indolicidin: ILPWKWPWWPWRR-NH₂; LL37: LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES; Magainin II: GIGKFLHSAKKFGK-AFVGEIMNS; ARVA: RRGWALRLVLAY-NH₂; VVRG: WVVLRLGY-NH₂; NATT²⁴: RRGWNLALTLTYGRR; MSI-78: GIGKFLKKAKK-FGKAFVKILKK-NH₂; MelP5²⁶: GIGAVLKVLATGLPALISWIKAAQQL-NH₂; WLBU2²⁵: RRWVRRVRRWVRRVVR-VVRRWVRR.

Bacterial strains and growth conditions

Escherichia coli strain ATCC 25922 and *Staphylococcus aureus* strain ATCC 25923 were used in this study. Subcultures, prepared by inoculating 25 ml of fresh tryptic soy broth (TSB) with 200 µl of an overnight culture, were grown to log phase (OD₆₀₀ = 0.3 – 0.6) after which cell counts were determined by measuring the OD₆₀₀ (1.0 = 1.5x10⁸ CFU/ml for *S. aureus*, 5x10⁸ CFU/ml for *E. coli*). Bacterial cells were diluted to appropriate concentrations in either TSB or PBS depending on the assay.

Human serum and erythrocytes

Fresh human serum (OTC) and human O+ erythrocytes were obtained from Interstate Blood Bank, Inc. Serum was vacuum-filtered through 0.45 μm filter to remove precipitates. RBCs were subjected to four cycles of centrifugation at 1000x *g* with resuspension in fresh DPBS. Following the final wash step, the supernatant was clear and colorless. RBC concentration was determined using a hemocytometer.

Minimum sterilizing concentration in the presence of RBCs

Antibiotics were prepared at 5x the final concentration needed in 0.025% acetic acid. The antibiotics were serially diluted by a factor of 2:3 horizontally across a 96-well, conical-bottomed plate from Corning, 25 μl per well. One column was reserved for controls. RBCs at 0, 2.5×10^9 , 2.5×10^8 , 2.5×10^7 cells/ml were added in 50 μl aliquots to all wells. Following a 30 minute incubation, 50 μl of TSB inoculated with 5×10^5 CFU/ml were added to all and plates were incubated overnight at 37° C. To assess bacterial growth, a second inoculation was performed with 10 μl of solution from the original plate added to 100 μl sterile TSB. Following overnight incubation at 37° C, the OD₆₀₀ was measured (values of less than 0.1 were considered sterilized).

Radial diffusion in the presence of RBCs

Underlay and overlay agarose solutions were prepared as previously described³⁵. To a rectangular, one-well petri dish from Nunc, 20-ml of underlay agarose inoculated with 8×10^6 CFUs of bacteria was added. A sterile, 96-well plate replicator from Sigma-Aldrich was set in the molten agarose and removed once the agarose solidified. Antibiotic was prepared at 4x the final desired concentration. For the antibiotic standard, a serial dilution of 3:4 across a 96-well plate was performed followed by 1:4 diluted with PBS. Otherwise, peptide was diluted to 20 μM with cells and/or serum to give between 2% (1×10^8 cells/ml) and 20% (1×10^9 cells/ml). Solutions were incubated with gentle shaking for 30 minutes at 37° C, prior to the addition of 10 μl to the wells in the underlay. Inverted plates were incubated at 37° C for 3 hours, overlay was added and the plate was incubated upside down overnight. Surface growth was cleared, the plates were sterilized with 25% methanol and 5% acetic acid. Zones of inhibition were photographed and analyzed using ImageJ.

Bacterial binding

Cells were grown to high density (OD₆₀₀ = 1.0 or greater), pelleted at 10000 x *g* and resuspended in 1x PBS. CFU were determined by OD, as above. Bacteria and peptide mixtures were rocked for 30 minutes at RT, and centrifuged at 10000 x *g*. The resulting supernates were injected onto an analytical reverse phase HPLC column Kromasil 100-5C18. The native tryptophan fluorescence of these peptides was used to determine how much peptide remained in solution following incubation with cells.

RBC binding

Stock RBC solutions were prepared and mixed with stock peptide solutions in a 3:1 ratio. The solutions were rocked gently for 30 minutes at RT prior to centrifugation at 10000 x *g*.

The resulting supernates were analyzed using HPLC as previously described for bacterial binding experiments.

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Figure 1.

Kinetic network model of the impediments to clinical utility of antimicrobial peptides (AMP). AMP accumulation on bacteria *in vivo*, which is required for useful activity, may be considered as a portion of a network of kinetic steps that include on and off rates for several binding phenomena and also irreversible steps, such as proteolytic degradation and bacterial growth. Here we test the hypothesis that interactions with host cells can act as an impediment to AMP activity and are thus an important part of the kinetic network.

Figure 2.

Effect of host cells on bacterial sterilization by antibiotics in broth dilution assays. **A:** Example primary plate. Antibiotics were serially diluted, right to left, into suspensions of human RBCs and incubated for 30 min. Media and 5x10⁵ CFU/ml of bacteria were added prior to overnight incubation at 37°C. Aliquots were then used to inoculate a secondary media plate (panel **B**) to help visualize bacterial growth in the primary plate. After overnight incubation of the secondary plate, clear wells (OD₆₀₀ < 0.1) indicate sterility in the first plate. **C:** Mean minimum inhibitory concentration (MIC) for all the peptides studied are averages of 5–8 independent experiments. Standard errors are 20–50% of the MIC values (median = 31%). NA: Antibiotic is known to be not active. >30: MIC was not observed, thus it must be higher than 30 μM. MICs are color-coded with darker red indicating higher MIC (lower activity). For each peptide, we also show % hemolysis caused by incubation of 1x10⁸ human RBC/ml with 100 μM peptide at 37° C for 1 hour. Darker colors indicate greater hemolysis.

Figure 3.

Effect of host cells and serum on antimicrobial activity in radial diffusion assays. **A:** An example 96-well radial diffusion plate using *E. coli* and the peptide ARVA-D. To each 3 mm diameter well, we add 10 μ l of buffer containing the indicated components, followed by overlay and overnight incubation. Clear areas in the opaque lawn of bacteria indicate inhibition. The smallest circles are the wells in the agarose. Row 1: Peptide is diluted in 3/4 serial dilution from right to left, starting at 20 μ M. Row 2: Peptide control. Each well has 10 μ l of 20 μ M ARVA-D. Rows 3–5: Each well has 20 μ M ARVA-D preincubated with RBC, serum or both. Blood components increase in 2% steps from 0 (left) to 20% (1×10^9 cells/ml) of whole blood values. Rows 6–8: Control wells with RBC, serum or both increasing in 2% steps from 0 (left) to 20% of the whole blood values. **B and C:** Each panel shows the relative activities for four AMPs and one conventional antibiotic in the presence of human RBCs for the organism indicated. Relative activity is the ratio of the radius of inhibition for 20 μ M peptide in the presence of RBC to the radius of inhibition in the absence of RBCs. Relative activity versus cell count are well fit by single exponential decay except for indolicidin. Each experiment was performed 5 times. Uncertainties are SEM.

Figure 4.

Measurement of binding of AMPs to bacteria and host cells. **Left column:** Peptides at 20 and 5 μM were incubated for 20 minutes with increasing concentrations of human RBCs, followed by centrifugation of the cells. The supernate was analyzed by HPLC and the peptide peak area was compared to that of an identically treated sample without cells to obtain fraction bound. Each measurement was repeated at least 3 times. The average $\pm$ SD are plotted. Curves are fitted with a hyperbolic binding equation (See Supplemental Data). **Middle Column:** Peptide binding to *E. coli* measured as described above. **Right Column:** Peptide binding to *S. aureus*.

Figure 5.

The number of bound peptides required to kill *E. coli*. A concentration of 20 μ M ARVA-D was incubated with increasing *E. coli* cell counts from 1×10^5 to 2×10^8 cells/ml. After 60 minutes, the mixture was serially diluted onto nutrient agar plates and viable colonies were counted after overnight incubation. At cell counts below 4×10^7 cells/ml (extrapolated from the curve) no viable CFUs were observed. Above 4×10^7 cells/ml viable CFUs were observed which increased with cell count.