Strategies and opportunities for engineering antifungal peptides for therapeutic applications

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Abstract

Antifungal peptides (AFPs) are widely described as promising prospects to treat and prevent fungal infections, though they are far less studied than their antibacterial counterparts. Although promising, AFPs have practical limitations that have hindered their use as therapeutics. Rational design and combinatorial engineering are powerful protein engineering strategies with much potential to address the limitations of AFPs by designing peptides with improved physiochemical and biological characteristics. We examine how rational design and combinatorial engineering approaches have already been used to improve the properties of AFPs and propose key opportunities for applying these strategies to push the design and application of AFPs forward.

Introduction

The number of fungal infections has been steadily rising, due to a variety of societal and medical advances that have increased the number of people susceptible to invasive fungal disease [1,2]. Climate change will likely lead to additional infections, due to the expansion of the range of endemic fungi and the emergence of new pathogens [3-6]. Although fungal infections are a serious health threat on a worldwide scale (as the World Health Organization recently noted in its list of "fungal priority pathogens" [7]), the number of antifungal drugs remains relatively small. Only four classes of drugs—azoles, polyenes, echinocandins, and one pyrimidine analog—are available [8,9]. Treatment of invasive fungal infections with this limited number of drugs can be complicated by side effects from off-target interactions, a lack of effective drugs for some pathogens, and growing resistance of infections to the drugs [8,9]. The challenge of resistance is exemplified by the recent emergence of the pathogen *Candida auris*, with one study showing 41% of *Ca. auris* isolates were resistant to two or more of the four classes of antifungal agents [10]. These challenges highlight the need for new antifungal agents, particularly those with a novel mechanism of action.

One class of molecules that could serve as a potential source for new antifungal agents is antimicrobial peptides (AMPs). While AMPs are more commonly studied for their antibacterial effects, a subset of AMPs have antifungal activity and are termed antifungal peptides (AFPs). In this review, we focus on AFPs formed only from the canonical amino acids, including ribosomally synthesized peptides and those formed by cleavage from larger proteins; we do not review peptides with non-canonical amino acids or peptides produced by nonribosomal peptide synthetases, such as those with lipid moieties (e.g., the echinocandin class of antifungal agents [11]). Of the nearly 18,000 peptides with antibacterial and antifungal activity listed in the Database of Antimicrobial Activity and Structure of Peptides (DBAASP; https://dbaasp.org/), 5,858 peptides are categorized as having antifungal activity [12]. The fungal targets of the AFPs in this database include a variety of fungal pathogens, such as *Cryptococcus neoformans, Candida* spp., *Aspergillus* spp., *Histoplasma capsulatum*, and *Fusarium* spp. AFPs commonly exert their activity by disrupting the cell membrane via the same mechanisms described for antibacterial peptides, including forming barrel-shaped pores in the membrane (barrel-stave model), accumulating on the membrane until the membrane is disrupted (carpet model), or forming toroidal pores in the membrane (toroidal pore model) [13]. AFPs can also exert antifungal activity through interactions with molecules not associated with the cell membrane, including nucleic acids and chitin, but the exact target or mechanism of action is often unknown [13]. Although AFPs are structurally diverse, they are often positively charged [14]. AFPs that interact with the cell membrane tend to adopt secondary structures (α-helical, β-sheet, or mixed) when associated with the cell membrane, and their amphiphilicity, percentage of hydrophobic residues (typically 30-60%), and length (typically 11-40 residues) affect their activity [14]. The novel mechanisms of action of AFPs compared to current antifungal agents and their potentially broad spectrums of activity lead to continued efforts to design new and improved AFPs.

Challenges of developing antifungal peptides as therapeutics

Although AFPs offer a potential source of antifungal agents with novel mechanisms of action, a number of challenges have limited their use, including physiochemical and biological properties. The biological stability of peptide therapies for systemic use has historically been a barrier to commercialization. Peptides are susceptible to proteolytic degradation, which decreases their activity and shortens their half-lives. Typically, proteolysis has been discussed in the context of the human proteases that limit efficacy [15], but the proteases produced by fungal pathogens may also be important in the context of AFPs. For example, the AFP histatin 5 is readily degraded and inactivated by secreted aspartic proteases produced by *Ca. albicans* [16]. An additional physiochemical challenge of AFPs (and AMPs more generally) is that some become inactive when exposed to ions and salt that naturally exist in human bodily fluids [17]. For example, Kerenga et al. showed that many plant defensins lose their effectiveness against *Ca. albicans* at elevated NaCl concentrations [18]. The toxicity of AFPs can also be limiting [19]. To be used as

therapeutics, AFPs need to show selective toxicity toward fungal cells over mammalian cells. While pore formation is involved in the mechanism of action of many AMPs, this route can be nonspecific because of similarities between fungal cells and mammalian cells [20]. A final limitation of AFPs is production at large scales. The chemical synthesis of AMPs is more expensive at large scales than small-molecule drugs, purification from natural sources cannot provide an adequate amount, and the success of recombinant production can be highly variable [14,21]. Protein engineering strategies offer a vast toolbox for overcoming these challenges of AFPs to make them more suitable for therapeutic use.

Engineering strategies

Rational design and combinatorial engineering are two overarching strategies used to engineer proteins and peptides (Figure 1). Both have applications in AFP design to improve on the properties of known AFPs.

Rational design

Rational design is a protein engineering method where modifications are made to an amino acid sequence based on knowledge of a peptide's structure or function. Rational design typically generates a small library of peptide variants that are chemically synthesized, and, due to the limited size of the library, low-throughput assays for peptide characteristics or activity can be used to assess the impact of a design hypothesis (e.g., amino acid substitution, length). Rational design has been widely used to improve the characteristics of AFPs, including antifungal activity, proteolytic stability, and thermal stability.

Commonly, rational design is applied to AFPs by first developing an understanding of structure-function relationships and using this understanding to predict promising peptide sequences. Gu et al. used rational design to improve cremycin-5 by comparing cremycin-5 and several paralogs to determine residues potentially important in antifungal activity [22]. They further isolated important sites using alanine scanning mutagenesis and used this information to design variants with increased fungicidal potency, higher thermal stability, and higher serum stability. In our lab, we have used rational design to improve the proteolytic stability and antifungal activity of histatin 5 [23,24]. We noted that secreted aspartic proteases produced by *Ca. albicans* cleaved histatin 5 at its lysine residues [16], so we designed variants with amino-acid substitutions at these sites. Our work identified variants with little to no proteolytic degradation in the presence of the proteases, and these variants also had improved antifungal activity [24,25]. These examples present only a small number of the many ways that knowledge of peptide structure and function can be used to rationally design peptides with improved characteristics.

Computational tools and methods can facilitate the rational design of AFPs. Web-based tools that report and predict the physicochemical properties of peptides can guide engineering to improve AFPs or evaluate *de novo* peptide designs. Using ProtParam [26], Li et al. predicted the properties of modified versions of the CGA-N46 peptide, an AFP derived from human chromogranin A, and found the derivative CGA-N12 showed reduced hemolytic activity and higher antifungal activity [27]. Molecular dynamics (MD) simulations are an additional tool for rational peptide design, though they require specialized knowledge for implementation. MD simulations can model peptide-membrane interactions, which are important interactions for many AFPs. For instance, Pandey et al. used MD simulations to study the radish defensin peptide RsAFP2, which has activity against *Ca. albicans* [28], and two variants of RsAFP2 (Gly9Arg and Val39Arg) [29]. These simulations showed that the Gly9Arg and Val39Arg substitutions generated increased conformational stability and higher membrane deformation compared to RsAFP2, and that the variants with these substitutions targeted specific lipids in the fungal cell membrane. Incorporating computational tools to guide rational design and help interpret experimental results provides a powerful approach to improve rational design strategies for AFPs.

Combinatorial engineering

Combinatorial engineering of peptides involves the creation of a large library of different peptides, allowing larger portions of "sequence space" to be explored experimentally when screening the library to for desired peptides. For peptide engineering, combinatorial libraries can be a set of totally or partially random peptides with a defined length, and they are traditionally synthesized on polymer resins through solid-phase synthesis [30,31]. While not yet in common use for engineering AFPs and other AMPs, peptide libraries can also be synthesized recombinantly by introducing a library of genes encoding the peptide library into host cells for recombinant production [32].

The library screening method is an important aspect of combinatorial engineering of peptides. The method needs to have a throughput high enough to screen the desired library size effectively. Screening of AMPs commonly takes place in a simple multiwell plate assay, where a single peptide is present in each well. Two types of screening assays are common: the liposome leakage assay and the microbial sterilization method. In the liposome leakage assay (Figure 2A), liposomes containing a fluorescent dye are mixed with a peptide in wells, and the leakage is monitored by measuring fluorescence intensity from the mixture [33,34]. This assay has been widely used to screen AMP libraries [35-37], and, although the focus is typically on AMPs with antibacterial rather than antifungal activity, it has yielded peptides with antifungal activity [36]. The microbial sterilization method (Figure 2B) involves adding a microbial cell suspension to wells that each contain a single member of a peptide library, incubating the cell suspension with the peptides for a period of time, adding growth medium for an overnight incubation, and analyzing which peptides led to growth inhibition [38]. The application of the sterilization method has also focused on antibacterial peptides, but analogous methods would be straightforward for AFPs.

If multiple rounds of library generation and screening are used to iteratively improve peptide characteristics, the process mimics natural selection and is called directed evolution. Work from the Wimley lab provides an excellent example of how directed evolution can be applied to AMPs engineering. In initial work [39], a combinatorial library of 16,384 peptides was designed and synthesized on polymer beads (one peptide per bead) with a photocleavable linker. The library was screened for liposome leakage and antimicrobial activity (including activity against *Cr. neoformans*) and promising peptides were sequenced [38,39]. Based on the results of the screening, two peptides were identified to serve as parents for a second-generation combinatorial library of 28,800 members, and the second-generation library was screened in parallel for antibacterial activity and a lack of hemolysis, yielding five particularly promising peptides [40]. One of these peptides was tested in a mouse surgical wound model and was highly effective at reducing infection [40], highlighting the promise of directed evolution for identifying useful AMPs.

Surface display is a technology for library screening where peptide variants are anchored to the surface of a cell or phage particle via a protein on the surface (Figure 2C), with yeast surface display [41] and phage display [42] being the most common types. The peptides are genetically fused to the surface protein and expressed from a plasmid, with each cell or particle displaying only a single peptide variant. Because the cell/particle retains the plasmid encoding for the peptide expressed on the surface, the sequence of the peptide can easily be recovered by sequencing. The use of surface display technologies in AMP engineering has focused on identifying peptides based on their binding to the surface of bacterial or fungal cells [43-48]. The identified peptides have rarely been evaluated for antifungal activity, but the extension of this strategy to identifying AFPs is straightforward.

As with rational design, computational strategies can be employed in combinatorial engineering, though they are less common than computational strategies using rational design principles. One example that illustrates the application of computational strategies to combinatorial design of AMPs is the work of Yoshida et al., who combined a genetic algorithm (which introduces variation into a parent sequence), machine learning, and experimental evaluation to direct the evolution of an AMP [49]. They started by synthesizing and testing the antibacterial activity of 93 peptides with similarity to a temporin peptide with moderate antibacterial activity. These data were used to train a model to identify the approximate effects

of amino acid substitutions on improvement of antibacterial activity and generate a fitness matrix based on amino acid residue and location in the peptide. Using this matrix, beneficial amino acid substitutions were predicted, a new set of 90 peptides was synthesized and tested, and the fitness matrix was updated. Three rounds of this optimization process were performed, generating peptides with up to a 160-fold increase in antibacterial activity compared with the starting peptide. This example highlights the success of computationally incorporating diversity into AMP sequences, though additional work is needed to direct these strategies toward AFPs.

Opportunities to improve antifungal peptides via peptide engineering

Peptide engineering strategies will continue to address the challenges associated with AFPs and push their application toward the clinic. In general, more emphasis needs to be placed on peptides that target fungal pathogens, given work on AMPs that target bacterial pathogens is much more prevalent. Here, we describe key opportunities to apply protein engineering strategies to improve AFPs and their practical use.

Targeting molecules specific to fungal cells

As discussed earlier, fungal cells have many potential targets for AFPs, but a primary target has been the fungal cell membrane. Fungal and mammalian membranes have very similar lipid compositions with a few differences in sphingolipids and phosphatidylinositol concentrations and different major sterols (ergosterol in fungal cells and cholesterol in human cells) [20]. Because of these similarities, specificity of AFPs is critical for their commercialization. Designing AFPs that exclusively target phosphatidylinositol or ergosterol can translate into toxicity in mammalian cells due to insufficient specificity [20]. To help avoid undesired interactions, AFPs could be engineered to target other components of the fungal cell membrane, such as glucosylceramide, mannosyl-di-(inositol-phosphoryl)-ceramide, and inositol phosphorylceramide, which are not produced in mammalian cells [20]. The naturally evolved AFP histatin 5 provides inspiration for additional fungal-specific targets. While its mechanism of toxicity to fungal cells is not fully elucidated, histatin 5 binds to the Ssa1p and Ssa2p cell wall proteins and is transported into the cell via the fungal polyamine transporters Dur3p and Dur31p [50]. Specifically engineering peptides to target these proteins or others specific to the fungal cell wall or cell membrane could lead to novel peptides and distinct mechanisms of action compared to those of known peptides and antifungal agents. An additional opportunity for expanding the targets available to AFPs is to engineer peptides with cell-penetrating motifs [51-54], which would make intracellular targets accessible to peptides that are not capable of crossing the cell membrane on their own. For example, the resistance of *Ca. glabrata* cells to histatin 5 can be overcome by expressing the *Ca. albicans* Dur3p and Dur31p transporters in *Ca. glabrata* [55], suggesting that attaching a cell-penetrating motif to histatin 5 could similarly improve transport into the cell and overcome resistance. The strategy of using a cell-penetrating motif could expand the peptides that could be used as AFPs to peptides that bind to novel intracellular targets but lack cell permeability on their own. Identifying new targets for AFPs could help alleviate the challenges associated with antifungal drug resistance that are amplified by the limited cellular targets of current antifungal agents.

Development of novel screening methods for directed evolution of antifungal peptides

Few combinatorial methods have applied directed evolution to engineering AMPs, and even fewer have focused on peptides that target fungal pathogens. In general, directed evolution of AMPs has not been able to take advantage of the large peptide libraries possible with surface display, due to a lack of high-throughput screening methods to identify peptides that cause cell death. Screens that require a separate peptide in each well of a multiwell plates are not practical for taking advantage of the large library sizes that can be generated for use in phage display (10^{10}–10^{11} variants) or yeast surface display (10^{8}–10^{9} variants) [56,57]. Additionally, AFPs require interactions with the cell membrane or intracellular targets that may not be possible while attached to a surface. Releasing the candidate AFPs from the surface via

cleavage allows peptides to readily interact with and enter cells, but it also destroys the physical linkage to the gene encoding for the peptide that allows simple determination of the peptide sequence. Development of new high-throughput screening workflows that can incorporate very large libraries and assay for death of fungal cells, while maintaining a facile and inexpensive way to recover the peptide sequence, will allow the true capabilities of directed evolution to be applied to AFP engineering.

Tucker et al. have developed a workflow for AMPs with antibacterial properties that makes progress toward this goal [58]. A library of ~800,000 peptides was displayed on the surface of *E. coli* cells by genetically tethering the library members to an outer membrane protein via a long peptide linker. The linker allowed the displayed peptides to interact with the membrane of the cells they were displayed on. By using deep sequencing to study the cells displaying the library before and after expression of the library was induced, they were able to identify the library members that were depleted after expression; this depletion indicated stronger antimicrobial activity. While the screen would not work for peptides with intracellular targets, continued development of creative approaches like this and applying them explicitly to AFPs will be important to harness the power of combinatorial design for engineering AFPs.

Expansion of the use of computational tools in engineering antifungal peptides

Experimental studies of AFPs are necessary for understanding *in vitro* and *in vivo* activity, but computational tools complement these experimental studies to help design new AFPs and understand mechanisms of action. As we gain more experimental data for structure-function relationships for AFPs, machine learning will become a powerful tool to sift through the data and identify patterns that can be applied to improve existing AFPs and design *de novo* peptides. Machine learning has already been implemented in AFP engineering [49,59], but the relatively small amount of data for AFPs compared to peptides that target bacterial species limits its capabilities. The use of MD simulations in AFP design will also enable improved AFPs, through an improved understanding of their molecular interactions with cells. MD simulations could be particularly useful in understanding the molecular basis for specificity (or lack of specificity). For example, MD simulations can study how peptides interact with membranes containing different lipids and different lipid compositions [60,61], ultimately leading to an understanding of the peptide characteristics that lead to highly specific activity. Computational tools that are accessible to non-expert users would further improve their ability to contribute to AFP engineering.

Conclusion

AFPs are widely accepted as promising prospects to treat and prevent fungal infections; however, naturally existing AFPs have not been able to fulfill this promise. By employing peptide engineering strategies, including rational design and combinatorial engineering, we can overcome some of the barriers to clinical applications of AFPs and design new, improved AFPs to address the clinical need for an expanded repertoire of antifungal agents.

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The authors developed a high-throughput workflow for engineering AMPs that combines surface display on bacterial cells with deep sequencing to identify peptides that are active against the cells they are displayed on. This work could serve as the inspiration for new approaches to combinatorial AFP engineering.

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Figures and figure captions

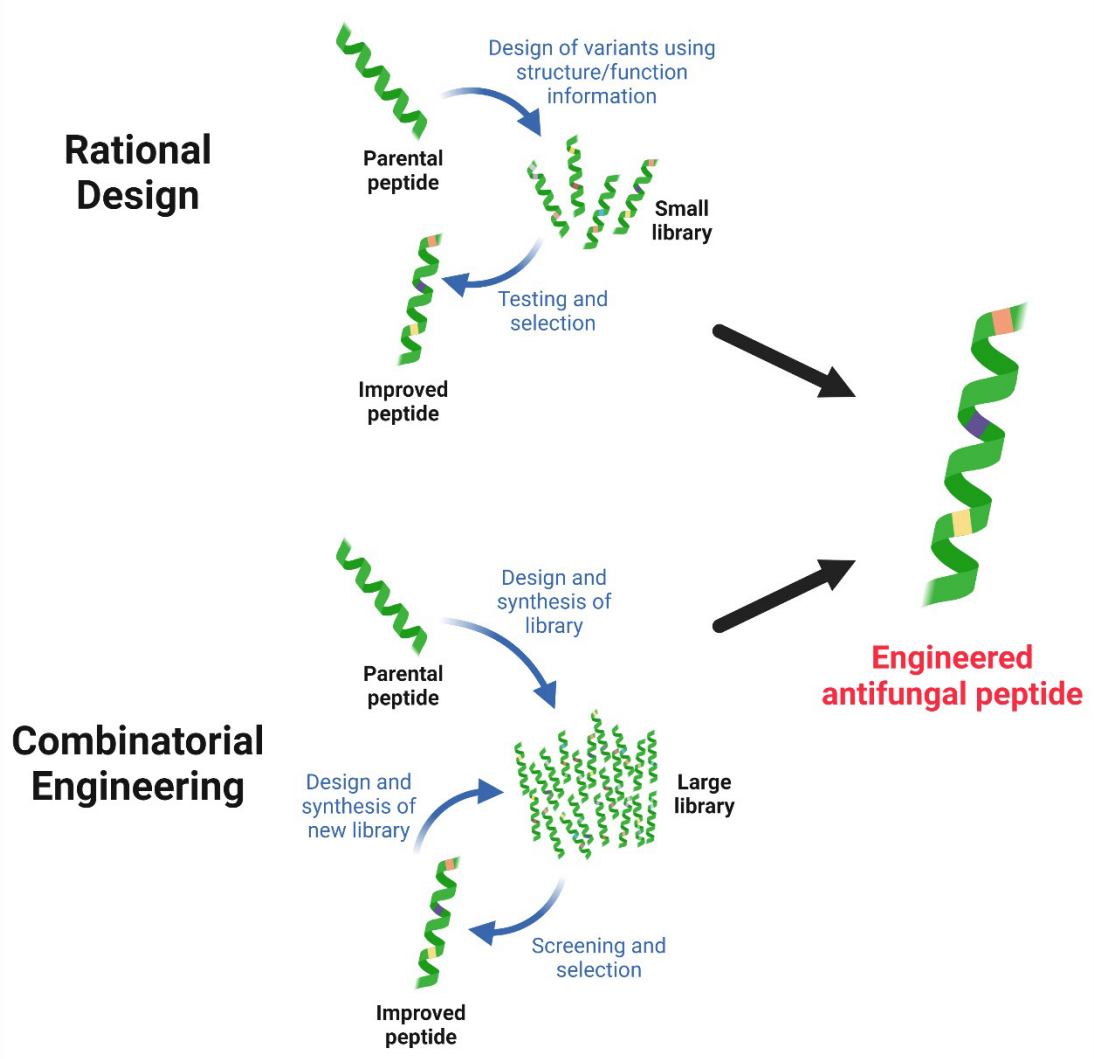

Figure 1. Engineering strategies for AFPs. Rational design and combinatorial engineering approaches can be used to improve the properties of AFPs.

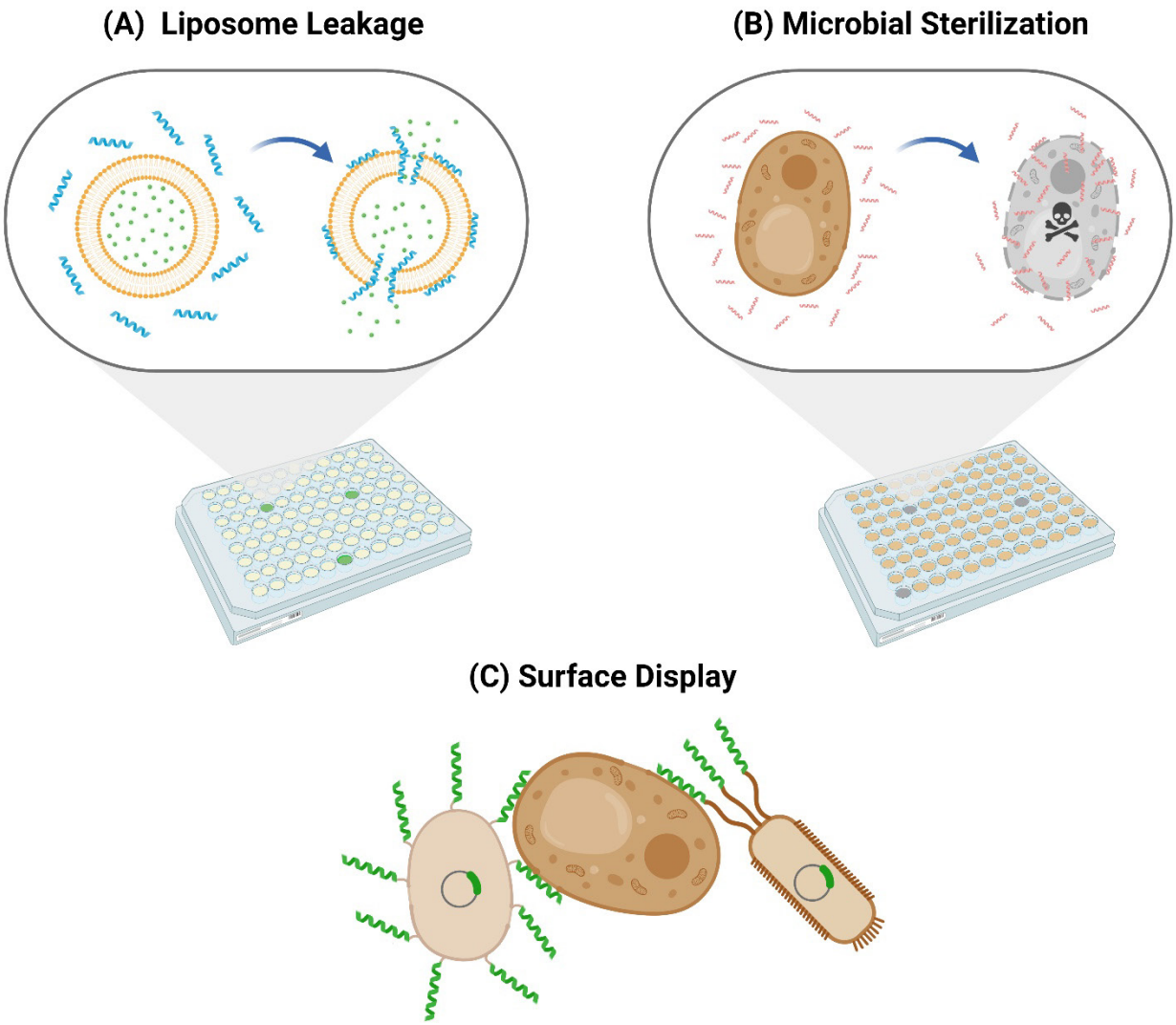

Figure 2. Screening methods for combinatorial libraries. (A) Liposome leakage assays identify peptides that cause membrane disruption by looking for leakage of a fluorescent dye from the liposomes following incubation with a peptide. The assay is performed in a multiwell plate, with one peptide per well. (B) The microbial sterilization method involves mixing fungal cells and peptides in a multiwell plate, with one peptide in each well. Wells containing peptides with antifungal properties are identified by looking for reduced growth of fungal cells. (C) Cell surface display approaches tether the library to the surface of cells or phage, with one peptide displayed on the surface of each cell or phage particle. Displayed peptides that bind to target cells are isolated, allowing recovery of the peptide sequence from the encoding plasmid in the cell or phage particle.