Bioconjugate Chemistry

Communication

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Simple Strategy for Taming Membrane-Disrupting Antibiotics

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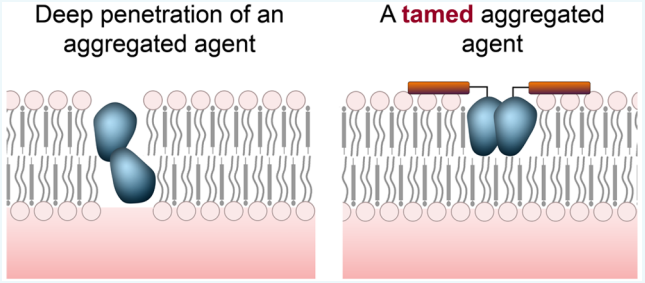
Supporting Information

ABSTRACT: A strategy has been devised for increasing the cellular selectivity of membrane-disrupting antibiotics based on the attachment of a facially amphiphilic sterol. Using Amphotericin B (AmB) as a prototype, covalent attachment of cholic acid bound to a series of α,ω-diamines has led to a dramatic reduction in hemolytic activity, a significant reduction in toxicity toward HEK293T cells, and significant retention of antifungal activity.



The need to create new antibiotics to combat drug resistant forms of microbes has now become urgent.[1,2] This need has presented medicinal chemists with a formidable challenge, i.e., how to rationally design antibiotics that are highly active in destroying disease-causing microbes without being highly toxic to mammalian cells.

With this challenge in mind, we have focused our efforts on those agents that are capable of killing microbes by disrupting their outer membranes. Our working hypothesis has been that the development of resistance toward such drugs should be less than for those that must be internalized to exert their toxic effects. More specifically, we believe that export mechanisms of resistance and enzymatic degradation should be less important for drugs that operate within biological membranes.[3] A classic example of a membrane-disrupting drug that supports this thinking is the naturally occurring heptaene macrolide antibiotic, Amphotericin B (AmB).[4−6] Thus, despite its broad use in treating fungal infections for more than 50 years, resistance to this antibiotic has proven to be extremely rare. Unfortunately, AmB remains as one of the most toxic drugs in clinical use.[7,8] One feature that is common to all membrane-disrupting antibiotics is their reliance on hydrophobic interactions for biological activity. In the case of AmB, a specific affinity appears to exist toward ergosterol, i.e., the dominant sterol found in fungal membranes. However, the basis for this affinity, and the precise mechanism (or mechanisms) of action of AmB remain as a matter of debate.[4,9,10]

Several years ago, we and others reported that *aggregated forms of AmB have very different biological action as compared with its monomer*.[11,12] Thus, whereas monomers were found to be highly selective in destroying fungal cells, aggregated forms were highly toxic to both mammalian and fungal cells. Recently, the selectivity of certain membrane-disrupting antibacterial agents has also been found to be dependent on their aggregation state, where monomers were the more selective species.[13] We believe these sharp differences in biological action between monomer and aggregates is directly related to the difference that we found for the action of monomers and aggregates of Triton X-100 on cholesterol-rich liposomes.[14] Specifically, whereas attack by monomers resulted in membrane leakage, attack by aggregates led to a catastrophic rupture of the membrane. Taken together, these findings have led us to posit that the low selectivity of membrane-disrupting drugs, in general, is a likely consequence of rupturing action by aggregated forms.

Based on this hypothesis, we have been pursuing two distinct approaches for increasing the cellular selectivity of membrane-disrupting agents: (i) raising their critical aggregation concentration (cac) via chemical modification such that the monomers are favored at therapeutically relevant concentrations, and (ii) chemically modifying them so that their aggregates have weaker rupturing power.[12,15] We refer to the latter as "taming".[15] Here, we report a minimalistic approach for taming a membrane-disrupting antibiotic that is much simpler than a design principle and modifying agents (i.e., molecular umbrellas) previously used.[15]

A detailed monolayer study of a variety of bile acid/phosphocholine mixtures has provided compelling evidence that these sterols are oriented with the long axis of the steroid nucleus lying parallel to the surface of the monolayer; i.e., the bile acids lie flat at the membrane/water interface.[16] This finding suggested to us that such molecules could be used as "floats" that would help prevent deep penetration of aggregates of membrane-disrupting agents, thereby reducing their rupturing power (Figure 1).

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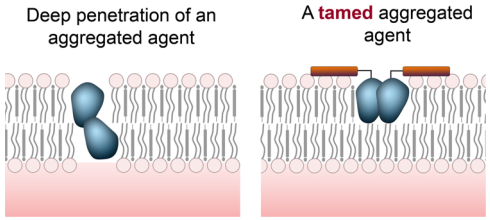
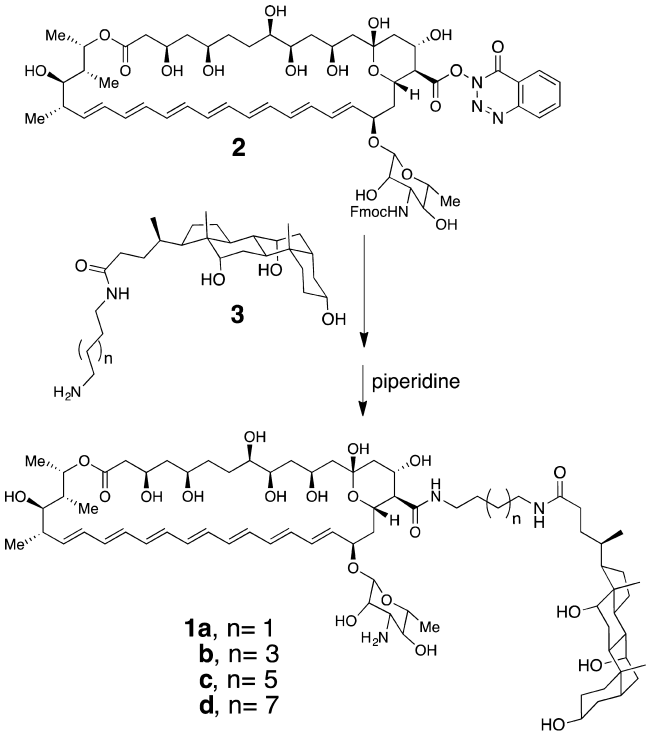


Figure 1. Stylized illustration showing (left) an aggregate of a membrane-disrupting agent penetrating deeply into a cholesterol-rich mammalian membrane and (right) a tamed aggregated agent that is held close to the membrane surface by a facial amphiphile.

To test this concept using a clinically important antifungal agent, we synthesized four cholic acid derivatives of AmB, where the length of the hydrophobic linker was varied, systematically, i.e., **1a**, **1b**, **1c**, and **1d** (Scheme 1). Although

Scheme 1



we suspected that the biological properties of such conjugates would be sensitive to the length of the linker used, exactly how this variable would influence antifungal activity and membrane selectivity was difficult to predict, hence the need for testing. Each conjugate was readily prepared by condensing an activated and protected form of AmB (**2**) with the corresponding α,ω-diamine that had been monoacylated with cholic acid.[15]

In Figure 2 is shown a plot the apparent molar absorptivity (λ_{max} 409 nm) of **1a** as a function of the reciprocal of its concentration in PBS at 37 °C. As discussed elsewhere, the intercept of two straight lines that can be drawn from such a plot affords an estimate of the conjugate's critical aggregation concentration (cac).[12] Based on such measurements, the cac values for **1a**, **1b**, **1c**, and **1d** were all found to be ca. 1 μM, which is the same as that found for AmB, itself.[12]

An evaluation of the minimum inhibitory concentrations (MICs) for AmB, **1a**, **1b**, **1c**, and **1d** against *C. albicans*, *C. glabrata*, *C. neoformans*, and *C. gatti* was then carried out. Our results are shown in Table 1. The two conjugates having the shortest chains (**1a** and **1b**) exhibited broad-spectrum antifungal activities that compared favorably with AmB for all of the fungi tested. Those conjugates having longer hydrocarbon spacers showed high antifungal activity against some, but not all, of the fungi tested. Three of the conjugates (**1a**, **1b**, and **1c**) exhibited clinically relevant antifungal and fungicidal activity (Supporting Information).

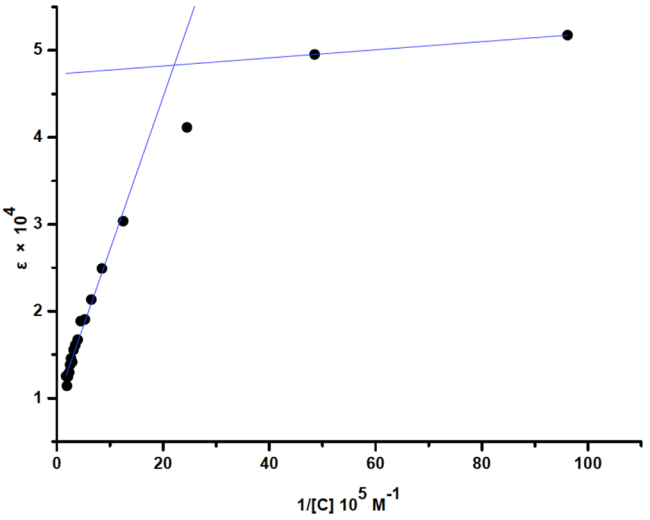


Figure 2. Plot of apparent molar absorptivity (λ_{max} 409 nm) as a function of the reciprocal concentration of **1a** in PBS at 37 °C.

Table 1. Antifungal Activity

	MIC (μg/mL)[a]				
Microbe	AmB	**1a**	**1b**	**1c**	**1d**
C. albicans	0.5	1	2	2	>16
C. glabrata	0.5	2	2	>16	>16
C. neoformans	0.3	1	1	1	2
C. gatti	0.3	1	1	1	1

[a]MIC values are the lowest concentrations required for completely inhibiting fungal growth.

In Figure 3 are shown plots of the percent release of hemoglobin from sheep red blood cells as a function of the concentration of AmB, **1a**, **1b**, **1c**, and **1d**. Whereas AmB was highly hemolytic (requiring 4 μM of the conjugate for 50% hemolysis, EH_{50}), the corresponding EH_{50} value for **1a** was more than 2 orders of magnitude higher; i.e., 465 μM. An even greater reduction in hemolytic activity was found for conjugates **1b**, **1c**, and **1d**, where close to negligible hemolysis was observed at concentrations as high as 600 μM.

A comparison of the toxicities of AmB, **1a**, **1b**, **1c**, and **1d** toward HEK293T cells is shown in Figure 4. In brief, AmB exhibited significant toxicity at ca. 1 μg/mL and was highly toxicity at ≥25 μg/mL. In sharp contrast, both **1c** and **1d** had negligible toxicities at concentrations as high as 100 μg/mL. In the case of **1a**, cytotoxicity was apparent at ca. 1 μg/mL, but its toxicity was significantly lower than AmB at all concentrations tested. At concentrations up to 25 μg/mL conjugate **1b** was even less toxic than **1a**. However, at concentrations greater than 25 μg/mL, the toxicity of **1b** approached that of **1a**.

The large separation of hemolytic activity from antifungal activity found in **1a**, **1b**, **1c**, and **1d** clearly demonstrates that

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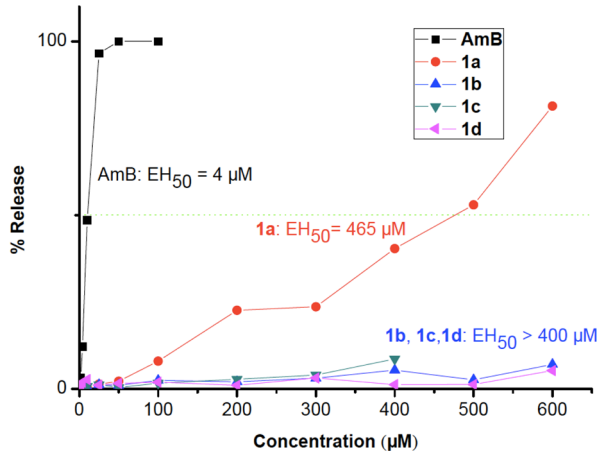

Figure 3. Plot of percent release of hemoglobin from sheep red blood cells as a function of concentration of AmB, **1a**, **1b**, **1c**, and **1d** at 37 °C in PBS, pH 7.4. AmB data taken from ref 15.

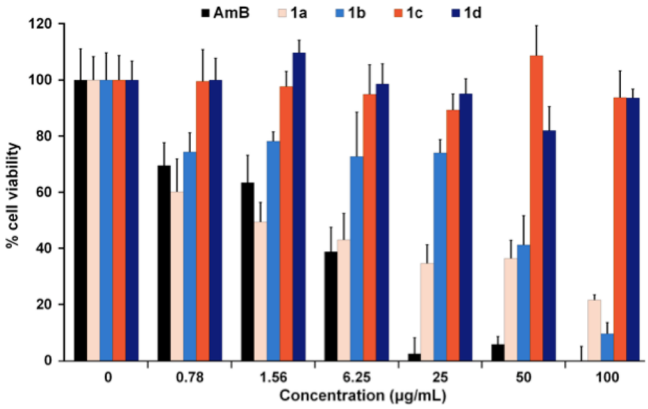

Figure 4. Bar graph showing the viability HEK293 T cells in the presence of varying concentrations of AmB and **1a**, **1c**, and **1d**.

the AmB molecule has been tamed by covalent attachment to the choloyl moiety. The fact that there is negligible hemolytic activity for **1b**, **1c**, and **1d** at concentrations that are well in excess of their critical aggregation concentrations further implies that the rupturing power of their aggregated forms (i.e., their ability to release the large hemoglobin molecule of ca. 65 kDa) has been greatly reduced. In the case of **1a**, this taming effect is not as dramatic as with **1b**, **1c**, and **1d**. One possibility for weaker taming action may be that the relatively short linker causes individual AmB moieties to be misaligned in the bilayer, resulting in greater rupturing power (Figure 5). Finally, it should be noted that the taming associated with **1a**, **1b**, **1c**, and **1d** with respect to HEK293T cells shows the same trend as that found with red blood cells; i.e., those conjugates having the longer hydrocarbon spacers (**1c** and **1d**) show the greatest degree of taming.

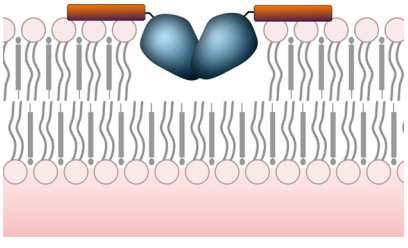

Figure 5. Stylized illustration showing a hypothetical misaligned aggregate of a membrane disrupting agent held near the surface of a lipid bilayer.

Although the generality of this taming strategy remains to be established, the effects seen here with AmB (where toxicity toward mammalian cells has been dramatically reduced while significant antimicrobial activity has been retained), together with its simplicity, provides considerable incentive for exploring its applicability to other classes of membrane-disrupting antibiotics. Such efforts are currently underway in our laboratories.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.6b00629.

Experimental procedures used for chemical synthesis and physical measurements (PDF)

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Notes

The authors declare no competing financial interest.

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