Membrane-Disrupting Molecules as Therapeutic Agents: A Cautionary Note

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ABSTRACT: Mechanistic studies have shown that aggregates of a common membrane disrupting molecule, Triton X-100, destroy the integrity of cholesterol-rich phospholipid bilayers via a catastrophic rupture process. In sharp contrast, attack on such membranes by monomers of Triton X-100 destroys their integrity through mild leakage events. This discovery of duplicity in the destruction of membrane integrity by a membrane-disrupting molecule has led to the design of derivatives of Amphotericin B that exhibit a lower tendency to aggregate and antifungal and hemolytic activities that are well-separated. An animal study with one such derivative has shown that its efficacy is similar to that of Amphotericin B but with substantially reduced toxicity. A related study of a series of derivatives of \( \text{L-phenylalanine} \) has revealed that monomers possess significant antibacterial activity, while aggregates of these same molecules exhibit hemolytic as well as antibacterial activity. Taken together, these experimental findings point to the need for paying special attention to differences in the selectivity between monomeric and aggregated forms of membrane-disrupting molecules as therapeutic agents, where monomers are expected to be the more selective species. Whether improving the selectivity of antimicrobial peptides and other antimicrobial agents is also possible by reducing their tendency to aggregate, and whether membrane-disrupting molecules can be created that exploit differences in the lipid composition between coronaviruses and mammalian cells, are two important questions that remain to be answered.

KEYWORDS: biomembrane, antibacterial, antifungal, antiviral, antibiotic, monomer, drug design, therapeutics

INTRODUCTION

The need for new classes of drugs is apparent by the recent emergence of SARS-CoV-2, the virus that has led to the COVID-19 pandemic. While not at the same level of urgency as SARS-CoV-2, the need for creating new classes of antibacterial and antifungal agents has also become urgent due to the evolution of drug-resistant forms of bacteria and fungi. The motivation for this Perspective is based on both of these needs. In essence, I wish to focus on one aspect of the design of membrane-disrupting molecules as therapeutic agents that has received surprisingly little attention, i.e., the consequences of aggregation of an attacking species on its selectivity features. Although emphasis is placed on antibacterial and antifungal agents, this Perspective also includes some brief discussion of coronaviruses (CoVs) and the possibility of exploiting modest differences in their lipid composition relative to that of the plasma membranes of mammalian cells.

THE ANTIMICROBIAL RESISTANCE PROBLEM

The world is now facing a crisis in treating infectious diseases due to the emergence of drug-resistant forms of bacteria and fungi. Of special concern are certain Gram-negative bacteria that have become resistant to virtually all antibacterial agents that are
agents are known to exert their cytotoxic effects on organisms, leaving the hands of small companies and academic laboratories who rely on public and philanthropic funding. A special issue of *ACS Infectious Diseases* has discussed this challenging problem in some detail.3−9

### WHY MEMBRANES AS TARGETS?

Our own efforts regarding antimicrobial agents have been built around one central hypothesis, i.e., the development of antimicrobial resistance toward agents that operate at the membrane level should be less significant than those agents that must be internalized to exert their toxic effects. Thus, by not having to enter a fungal or a bacterial cell to destroy it, a therapeutic agent should circumvent two of the common mechanisms of resistance, i.e., efflux mechanisms and enzymatic degradation within the cell. An example of an antimicrobial agent that supports this thinking is the membrane-disrupting macrolide antibiotic Amphotericin B (Chart 1). Thus, despite its broad use in treating systemic fungal infections for more than 50 years, resistance toward this antibiotic has proven to be extremely rare.10,11

Because antimicrobial peptides are naturally occurring host defense agents, one might expect them to be robust in terms of fending off resistance.12,13 Similar to Amphotericin B, these agents are known to exert their cytotoxic effects by destroying the integrity of plasma membranes. One class of antimicrobial peptides that has attracted special interest in this regard is the pentacationic lipopeptides. For example, Polymyxin B is now considered to be a "last resort" antibiotic for the treatment of multidrug resistant Gram-negative bacterial infections (Chart 2).

What is common to Amphotericin B and all of these antimicrobial peptides is that they are known to act via membrane disruption. Another common feature is that the selectivity of these agents in destroying target membranes is generally low. Thus, despite its importance in treating systemic fungal infections, Amphotericin B is now regarded as one of the most toxic antibiotics that is currently in use.

While many quaternary ammonium compounds are effective in destroying Gram-negative bacteria by disrupting their plasma membrane, such molecules are not considered as therapeutic agents, also because of their low membrane selectivity and high toxicity. Instead, most quaternary ammonium compounds are of special interest for use as disinfectants.14 In principle, if it were possible to improve the membrane selectivity of quaternary ammonium compounds through derivatization, they could become an important new class of antimicrobial drugs. One recent report appears to add some encouragement along these lines. Thus, it has been shown that Polymyxin B can be coupled to a "membrane-active quaternary ammonium warhead" and used to target Gram-negative bacteria with reduced toxicity toward mammalian cells.15

### MEMBRANE DISRUPTION VIA SELF-ASSEMBLY

Most of the current models for membrane disruption by antimicrobial agents involve self-assembly processes in one form or another. For example, a popular hypothesis for the disruption of fungal cell membranes by Amphotericin B is based on the "barrel-stave" model in which several Amphotericin B molecules combine with ergosterol to form lethal pores (Figure 1).16,17

![Figure 1. Barrel stave models where Amphotericin B (gray oval) combines with ergosterol (red rectangle) to form (A) a single pore or (B) two aligned water-filled pores.](https://dx.doi.org/10.1021/jacsau.0c00037)

Another popular model for membrane disruption is the "carpet" model. Here, a series of disruptive molecules is thought to aggregate on the surface of a target membrane, insert into the bilayer, and create defects or pores along with mixed-micelle-like structures (Figure 2).18

### MEMBRANE ATTACK BY MONOMERS VERSUS AGGREGATES

Although a great deal of attention has focused on the types of self-assembled aggregates that may be responsible for destroying the integrity of bacterial and fungal cell membranes, remarkably little attention has been paid to the steps leading up to their formation, i.e., whether the initial attack on the membrane comes in the form of monomers or aggregates and whether it makes any difference which is the attacking species in terms of the agent’s selectivity features.

In previous mechanistic studies with model systems, we have shown that the common membrane-disruptive agent Triton X-100 can destroy the integrity of compact lipid bilayers (e.g., cholesterol-rich liposomes made from 1-palmitoyl, 2-oleoyl-sn-glycerol-3-phosphocholine/cholesterol) by two distinct path-
ways depending on whether it attacks as monomers or as aggregates. Specifically, monomers were found to promote leakage processes, while aggregates favored the catastrophic rupture of the membrane. These conclusions were based on experiments that were carried out in which the concentration of Triton X-100 and the target membranes were carefully controlled, and the release of encapsulated 5(6)-carboxyfluorescein (CF) identified as being due to (i) “all or none” processes (where some of the liposomes release all of their encapsulated CF and others release none, and (ii) leakage processes in which all of the liposomes release some of their encapsulated CF. In our report, we noted related findings by Bolard and coworkers where aggregates of Amphoterin B were found to be toxic to both human erythrocytes and fungal cells, but monomers were toxic only to fungal cells.

**SEPARATING ANTIMICROBIAL FROM HEMOLYTIC ACTIVITY BY RATIONAL DESIGN**

Based on our mechanistic studies and also Bolard’s related findings, we hypothesized that the hemolysis of red blood cells by Amphoterin B involves rupture pathways that result from attack by aggregates of this agent. We also hypothesized that derivatives of Amphoterin B, which are less prone to aggregation, should exhibit higher selectivity in destroying fungal cells. To test these hypotheses, we synthesized a series of conjugates of Amphoterin B (i.e., 1, 2, and 3) in which an increase in the number of pendant ethylene glycol units resulted in a steady increase in hemolysis. Potassium release measurements confirmed that concentrations below the cac of each of these macrolides were cytotoxic toward C. albicans. These encouraging results led us to carry out an animal study to test the in vivo efficacy of one of these conjugates, 2, in treating mice that were infected with C. albicans. In brief, in vivo testing of five clinical isolates of C. albicans revealed a potency for 2 that was similar to that of Amphoterin B (Table 1). Of special significance was the fact that 2 was well-tolerated up to 30 mg/kg of body weight per dose, a dosage that would be lethal in the case of Amphoterin B.

It should be noted, in this regard, that liposomal formulations of Amphoterin B that are currently in use (e.g., Ambisome) are generally favored over classic micellar solutions derived from sodium deoxycholate because they are less toxic. Although the
Gram-negative bacteria is significant for bacteria where the potential across the inner membrane of potential is another important factor for the selective destruction tendency to aggregate. It should also be mentioned that membrane appear that considerable progress in this area should be possible.

Membrane (M), and nucleocapsid (N) proteins (Figure 4).28 targets for SARS-CoV-2, including the spike (S), envelope (E), and not the plasma membrane, their cholesterol content may be significantly lower than that found in plasma membranes.31,32 As a frame of reference, the cholesterol content in H9 plasma membranes corresponds to ca. 33 mol % of the total lipids present.33 To the best of my knowledge, actual cholesterol concentrations in coronaviruses have not been firmly established. In past studies, we have shown that certain surfactants (e.g., 4) are capable of destroying the membrane integrity of liposomes having relatively low cholesterol concentrations (ca. 25 mol %) (Chart 4).34 We have also shown that with modestly higher cholesterol concentrations (ca. 33 mol %), the membrane-disrupting activity of 4 was sharply reduced.34 In these earlier studies, no effort was made to distinguish between monomer versus aggregate activity. Whether agents of this type could be used to disrupt the integrity of coronavirus membranes, selectively, is an important question that should be answered. One possible mechanism for antiviral action in such a case would involve changes in the lateral pressure in the lipid envelope and changes in conformation and/or the lateral organization of membrane proteins that are necessary for fusion with mammalian cells.35 Another possible mechanism could involve the transport of ions across the lipid envelope and the removal of an osmotic gradient that is needed to drive membrane fusion. While these ideas are speculative, given the seriousness of the COVID-19 pandemic, I believe they are well worth exploring.

### The Lipid Envelope of Coronaviruses as Targets

Over the past three decades, SARS, MERS, and most recently COVID-19 have emerged from novel CoVs, and all three have resulted in varying degrees of mortality in humans. The three causative CoVs (SARS-CoV, MERS-CoV, and SARS-CoV-2) have all demonstrated pandemic potential and, tragically, this potential has now been realized in the case of COVID-19. An urgent need for therapeutic agents that can combat COVID-19 as well as future CoV outbreaks is apparent.27

To date, several viral proteins have been identified as potential targets for SARS-CoV-2, including the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins (Figure 4).28 However, little attention has been paid to the virus’s lipid envelope as a possible target.28–30

Because coronaviruses bud from a pre-Golgi compartment and not the plasma membrane, their cholesterol content may be significantly lower than that found in plasma membranes.31,32 A frame of reference, the cholesterol content in H9 plasma membranes corresponds to ca. 33 mol % of the total lipids present.33 To the best of my knowledge, actual cholesterol concentrations in coronaviruses have not been firmly established. In past studies, we have shown that certain surfactants (e.g., 4) are capable of destroying the membrane integrity of liposomes having relatively low cholesterol concentrations (ca. 25 mol %) (Chart 4).34 We have also shown that with modestly higher cholesterol concentrations (ca. 33 mol %), the membrane-disrupting activity of 4 was sharply reduced.34 In these earlier studies, no effort was made to distinguish between monomer versus aggregate activity. Whether agents of this type could be used to disrupt the integrity of coronavirus membranes, selectively, is an important question that should be answered. One possible mechanism for antiviral action in such a case would involve changes in the lateral pressure in the lipid envelope and changes in conformation and/or the lateral organization of membrane proteins that are necessary for fusion with mammalian cells.35 Another possible mechanism could involve the transport of ions across the lipid envelope and the removal of an osmotic gradient that is needed to drive membrane fusion. While these ideas are speculative, given the seriousness of the COVID-19 pandemic, I believe they are well worth exploring.

### The Take-Home Message

Membrane-disrupting molecules in their monomeric state can have very different selectivity features as compared with aggregated forms. At present, all of the existing evidence points toward monomers as being the more selective membrane-disrupting species.

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Notes
The author declares no competing financial interest.

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