Bioconjugate Chemistry

Communication

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Taming Amphotericin B

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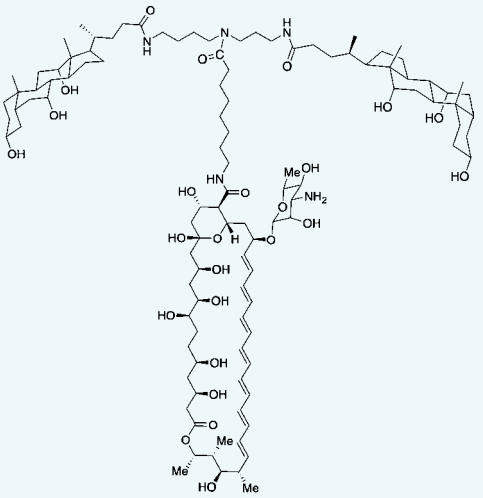
Supporting Information

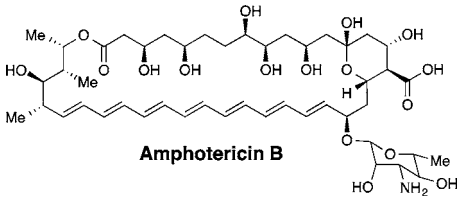
ABSTRACT: A strategy is introduced for enhancing the cellular selectivity of Amphotericin B (AmB) and other classes of membrane-disrupting agents. This strategy involves attaching the agent to a molecular umbrella to minimize the disruptive power of aggregated forms. Based on this approach, AmB has been coupled to a molecular umbrella derived from one spermidine and two cholic acid molecules and found to have antifungal activities approaching that of the native drug. However, in sharp contrast to AmB, the hemolytic activity and the cytotoxcity of this conjugate toward HEK293 T cells have been dramatically reduced.



Molecular Umbrella-Tamed Amphotericin B

Amphotericin B (AmB) has been widely used to treat systemic fungal infections and fungal infections in the central nervous system for more than 50 years (Chart 1).[1−11]

Chart 1



Amphotericin B

Despite its clinical importance and its stature as the "gold standard" for antifungal chemotherapy, this natural heptaene macrolide antibiotic is generally regarded as one of the most toxic drugs that is used in modern medicine. Very recently, it has been reported that the insertion of an NH unit between the C16 and the C41 (carboxyl) carbons leads to a dramatic reduction in toxicity.[12] The basis for this remarkable behavior, however, remains to be established.

The mechanism by which AmB kills fungal cells continues to be debated.[2] In the classic barrel stave model, several AmB molecules combine with ergosterol to form pores. Subsequent alignment of two such pores across the plasma membrane, or a thinning of the membrane around individual pores, is thought to produce lethal water-filled channels through which ions are free to pass (Figure 1A and B). Recently, however, evidence has begun to emerge in support of an entirely different mechanism—one in which AmB extracts ergosterol from the hydrocarbon interior of fungal membrane and deposits it on the cell's surface as a single complex or a "pile" of complexes; i.e., the "sterol sponge" model (Figure 1C and 1D, respectively).[13,14]

What has complicated virtually all mechanistic investigations of AmB is the fact that this antibiotic exists in two discrete forms, having different biological properties. Specifically, Bolard and co-workers have shown that, whereas water-soluble *aggregates* of AmB are toxic to erythrocytes and fungal cells, the *monomers* are toxic only to fungal cells.[15] Similar complexity is likely to exist with all derivatives of AmB that have been reported to date.

In a past study, we provided additional evidence that AmB *monomers* exhibit much greater cellular selectivity than aggregated forms by showing that the attachment of poly(ethylene glycol) chains allows one to separate antifungal from

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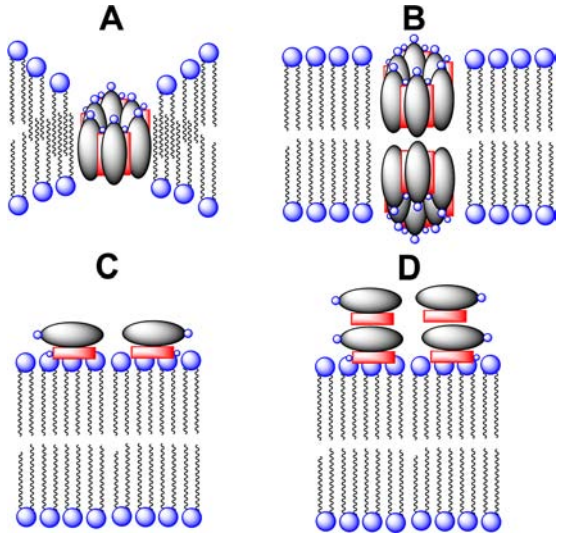


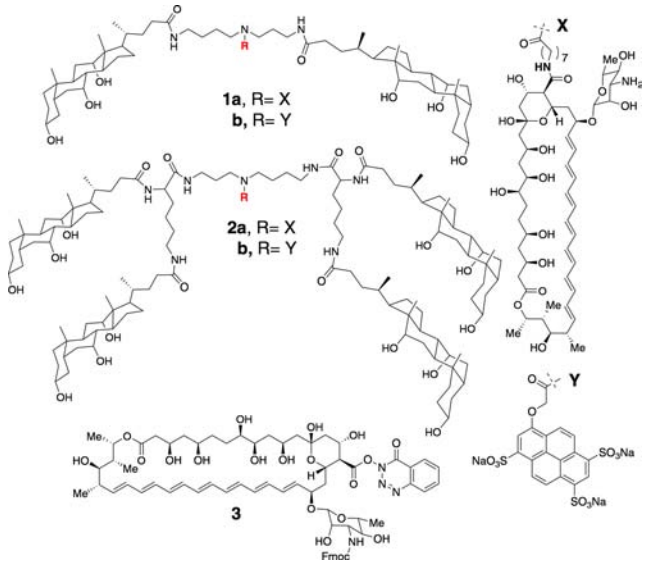
Figure 1. Barrel stave model in which AmB (gray oval) combines with ergosterol (red rectangle) to form (A) a single pore, (B) two aligned water-filled pores, (C) a single adsorbed complex, and (D) a "pile" of complexes.

hemolytic activity. Specfically, we showed that the attachment of poly(ethylene glycol) chains to AmB increases its critical aggregation concentration (cac), and that hemolytic activity is observed only at concentrations that are in excess of these concentrations—where aggregates and monomers coexist.[16] Below their cac concentration, where monomers are dominant, only antifungal activity was significant. As previously discussed, this behavior closely resembles the action of a common detergent (Triton X-100) on cholesterol-rich liposomes where attack by aggregates results in a catastrophic rupture of the membrane, while attack by monomers leads to mild leakage.[17] In fact, this dichotomy in membrane-disrupting behavior (catastrophic rupture by aggregates versus mild perturbation by monomers) can account for the reduced toxicity of AmB that has been found with liposomal formulations; i.e., the liposomes merely serve as a reservoir that release highly cell-selective *monomers*.[17,18]

Based on these earlier studies, our working hypothesis has been that (i) the "sponge" mechanism of action is the dominant pathway by which AmB monomers destroy fungal cells, and (ii) *nonselective membrane disruption by aggregates is responsible for the high toxicity of this antibiotic*. This hypothesis has also led us to speculate that if one could reduce the ability of AmB to insert into cell membranes, the rupturing power of its aggregates would be greatly reduced, while the monomers would retain significant antifungal activity. In other words, AmB would be "tamed".

To explore this line of reasoning, we chose molecular umbrella-AmB conjugates **1a** and **2a** as synthetic targets (Chart 2). Our rationale was based on previous fluorescence quenching measurements that were made with certain diwalled and tetrawalled "molecular umbrellas" bearing Cascade blue; i.e., **1b** and **2b**, respectively.[19] Specifically, these measurements provided support for a model in which such molecules favor binding at the surface as opposed to the interior of lipid membranes (Chart 2 and Figure 2). Thus, we posited that analogous molecules would localize a pendant AmB moiety close to the cell surface, thereby favoring the killing of fungal cells via a "sponge" mechanism.

Chart 2



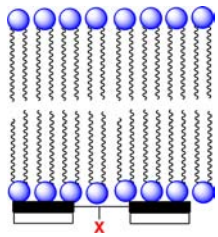


Figure 2. Stylized illustration of a diwalled molecular umbrella bearing Cascade Blue (X) adsorbed on the surface of a lipid bilayer. The open and filled rectangles represent the hydrophilic and hydrophobic face of facially amphiphilic moieties, e.g., a choloyl group.

In synthesizing derivatives of AmB, we have found an Fmoc-carbamate of AmB that has been activated by *N,N,N′,N′*-tetramethyl-*O*-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)-uronium tetrafluoroborate (TDBTU) (i.e., **3**) to be a convenient and stable precursor (Chart 2).[12] Thus, direct coupling of **3** with a diwalled molecular umbrella bearing a pendant amine group (**4**), followed by removal of Fmoc with piperidine afforded **1a** (Scheme 1). A similar sequence of reactions that was carried out using lysine dicholamide in place of cholic acid afforded **2a** (not shown).

To assess the antifungal properties of **1a** and **2a**, we examined their *in vitro* activity toward four clinically relevant microbes, i.e., *C. albicans*, *C. glabrata*, *C. neoformans*, and *C. gatti* (Table 1). On a molar basis, **1a** exhibits a potency that approaches that of the native antibiotic. However, **2a** showed negligible antifungal activity at concentrations as high as 11 μM. We suspect that strong intramolecular association of the pendant AmB moeity with the molecule's *four* choloyl groups limits its ability to bind ergosterol, thereby reducing its activity. Our attention then focused on **1a** for more detailed investigation.

Similar to AmB, **1a** exhibits a characteristic absorption at 409 nm with an apparent molar absorptivity that decreases upon aggregation.[16] If one lets (i) T, m, and P represent the total, the monomeric, and the aggregate concentrations of this macrolide, respectively, (ii) ε represent the apparent molar absorptivity and (iii) ε_m and ε_p represent the molar absorptivity for the monomeric and aggregate components, respectively, then it can

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Scheme 1

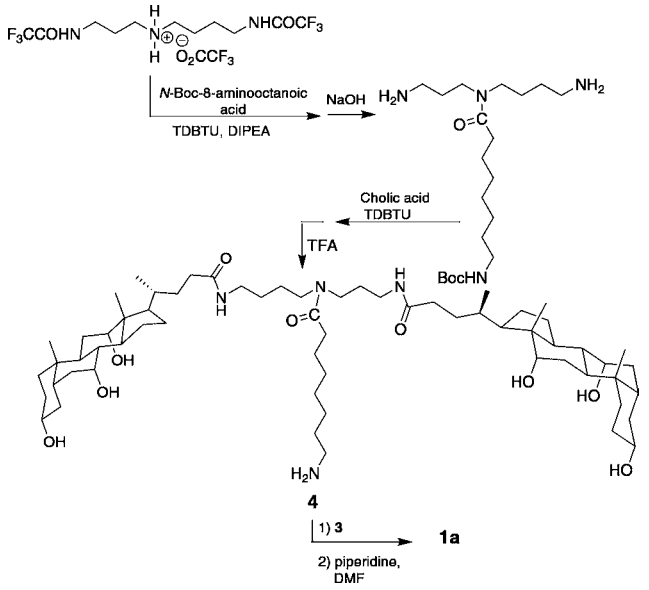

Table 1. Antifungal Activities

	MIC/MFC[a] (μM)		
Microbe	AmB	**1b**	**2b**
C. albicans	0.5/1	1/2	>11/--
C. glabrata	0.5/1	2/4	>11/--
C. neoformans	0.3/0.5	1/2	>11/--
C. gatti	0.3/0.5	1/2	>11/--

[a]MIC and MFC values are the lowest concentrations required for completely inhibiting growth, and killing at least 99% of the fungi, respectively.

be shown that $\varepsilon = \varepsilon_p + (\varepsilon_m - \varepsilon_p)m/T$.[16] At concentrations that are in excess of its cac, m is constant, and ε is expected to be inversely proportional to T. Thus, by measuring the apparent molar absorptivity as a function of the reciprocal of the concentration of **1a**, its cac value is estimated to be 0.9 μM (from the intercept of two straight lines). This is essentially the same cac as that of AmB (Figure 3).[16]

Significantly, aggregates of **1a** showed dramatically reduced hemolytic activity relative to aggregates of AmB (Figure 4). Thus, whereas the concentration of AmB that was required to induce 50% release of hemoglobin from erythrocytes was 4 μM, the concentration of **1a** that was required for such release was ca. 2 orders of magnitude higher, i.e., 375 μM.

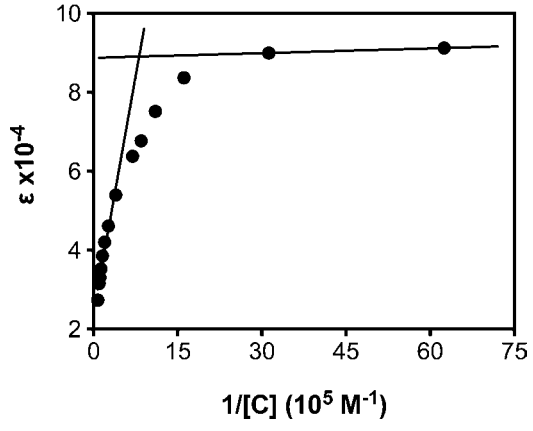

Figure 3. Plot of molar absorptivity (λ_{max} 409 nm) as a function of the reciprocal concentration of **1a** [**C**]in PBS at 37 °C.

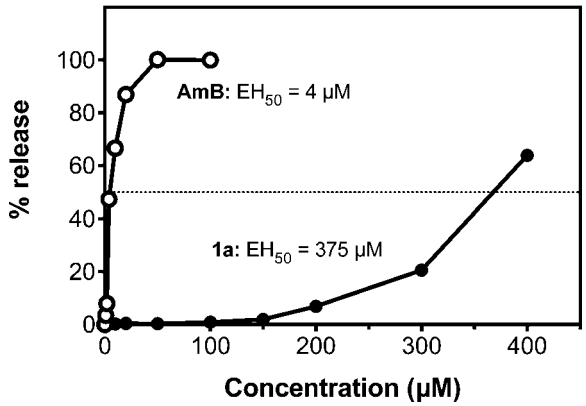

Figure 4. Plot of percent release of hemoglobin from sheep red blood cells as a function of concentration of AmB (○) and **1a** (●) at 37 °C in saline, pH 7.4.

As further evidence that AmB has been tamed in the form of **1a**, we have compared its toxicity toward HEK293 T cells with that of the native AmB molecule. As shown in Figure 5, its toxicity toward these cells has been dramatically reduced.

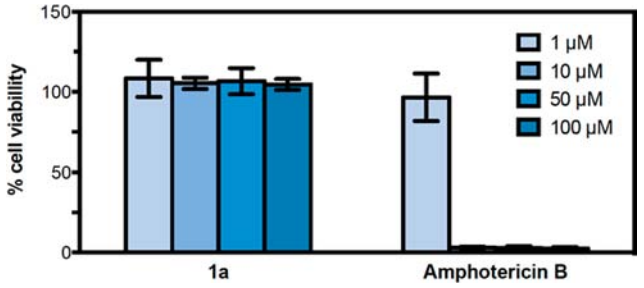

Figure 5. Bar graph showing the viability HEK293 T cells in the presence of 1, 10, 50, and 100 μM concentrations of AmB and **1a**.

In a broader context, this taming strategy that we have described offers a unique opportunity for enhancing the cellular selectivity and therapeutic potential of a variety of membrane-disrupting agents. As we noted previously, those agents that operate at the membrane level are particularly attractive as drugs because they circumvent two of the more common mechanisms of drug resistance, i.e., export mechanisms and enzymatic degradation within the cell.[20]

The ability of molecular umbrellas to cross lipid membranes raises the possibility that **1a** may allow for more efficient transport of this heptaene macrolide antibiotic across the blood-brain-barrier via passive diffusion.[21−24] Studies currently in progress are aimed at assessing the in vivo toxicity and efficacy of **1a** and related analogs in treating systemic fungal infections and fungal infections in the central nervous system. Efforts are also underway to explore the scope of this taming strategy by examining its applicability to other classes of membrane-disrupting agents (e.g., the polymyxins, the magainins, and quaternary ammonium compounds) using molecular umbrellas and a variety of other facially amphiphilic molecules.[25,26] In this regard, it should be noted that there are recent indications that monomers of quaternary ammonium compounds are much more selective in killing bacterial cells

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than aggregated forms.[27] Thus, this taming strategy could lead the way to important new classes of therapeutic agents in the fight against drug-resistant bacteria.[28]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00463.

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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■ NOTE ADDED AFTER ASAP PUBLICATION

This paper was published to the Web on Sepember 10, 2015, with errors in the TOC and abstract graphics, Scheme 1, Chart 2, and the Supporting Information. These errors were corrected in the version published to the Web on October 2, 2015.

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