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Application of Synthetic Molecular Evolution to the Discovery of Antimicrobial Peptides

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Introduction

Antimicrobial peptides were first described in the 1980's, having been found in insect hemolymph [4, 5], mammalian neutrophil granules[6] and frog skin secretions[8]. Subsequently, more than a thousand natural AMPs have been found in many tissues of many different species[9, 10]. Hundreds of synthetic AMPs have been also been created, often by mimicking natural sequences in combination with trial and error experimentation[9,10], and sometimes by screening or computer aided design[2, 3, 11–14]. Since the beginning of the field, AMPs have been promoted as novel antibiotics that might improve human health and well-being. Yet, at the time of their initial discovery, there was little urgency to the translational applications of AMPs because it was not known, at the time, that drug resistant bacterial infections would grow over the next 30 years to become a global health crisis in morbidity and mortality[15–17]. We are now urgently and ever increasingly in need of novel antibiotic treatment options against drug-resistant bacteria. While some AMPs have been developed into potential topical drugs, and some are nearing or are in clinical trials[18], AMPs have not succeeded, recently or in past decades[19], to have any real impact on the systemic treatment options for drug resistant bacteria.

Thousands of AMPs are known that have good antibiotic activity in the culture tube, microwell plate, and petri dish. At least under standard laboratory conditions, many AMPs have potent, sterilizing activity at low μM concentrations against multiple species of bacteria[20,21], often including both Gram positive and Gram negative species. AMP activity is observed at the same concentrations at which most conventional antibiotics are active under the laboratory conditions. AMPs often have equally potent activity against drug-susceptible, drug-resistant and multidrug resistant bacteria[22–25] showing that the conventional mechanisms of drug resistance do not apply to AMPs. AMPs can also act against biofilms[22, 26], and they can create antibacterial surfaces by covalent tethering or physical adsorption[27]. Importantly, AMPs may be less likely to induce resistance than conventional antibiotics[28–30] although resistance to AMPs does occur[31]. Despite their potent, broad spectrum activity in the laboratory at low concentration, AMPs have not succeeded in the ultimate goal: the development of novel antibiotics that can be used systemically to prevent or treat drug resistant bacterial infections.

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This dearth of systemically active AMPs has many causes, but may be due in part, to impediments to bioavailability and dosing such as host cell inhibition[1], serum inhibition[22], residual toxicity[32], and proteolytic degradation[7]. Systemically active AMPs are almost certainly possible, as none of these impediments seems to be unsurmountable on its own. In fact, the possibility of a life-saving anti-infective, peptide drug has been proven by the record of the anti-HIV peptide enfuvirtide that has been approved for use in the US and Europe since 2003[33]. Enfuvirtide is a linear 36-residue peptide drug with over $1 billion in net sales[33–35]. This peptide is typically administered subcutaneously in 90 mg doses and has extended the lives of many patients infected with HIV that had become resistant to other drugs. Enfuvirtide has a long half-life in the human body[34] despite the lack of specific modifications to increase bioavailability or decrease proteolytic sensitivity. The peptide is an amphipathic α-helical peptide[36] that likely binds to cells and serum proteins, and this may help it to remain intact and in circulation. Despite this, enfuvirtide has low toxicity and is able to effectively inhibit the fusion of HIV viruses with cell membranes in vivo, probably by interfering with the structure-function relationships of the GP41 fusion protein, from which enfuvirtide was obtained[37].

If there are no insurmountable barriers to systemically active AMPs and we have thousands of known AMPs with activity in the laboratory, it is reasonable to ask: Why are there no systemically active AMP drugs? Why does there seem to be few in the development pipeline? In this chapter we hypothesize that the number of potential systemically active AMPs drugs in the drug development pipeline is small because i) the thousands of AMPs known have not evolved to be systemic AMPs or have not been discovered under the most relevant conditions, and because ii) rational engineering of AMP properties is not possible due to the fact that we do not have good sequence-function relationships for any of these impediments, including antibacterial activity. Below, we discuss an approach that may be especially well suited in this situation: Discovery of novel AMPs by iteratively screening small peptide libraries under experimental conditions that are increasingly relevant to physiological conditions. We have referred to this approach as “synthetic molecular evolution” [38–40]. By these means we suggest approaching the most clinically relevant conditions in a stepwise manner and doing so as a first stage in the preclinical identification of peptide antibiotic drugs to feed a larger number of relevant candidates into the development pipeline. Below we detail some of the major impediments to systemic activity of AMPs, and then describe how screening for AMP activity can be done under conditions that much more closely mimic *in vivo* conditions to identify peptides without these impediments.

Impediments to systemic activity

Compared to conventional antibiotic drugs, AMPs have a fundamentally different mode of action on bacteria. Most conventional antibiotics inhibit a critical biochemical process by targeting one molecule (e.g. an enzyme or ribosome). The number of functional molecules decreases until the microbe loses so much of that essential function that it cannot replicate or survive. The rate or degree to which critical activity is lost depends on local drug concentration. AMPs on the other hand kill microbes in a cooperative, saturation-like process in which the peptides must massively accumulate on bacteria to levels that

essentially saturate the cell in order to kill them through the effect of their interfacial activity[41] on membrane integrity. Various measurements in the literature[1, 42–45] have shown that the number of bound AMPs required to kill a bacterial cell is extremely high, from 10^7 to 10^8 peptides *per cell*. Since an E. coli cell can be expected to have perhaps 2×10^7 total lipids, this means that killing does not occur until there around one peptide bound for every bacterial lipid, which is more peptide than can possibly bind to the cytoplasmic membrane alone. This lethal amount of peptide is also equivalent to roughly one peptide per DNA base. If we assume that 10^8 peptides are evenly distributed in the volume of the cell, the local concentration of AMP is about 80 mM[1]. In reality, AMPs will specifically accumulate on anionic structures such as cell wall, LPS, cytoplasmic membrane, and DNA and could have local concentrations that approach molar. As a result, the mode of action of AMPs is a successive attack on the cell architecture. Membranes are permeabilized within minutes[2, 3], followed by leakage of macromolecules, including DNA. Within 60 minutes of treatment of bacteria with AMPs the entire cell architecture is compromised and individual cells are sometimes not discernable. It is thus reasonable that resistance would be more difficult to evolve, given that mechanism of action of AMPs may involve the entire cell architecture. When resistance is observed, it is usually due to changes in LPS or cell wall components[46, 47], making these structures less anionic so they do not accumulate large amounts of cationic AMPs.

Serum and Host Cell Inhibition

The need for accumulation of peptide means that systemic activity of AMPs faces different challenges than conventional drugs (Figure 1) and will exhibit threshold behavior. There may be only a narrow window between saturation/killing of bacteria and survivable accumulation of an AMP (Figure 2). Thus, any factor that competes for bacterial binding has the capacity to decrease accumulation on bacteria. Contrary to the commonly stated fact, external eukaryotic membranes are highly anionic overall, due to the large amount of anionic glycoconjugates on lipid and proteins. Thus, cationic AMPs bind to eukaryotic cells and tissues, at least moderately, and because host cells and tissue will always be orders of magnitude more abundant than pathogens, even weak competition can be problematic. Using a set of 12 natural and synthetic AMPs, we have shown that even a few minutes of preincubation of AMPs with human erythrocytes strongly reduced the activity of most of them[1], see Figure 3, through some combination of host cell binding and proteolysis by the cytosolic proteases of RBCs[7]. This effect is not always observed, indicating that it is a surmountable impediment. For example, the insect peptide cecropin A was not affected in our study by human RBCs[1]. Similarly Stella and colleagues carefully examined the effect of RBCs on the activity of an AMP and found little inhibition[43].

Serum proteins, especially serum albumin, can also bind cationic AMPs and are also highly concentrated in the body (35–50 mg/ml in blood), further potentially reducing the effective concentration of peptide available to bind to bacteria[22]. As we discuss in detail below, host cell and serum protein binding are rarely considered in the early stages of novel AMP discovery or design. If they are tested at all, it is determined how much these factors interfere with AMP activity only very late in the preclinical development pipeline. Here we are proposing that these factors be included during initial discovery of AMPs by screening.

This will presumably give rise to a large number of relevant AMPs that can enter the pipeline, which will increase the probably of finding a few that can be developed into systemic drugs.

Toxicity Against Mammalian Cells

AMPs bind to anionic mammalian cells through electrostatic interactions[1, 48, 49, 50] and have interfacial activity[41]. As a result, many have at least some acute toxicity due to permeabilization of the plasma membrane. Toxicities vary significantly among known AMPs, yet those with relatively little toxicity still have relatively poor therapeutic indices compared to conventional antibiotics. Some AMPs may become less toxic in the presence of serum or when host cells are highly concentrated, although this effect is rarely tested.

In the abundant literature on AMPs, researchers often measure lysis of erythrocytes (hemolysis) as a surrogate for eukaryotic cell toxicity. This is an easy assay and its widespread use provides some uniformity in the AMP literature. However for the maximum sensitivity to toxicity, nucleated cells in culture may be a more sensitive and more informative model system. Nucleated cells can also respond to mechanisms of toxicity other than acute cytolysis. While some researchers have discussed how some AMPs may have useful selective activity against cancer cells[48, 51], we argue that cancer cells would make an especially stringent test system for selecting against toxicity because they are especially sensitive to AMPs. In other words, a synthetically evolved AMP that has potent antibacterial activity under relevant conditions and *no toxicity against mammalian cancer cells* would seem to be an ideal candidate for development into a systemic drug. We discuss how this can be done in a screen below.

Proteolytic Degradation

Chemical stability of peptides, i.e. resistance to proteolysis, which does not come into play in laboratory assays, is also a critical consideration for systemic activity of peptides. Some peptides are degraded rapidly by serum exopeptidases, dipeptidases and other proteases[7, 52, 53]. Sensitivity to serum proteolysis is partially predictable based on sequence. In peptide drug development, serum stability competes with synthetic complexity (i.e. manufacturing cost). Shorter, linear, L-amino acid peptides are most economical to produce, but are susceptible to rapid proteolysis, while cyclic, crosslinked or chemically modified peptides are more costly to produce but are resistant. Proteolysis is a pervasive threat to AMPs. For example, we have shown that washed human RBCs contain a very high concentration of multiple proteases in their cytosol[7]. Incubation of a set of natural and synthetic linear AMPs with dilute RBCs leads to rapid degradation of peptide if there is even a small amount of hemolysis, which is almost always true. Cytosolic amino-and carboxy-exopeptidases removed amino acids one or two at a time from both termini[7]. For this reason, even standard hemolysis assays in the laboratory may be strongly affected by the proteolytic sensitivity of peptides.

It is likely that host cell binding and serum protein binding will decrease susceptibility to degradation, but as stated above they may also interfere with activity. Cyclization or

crosslinking, as found in many natural AMPs will reduce proteolysis. For linear AMPs chemical modification of the termini[52, 54, 55] non-natural terminal amino acids or selective substitution with D-amino acids can increase stability[54]. Perhaps the simplest approach is to replace all residues with D-aminoacids, as this will provide complete resistance to proteolysis while not changing activity[1, 2, 42]

Synthetic Molecular Evolution

To create an AMP that could have useful, systemic (*in vivo*) antibacterial activity, the factors describe above will need to be simultaneously optimized. Specifically it will be necessary to i) maximize selectivity for binding to bacteria over serum proteins and host cells; ii) maximize bactericidal activity of bound peptide; iii) minimize susceptibility to proteolytic degradation; iv) minimize residual cytotoxicity and v) maximize solubility under physiological conditions. Thus, the design of a systemically-active AMP is like a puzzle in which each of these coupled factors must be simultaneously minimized or maximized without negatively affecting the others. Yet, other than proteolytic susceptibility, the sequence-structure-function relationships for none of these factors are understood well enough to make useful predictions or to enable rational engineering. This is why most new AMPs described in the literature are either identified from natural sources, or are discovered in the laboratory by simple trial and error under standard conditions.

How can one simultaneously optimize these various factors when they are incompletely coupled and when the molecular mechanism are not understood in enough molecular detail to enable rational design? In this chapter, we discuss how this might be done using synthetic molecular evolution (SME). By this we mean iterative screening of rationally designed peptide libraries that are based on known AMPs and are designed using the known physical principles to allow rational variations in library members. SME is useful for the development of AMPs when screening is done under conditions that mimic the environment in which a systemically-active peptide must function. We call it “evolution” because it is most economical to screen iteratively such that each “generation” of gain-of-function AMP is selected from a library built around an active sequence from the previous generation, and each iterative screen further refines the selected sequences to have the properties that are sought. In this case we seek bactericidal activity at low μM concentration in the presence of concentrated host cells and serum, without toxicity.

Design and Synthesis of Combinatorial Peptide Libraries

Although there are many ways to synthesize and screen peptide libraries[14, 56–63] we focus here on the approach we have taken recently to identify membrane active peptides with specific properties[2, 3, 38–40, 63–67]. We design small, iterative libraries of 10–30,000 members that are based on a template sequence with known activity. Using a library of this level of diversity means that we can design a library synthesis scheme that provides a relatively large amount of each library member to work with. This, in turn, enables us to use much more complex screens and it allows us to screen the same library member in multiple parallel assays, which is needed to screen for bactericidal activity against multiple microbes as well as toxicity against host cells.

Our approach to library synthesis and quality control is well described in many papers[2, 3, 38, 61, 63–69]. In short, a photocleavable linker is added to Tentagel-NH_2 Megabeads, followed by library construction using the split and recombine approach[58]. Quality control is assured with HPLC, mass spec and sequencing performed on multiple individual beads. Each bead contains about 1 nmol of peptide which is released by UV light providing 100 μl of a 10 μM solution. This is sufficient peptide to perform multiple parallel assays on each library member. Screening of such small iterative libraries has long been routine in the laboratory[2, 3, 38, 61, 63–69] and requires no special robotic instrumentation.

High Throughput Screening for Antibacterial Activity

As we envision the discovery of systemically active AMPs, screening must be done under conditions that most closely mimic the conditions experienced by a peptide antibiotic *in vivo*. At a minimum, there must be a high concentration of host cells and a high concentration of serum that has been heat inactivated to eliminate activated complement proteins. RBC also contain a very high concentration of proteases that are somewhat different that serum proteases[7], so any screen with RBCs and/or serum will contain many realistic proteases. We have been experimenting with using human red blood cells (RBCs) as a host tissue analog. This is advantageous because it is easy to procure large amounts of fresh, concentrated human RBCs.

In Figure 5 we show the effect of preincubation of 1×10^9 RBC/ml on the antimicrobial activity of a set of natural and synthetic AMPs. In many cases, but not all, RBCs inhibit the antimicrobial activity when AMP are preincubated for a few minutes with RBCs. We subsequently showed that this inhibition is due to two things: RBC proteases, released by background autolysis or by a small amount of direct hemolysis, and direct interactions of AMPs with the cells that reduce the pool of available AMP by competition. Some peptides are more susceptible to the former and some are more susceptible to the latter[1]. Any AMP that will maintain antibacterial activity *in vivo* will need to be resistant to both proteolysis and direct host cell inhibition.

Radial diffusion

There are a number of antimicrobial assays that can be used in a high throughput screen format. Here we will discuss the strengths and weaknesses of some of them in the context of SME. Radial diffusion is an assay in which a thin bacteria-seeded agar layer is overlaid with a sterile, nutrient rich agar enabling a lawn of bacteria to grow between the layers, except where growth is inhibited in a zone around a locally applied antibiotic. For peptides to be tested in the presence of host cells and serum, a small hole can be made in the lower agar layer and a small volume of peptide mixture can be introduced. After allowing some time for peptide diffusion into the agar, the nutrient overlay is added and the plate is allowed to grow overnight. The following day, the lawn of bacteria is visible and the zones of inhibition are readily observed and quantitated, as shown in Figure 5. Radial diffusion has the advantage that it is readily adapted to high throughput, and that it is quantitative. A larger zone of inhibition is related to better activity. However, the size of the zone of inhibition may also be strongly affected the ability of the antibiotic to diffuse in agar rather than by its inherent

antibacterial activity. MIC-based quantitation by radial diffusion using serial dilution circumvents this problem, but cannot be accomplished in a screen. Further, radial diffusion reports on inhibition of bacterial growth, which does not necessarily indicate sterilization, a more desired property. In Figure 5 we show some examples of radial diffusion-based screens of a peptide library simultaneously against the Gram negative *Escherichia coli* and against the Gram positive *Staphylococcus aureus.* Some library members inhibit only one or the other species, while some inhibit both.

Incorporation of concentrated host cells and serum is easily accomplished in radial diffusion, as they can be mixed with peptide prior to introduction of the whole mixture into the well in the agar layer. In Figure 6 we show the effect of concentrated human RBCs on Radial diffusion against *S. aureus*. While powerful and quantitative, we have found that the most significant fault of radial diffusion as a screening method is that it does not necessarily select for peptides with sterilizing activity. To overcome this barrier, we also have developed screens based on broth sterilization, which we describe next.

Broth sterilization

Broth sterilization is an unambiguous, all-or-none assay for *sterilization*. Broth assays are done in liquid media inoculated with bacteria and treated with antibiotic. After overnight incubation, cultures are assayed for absence or presence of live bacteria. Survival of any bacteria generally means that they will grow to a high density after overnight incubation, while sterilized cultures will remain sterile. These two outcomes can easily be measured with optical density, and sterility can be verified by plating the sterile culture on nutrient agar and noting the presence or absence of colony forming units (CFU). Broth sterilization assays can be modified by the addition of concentrated host cells and heat inactivated serum to test for sterilization under physiologically relevant conditions. When concentrated host cells, such as concentrated human RBCs, are used the growth of bacteria cannot be measured directly by turbidity, so a secondary plate can inoculated and allowed to grow overnight. Alternately, aliquots can be spotted on nutrient agar and CFUs can be counted.

Broth dilution assays are not quantitative in a high throughput screen. They are binary tests in which library members will either be positive or negative for sterilization under the conditions of the screen. Typically screens can only be done under one condition for each library member tested. We propose testing antibiotic activity against multiple species simultaneously limiting the amount of each library member available. Thus it is critical to adjust the stringency such that a small number of leads are identified. The stringency of a broth sterilization assay can be modified by adjusting the inoculum size, antibiotic concentration, incubation time or other factors. A possible scheme for a broth dilution screen is shown in Figure 7, along with the results of screening members of an AMP library using broth dilution.

Reduction of Colony Forming Units

CFU reduction is a hybrid assay that enables counting of live bacteria remaining in a solution after antibacterial treatment. It essentially reports on the same phenomenon as broth dilution, yet can be done with less labor and in less time. In this assay, which is readily

adapted to high throughput, bacteria and antibiotic, with host cells and/or serum, are incubated together for an amount of time that enables killing, and then are spotted on a nutrient agar plate at high nominal CFU counts and grown overnight. In the absence of bactericidal activity, a dense mat of bacteria will grow. However, when only a small fraction of bacteria survive, or none at all, a countable number of colonies will grow, a quantitative result that can be used to rank order AMPs in a screen. CFU reduction assays can readily be modified by the addition of host cells and serum, as above. In Figure 8 we show a possible scheme for SME using CFU counts along with the results of a test screen of AMPs from a library. Note that positive, sterilizing sequences can readily be identified by the absence of CFUs, which amounts to more than 4 logs of CFU reduction.

Cytotoxicity and hemolysis

In a screen for antimicrobial assays under physiological conditions, toxicity must also be measured simultaneously. This can be accomplished in assays that are done in the presence of RBCs as host cells by also measuring hemolysis. However, hemolysis may not be sensitive enough to be useful, especially in the presence of concentrated serum which can be protective protective. Here we suggest that toxicity be measured in parallel using sensitive human cancer cell lines, such as HeLa cells such that peptides with very low toxicity can be identified during the screen.

ESKAPE Pathogens

While many bacteria can infect humans and harbor drug resistance, there are a small set that account for the majority of morbidity and mortality[16, 70]. These include *Clostridium difficile*, often associated with gastrointestinal infections, and the *ESKAPE* pathogens, whose acronym indicates ***E****nterococcus faecalis*, ***S****taphylococcus aureus*, ***K****lebsiella pneumonia*, ***A****cinetobacter baumannii*, ***P****seudomonas aeruginosa*, and the **E**nterobacteriaceae, which includes *Escherichia, Salmonella, Vibrio* and *Shigella* species, among others. Since some AMPs have variable potencies against these different organisms, thus screening for the broadest activity must be done against multiple species simultaneously. We previously screened against two ESKAPE bacteria, *E. coli* and *S. aureus*, and a fungus, *Cryptococcus neoformans*, simultaneously and found very low overlap in activities. This enabled the identification of the rare peptides with broad spectrum activity[2]. We suggest screening in parallel against S. aureus two Gram negative ESKAPE pathogens.

Future Prospects

The physical chemistry-based action of AMPs on bacteria leads to broad-spectrum activity and difficulty in evolving resistance, which accounts for some of the appeal of AMPs as potential drugs. Yet, these same properties also drive nonspecific interactions with serum protein and host cells, which reduce the effectiveness of AMPs. In this chapter, we have presented the concept of synthetic molecular evolution as an tool to enable the discovery of AMPs, early in the development pipeline, that are less affected by host cell and serum protein binding. We remain hopeful that this new approach will finally enable AMP researchers to bridge the gap between the laboratory bench and the clinic.

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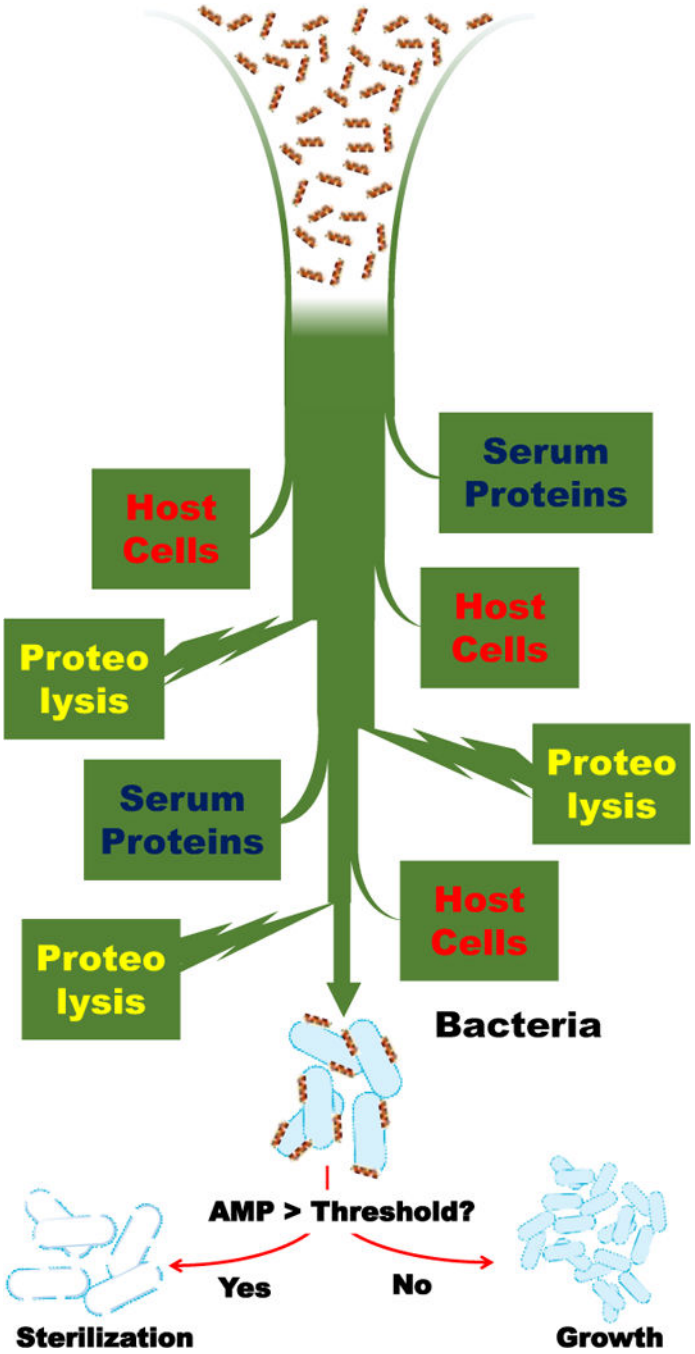


Figure 1.

Some impediments to the bioavailablity and systemic activity of antimicrobial peptides. Antimicrobial peptides must accumulate significantly on bacteria to have bactericidal activity. In the body, interactions with host cells and tissue, interactions with serum proteins and proteolytic degradation can decrease accumulation, and decrease activity.

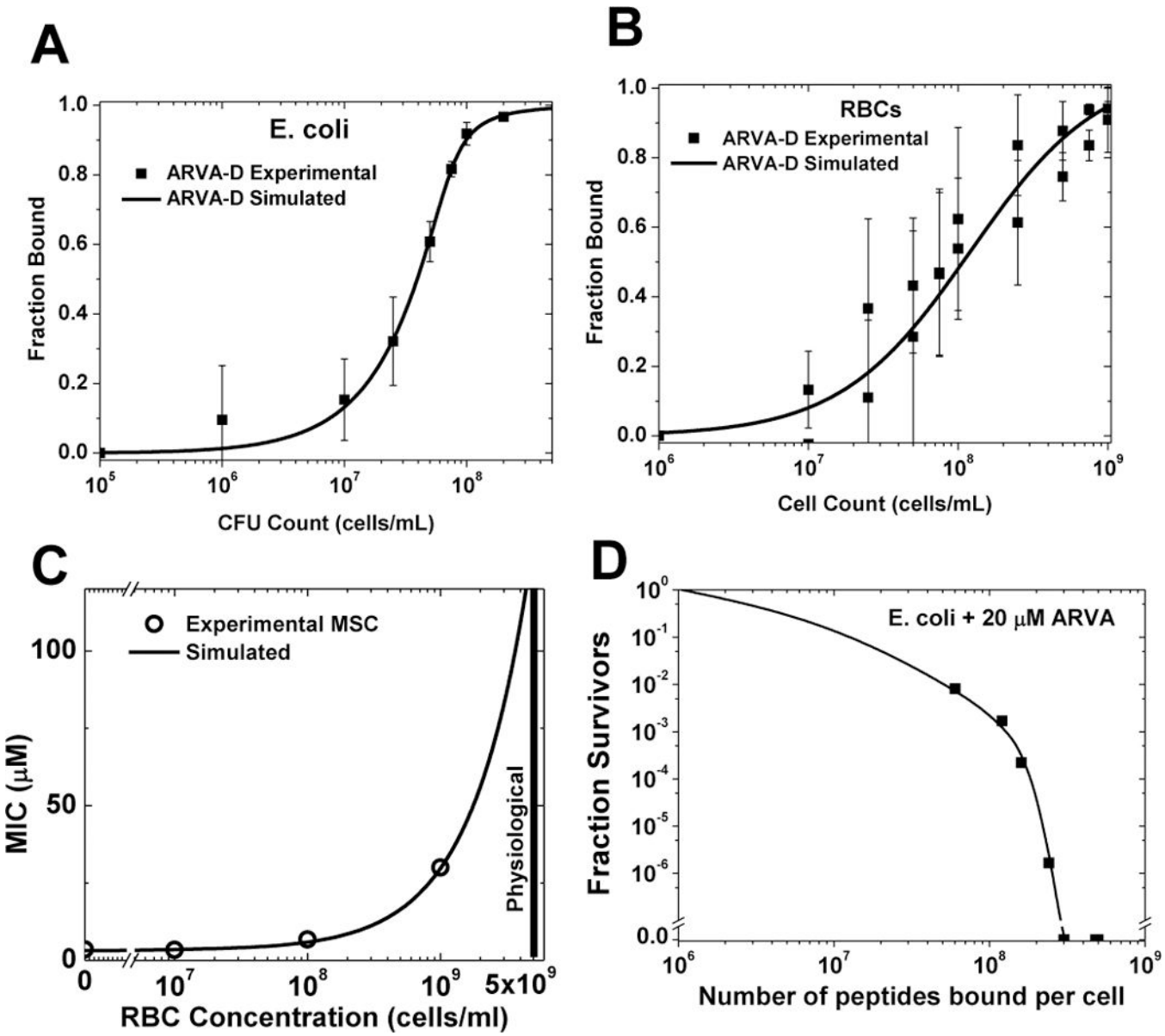


Figure 2.

Saturation-dependent activity of an antimicrobial peptide and its inhibition by host cells. The protease resistant AMP D-ARVA (rrgwalrlvlay-NH_2) was used in these experiments[1–3]. **A:** Measured binding of D-ARVA to E. coli cells. **B:** Measured binding of D-ARVA to human RBCs. **C:** Experimental measurements compared to simulation of a mixed experiment assuming simple competition between E. coli and RBC shows that the measured binding accounts for the loss of activity when host cells are present. **D:** Survival of different innocula of E. coli incubated with 20 μM D-ARVA in conjunction with the binding curve in panel A, enables comparison of peptide lethality and the number of peptides bound to each bacterial cell. More than 2×10^8 peptides bound per cell are required for sterility.

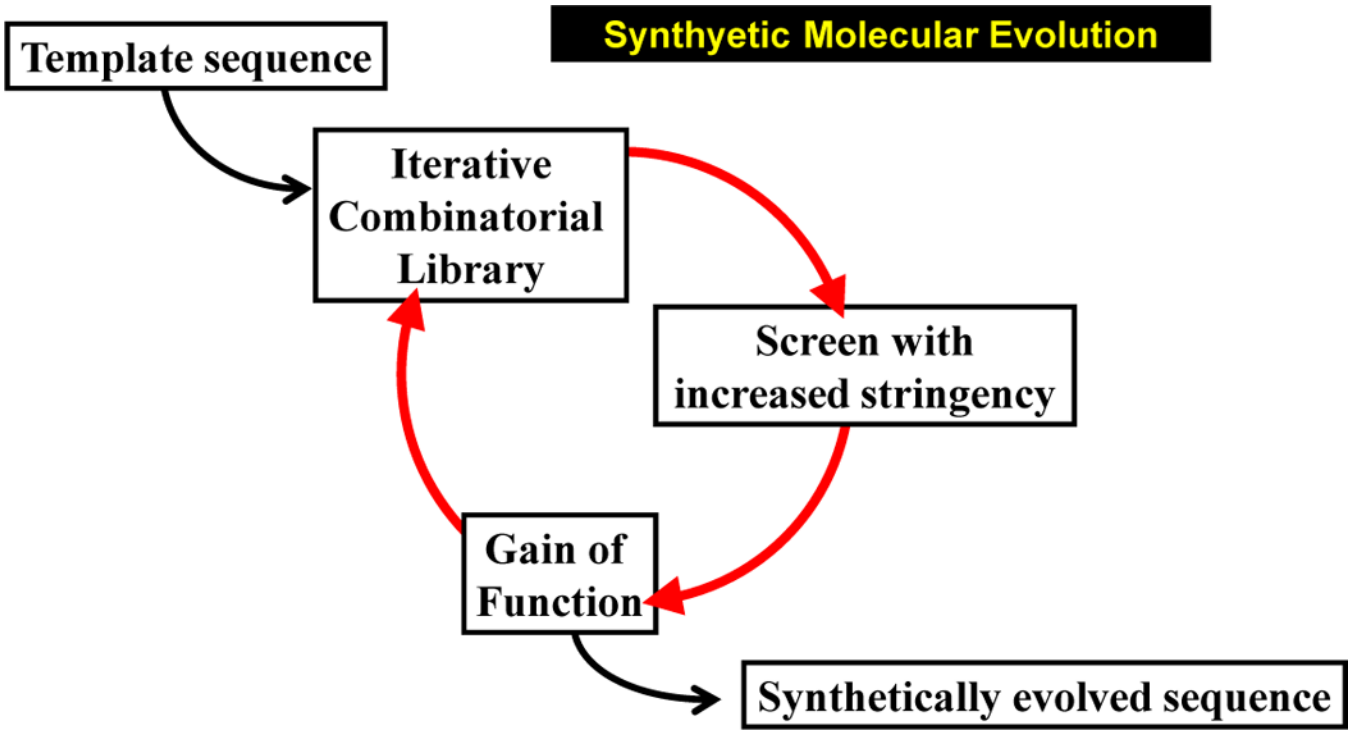
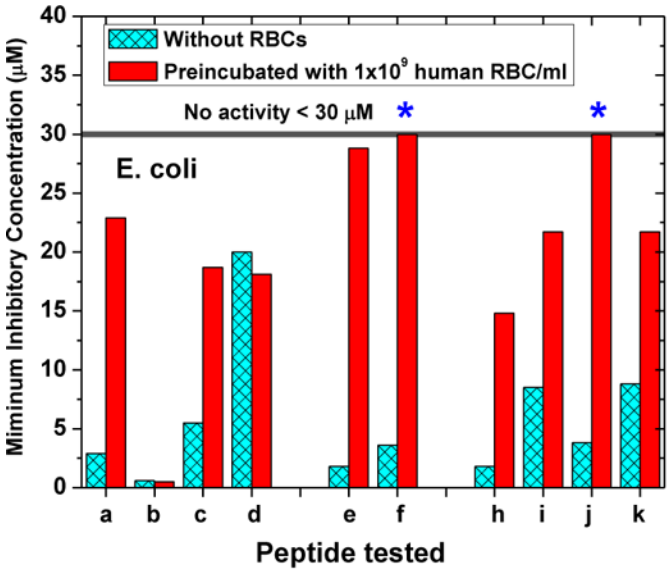
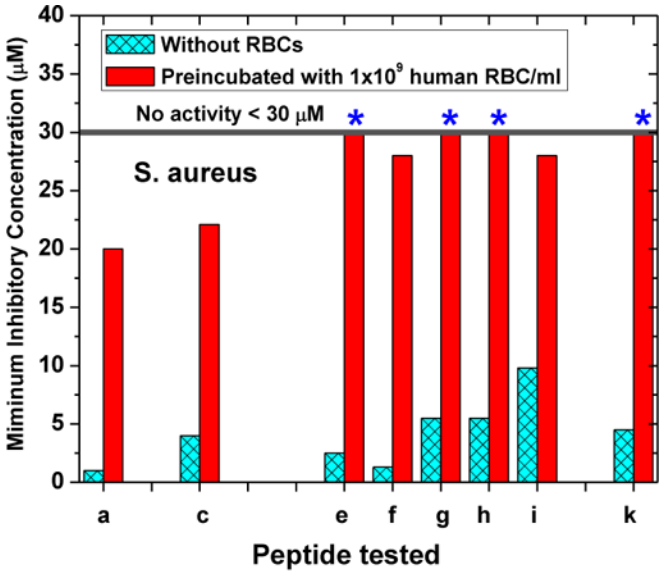


Figure 3.
Synthetic molecular evolution of peptides. As we practice it, SME utilizes multiple small libraries (generations) which are iteratively screened for gain of function daughter sequences.





	Natural Peptides	
a	Melittin	*Apis mellifera* (Honey Bee)
b	Cecropin A	*Hyalophora cecropia* (Moth)
c	Indolicidin	*Bos Taurus* (Mow)
d	LL37	*Homo sapiens* (Human)

	Synthetic Peptides	
e	ARVA-L	Library Screen
f	ARVA-D	Library Screen
g	VVRG	Library Screen
h	NATT	Library Screen
i	MSI-78	Magainin analog
j	MelP5	Melittin analog
k	WLBU2	LL37 derived

Figure 4.

Human Red Blood cells inhibit antimicrobial peptides. As described elsewhere[1] preincubation of natural and synthetic AMPs with 1×10^9 human RBC/ml (2% of physiological concentration) causes inhibition of most, but not all, of them. We have shown that such host cell inhibition is the result of direct RBC binding and also to proteolysis of the AMP by the cytosolic proteases found in human RBCs[7].

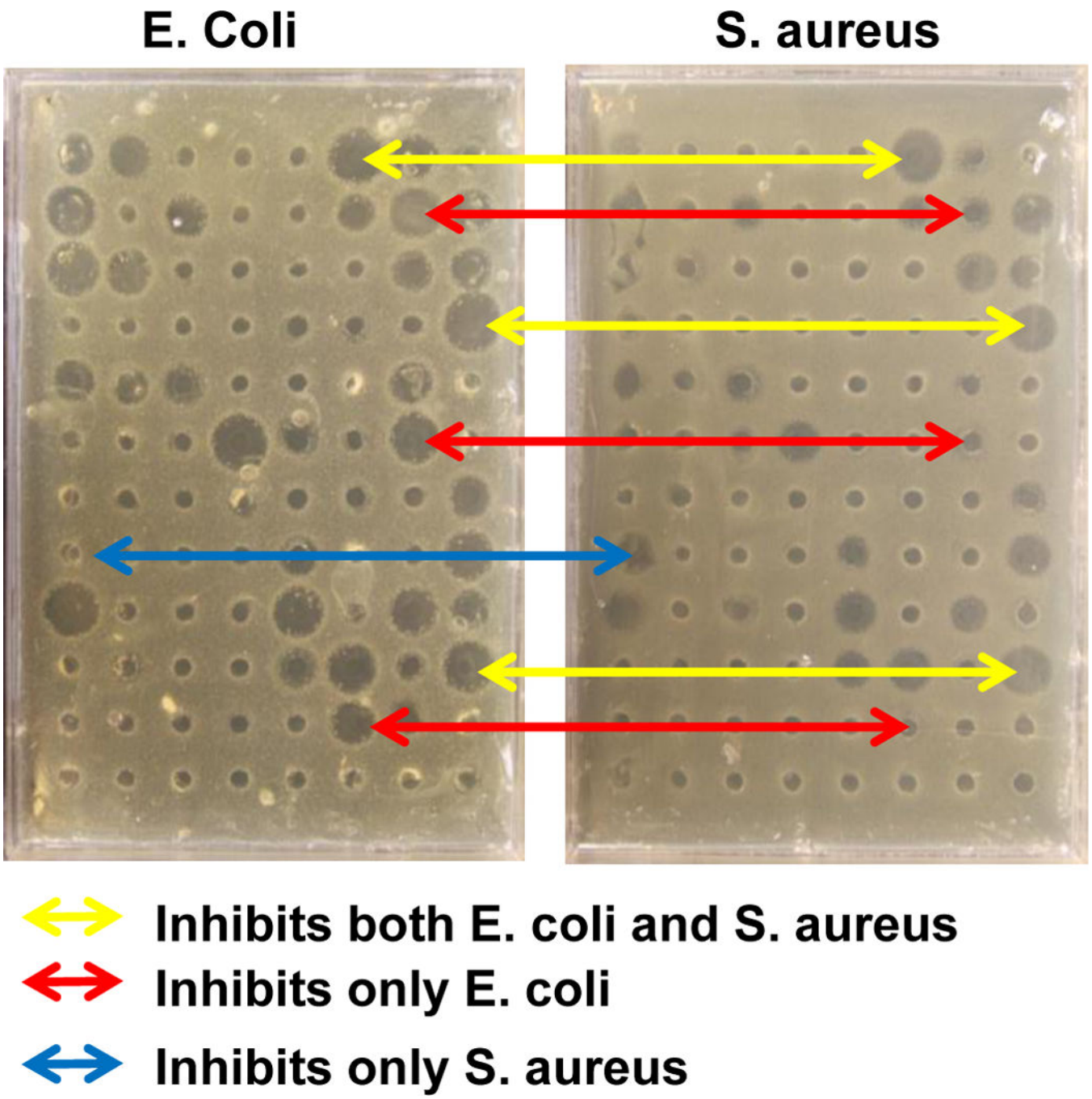


Figure 5.

Example screening of members of a peptide library against two organisms using radial diffusion. The same set of peptide library members were screened again the Gram negative *E. coli* and the Gram positive *S. aureus* with radial diffusion. Zones of inhibition are observed. Some active library members inhibit both bacteria (yellow) while others inhibit only one of the two (red and blue).

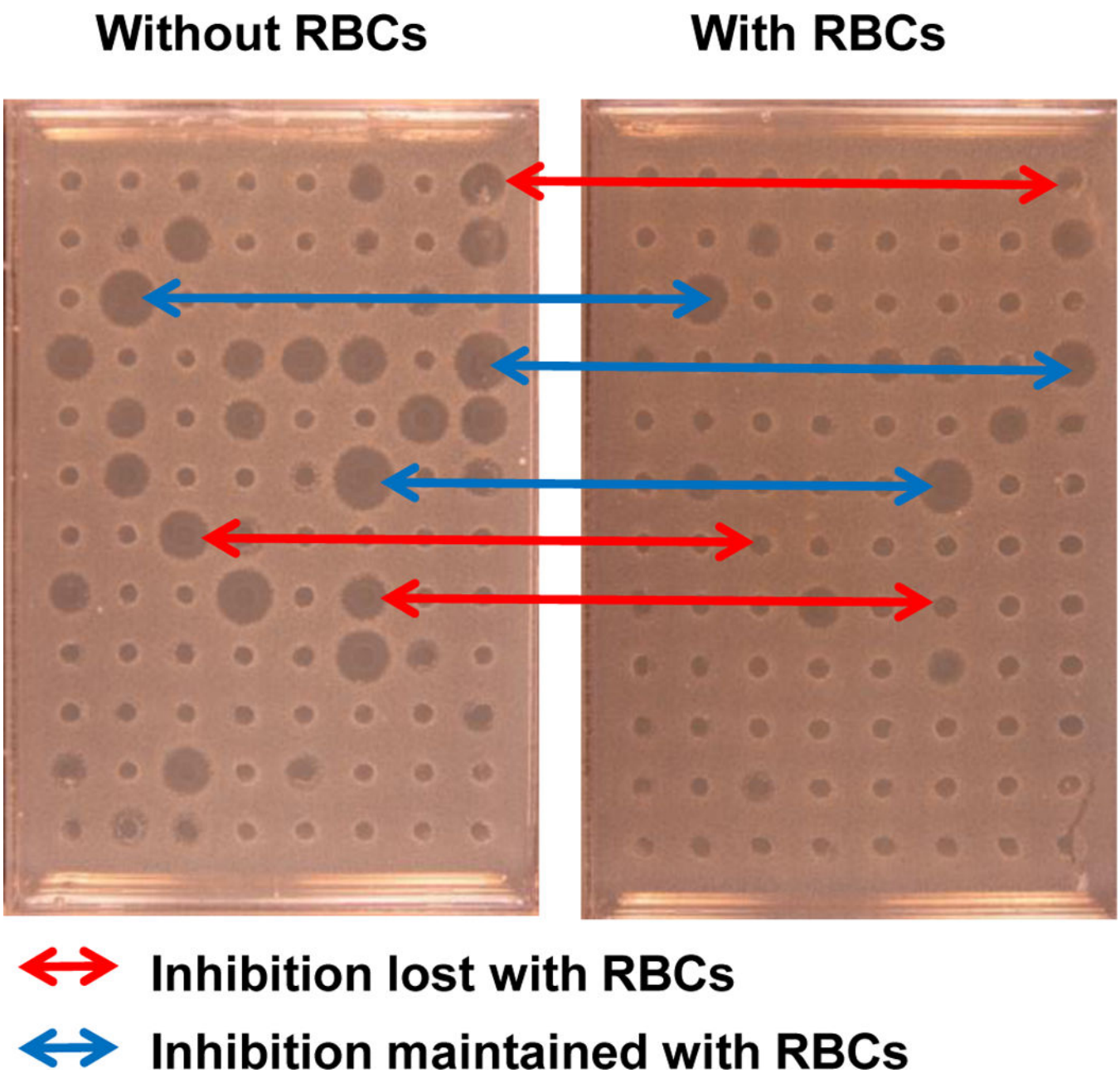
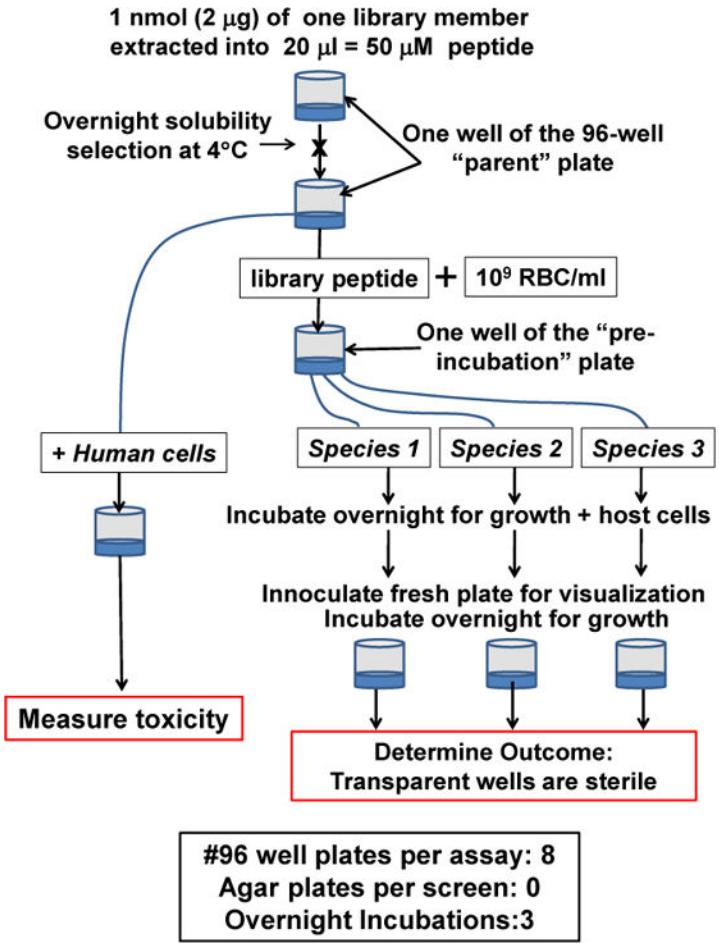


Figure 6.

The effect of human RBCs on the activity of peptide library members against S. aureus using radial diffusion. Example screening of a peptide library *S. aureus* with radial diffusion. Both plates were screened with the same library members. The samples added to the right plate had been incubated with 1×10^9 human RBC/ml prior to use. Some active library members are inhibited by RBCs, while others are not.



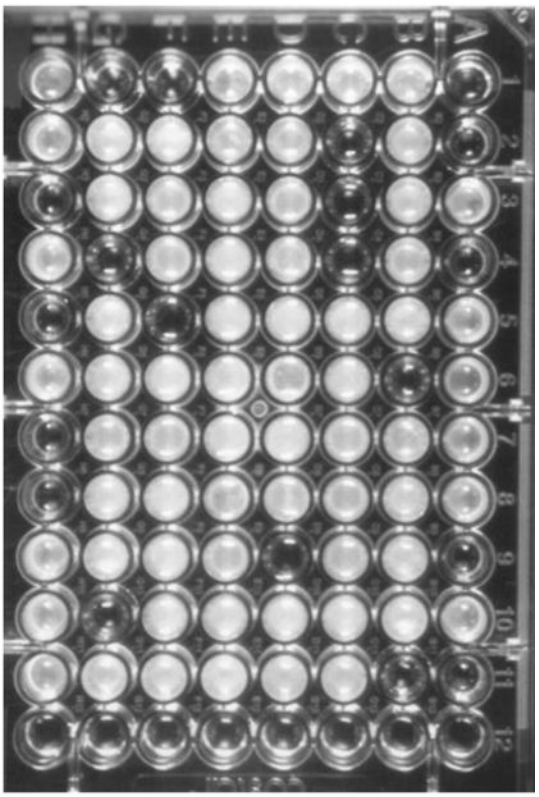
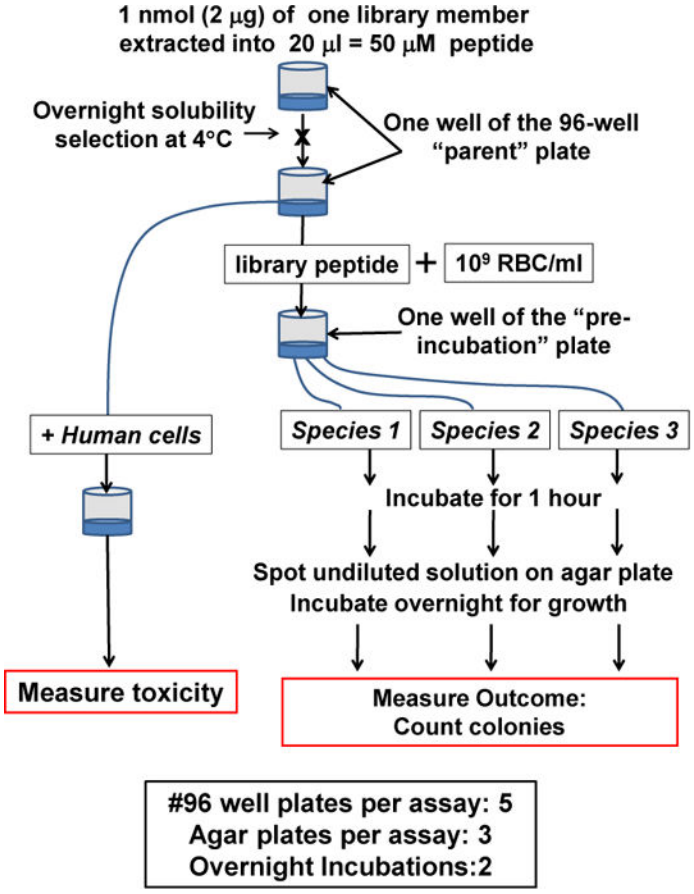


Figure 7.

Peptide library screening using broth sterilization. **Left:** One possible scheme for screening a peptide library for solubility, for physiologically relevant broad spectrum bactericidal activity, and for lack of toxicity. **Right:** Example 96 well plate after screening a library for sterilizing activity against E. coli. Wells are either transparent or opaque. Transparent wells have no colony forming units on nutrient agar, confirming sterility.



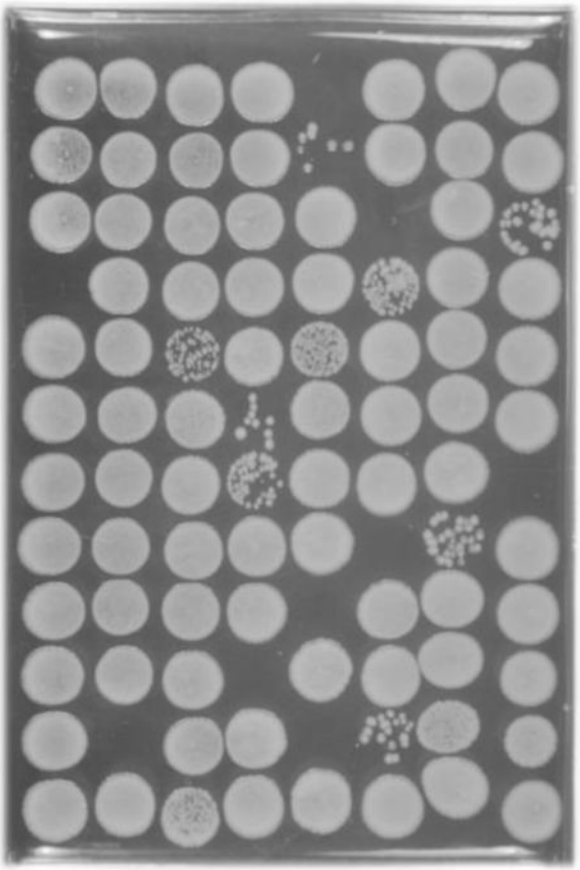


Figure 8.

Peptide library screening using the reduction in colony forming units (CFU). **Left:** One possible scheme for screening a peptide library for solubility, for physiologically relevant broad spectrum bactericidal activity, and for lack of toxicity. **Right:** Example nutrient agar plate after spotting library members mixed with bacteria for 1 hour. Clear spots with no colonies have been sterilized.