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The C2′–OH of Amphotericin B Plays an Important Role in Binding the Primary Sterol of Human But Not Yeast Cells

Brandon C. Wilcock, **Matthew M. Endo**, **Brice E. Uno**, and **Martin D. Burke**[*]
Howard Hughes Medical Institute, Roger Adams Laboratory, Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois, 61801

Abstract

Amphotericin B (AmB) is a clinically vital anti-mycotic but is limited by its severe toxicity. Binding ergosterol, independent of channel formation, is the primary mechanism by which AmB kills yeast, and binding cholesterol may primarily account for toxicity to human cells. The leading structural model predicts that the C2′ hydroxyl group on the mycosamine appendage is critical for binding both sterols. To test this, the C2′ hydroxyl group was synthetically deleted and the sterol binding capacity of the resulting derivative, C2′deOAmB, was directly characterized via isothermal titration calorimetry. Surprisingly, C2′deOAmB binds ergosterol and, within the limits of detection of this experiment, does not bind cholesterol. Moreover, C2′deOAmB is nearly equipotent to AmB against yeast but, within the limits of detection of our assays, is non-toxic to human cells in vitro. Thus, the leading structural model for AmB/sterol binding interactions is incorrect, and C2′deOAmB is an exceptionally promising new antifungal agent.

The polyene macrolide natural product, amphotericin B (AmB), is the archetype for both small molecules that form ion channels[1] and antibiotics that are inherently refractory to microbial resistance.[2] AmB is also, unfortunately, highly toxic,[3] which often limits its effective utilization as the last line of defense against life-threatening systemic fungal infections. As a result, the mortality rate for these infections remains near 50%.[2a,3b,c] Moreover, the incidence of such fungal infections and resistance to all other classes of antifungals are rising.[2] For all of these reasons, finding a way to improve the therapeutic index of AmB is a critically important problem. Some progress has been made with liposomal formulations,[4,5] but these are often prohibitively expensive,[4] and substantial toxicity remains.[5] Despite more than four decades of extensive efforts worldwide,[6] a clinically viable derivative of AmB with an improved therapeutic index has yet to emerge.

A major contributor to this lack of progress has been poor understanding of the mechanism(s) by which AmB impacts yeast and human cells. It has for half a century been widely accepted that AmB kills both types of cells primarily via ion channel-mediated membrane permeabilization.[2,6,7] Guided by this model, extensive efforts have focused on the challenging problem of selectively forming ion channels in yeast vs. human cells.[6,7]

In contrast to this classic model, we recently discovered that AmB primarily kills yeast by simply binding ergosterol; channel formation is not required.[8] This suggests that binding cholesterol may account primarily for the toxicity of AmB to human cells, and that efforts to improve the therapeutic index of this clinically vital antimycotic can focus directly on the simpler problem of maximizing the relative binding affinity for ergosterol vs. cholesterol.

Corresponding Author: burke@scs.uiuc.edu.

Supporting Information
Detailed synthesis, spectral data, and assay procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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In this vein, we have previously found that deletion of the mycosamine appendage from AmB eliminates its capacity to bind both ergosterol and cholesterol.[8] The resulting derivative, amphoteronolide B (AmdeB), was also found to be non-toxic to yeast.[8,9] The roles played by each heteroatom contained in the mycosamine appendage, however, have remained unclear. In the leading structural model, AmB interacts with both ergosterol and cholesterol via a similar binding mode in which the C2′ hydroxyl group of AmB forms a critical hydrogen bond to the 3β hydroxyl group on each sterol (Fig. 1).[10] Experiments designed to probe this hypothesis, however, have yielded conflicting results. Studies comparing the membrane permeabilizing activities of conformationally restricted derivatives of AmB concluded that such a hydrogen bond plays a key role with both sterols,[10b] whereas recent computations suggested that this hydrogen bond is not involved in binding cholesterol.[11] The results of a series of C41 methyl ester derivatives of AmB further modified at C2′ were mixed: epimerization at C2′ led to retention of both membrane permeabilizing and antifungal activities whereas epimerization and methyl etherification at C2′ resulted in substantial reductions in both activities.[10e] Most importantly, none of these prior studies directly measured sterol binding.

In pursuit of a definitive experiment, we aimed to delete the C2′ hydroxyl group from AmB and directly determine the impact on binding ergosterol and cholesterol. Synthesis of the targeted C2′deoxyAmB (C2′deOAmB), however, represented a major challenge. This is because, in addition to all of the other problems associated with chemically manipulating this complex and sensitive natural product,[8,9] 2-deoxy sugars are more acid-sensitive than their oxygenated counterparts.[12]

We pursued two different synthetic strategies toward this probe (Schemes 1&2). In the first approach, we targeted site-selective deoxygenation of the decahydroxylated natural product (Scheme 1). This led to the discovery that site-selective and site-divergent functionalizations can be achieved simply by modifying the electronic properties of achiral reagents.[13] Harnessing this phenomenon, we achieved site-selective acylation of the C2′ hydroxyl group to generate intermediate **1**, and subsequent persilylation, deacylation, and deoxygenation of the C2′ hydroxyl group generated protected C2′deOAmB **2**.[13] Deprotection of this intermediate was initiated by global desilylation with HF-pyridine. We then employed potassium hydroxide to deprotect the methyl ester and CSA to concomitantly remove the *p*-methoxybenzylidene acetals and methyl ketal. Consistent with the sensitivity of 2-deoxysugars, these studies revealed that C2′deOAmB derivatives are substantially less compatible with many chemical reagents than their mycosaminoylated counterparts, and this lack of compatibility manifested in low yields for these transformations. This problem was particularly evident in the final step. Specifically, removal of the phenylacyl group from the C3′ amine using penicillin G amidase (PGA), a reaction that was previously successful with both AmB and C35deOAmB,[8a] resulted in a low yield of C2′deOAmB as an inseparable mixture containing a variety of deglycosylated byproducts.

Importantly, extensive knowledge gained during these studies bolstered an alternative semisynthetic approach[14,10e] that ultimately proved to be much more productive (Scheme 2). In this route, we first generated a C2′-deoxygenated mycosamine (acosamine) donor from known intermediate **3**.[15] After much exploration, it was determined that the TBS-protected derivative of this 2,3 epoxy alcohol can be regioselectively opened at C2′ using lithium triethylborohydride in THF at 40 °C to give intermediate **4**. The resulting alcohol was mesylated and displaced by sodium azide and subsequent removal of the PMB group generated the deoxysugar donor **5**. Importantly, **5** is protected in such a way that the functional groups at C3′ and C4′ are inert to all of the subsequently required transformations yet readily unmasked at the end of the synthesis using mild conditions.

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We also prepared a similarly protected macrolide acceptor **7**,[14,9,10e] having suitably stable yet readily cleavable silyl ethers protecting all of the hydroxyl groups and the carboxylic acid at C41. Glycosylation of **7** with **5** proceeded smoothly to yield C2′deOAmB derivative **8** as a 2:1 mixture of α and β anomers. Importantly, derivative **8** proved to be much more amenable to deprotection than **2**. Concomitant cleavage of all nine of the silyl protecting groups was achieved with HF-pyridine, and the resulting α and β anomers were readily separated by HPLC. Finally, deprotections of the C3′ azide with trimethylphosphine and the C13 hemiketal with aqueous acid completed the synthesis of C2′deOAmB (94% pure as judged by analytical HPLC).

With several milligrams of this key probe in hand, we tested whether deletion of the C2′ hydroxyl impacts the capacity of AmB to bind ergosterol via an optimized isothermal titration calorimetry (ITC)-based assay (Fig. 2A). We first titrated an aqueous solution of AmB with a suspension of large unilamellar vesicles (LUVs) comprised of only 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), and the net exotherm was recorded. We then repeated this titration using POPC LUVs containing 10% ergosterol. Consistent with our previous results,[8] we observed a significant increase in net exotherm when switching to ergosterol-containing LUVs, indicating a direct AmB-sterol binding interaction. No such binding was observed when the same pair of titrations was repeated with AmdeB (Fig. 2A).[8] Surprisingly, when C2′deOAmB was subjected to the same experiments, a significant increase in net exotherm was observed demonstrating a retained capacity for this derivative to bind ergosterol. Thus, contrary to the leading model, the C2′ hydroxyl group on AmB is not required for ergosterol binding.

Even more surprisingly, when we repeated these same binding studies with cholesterol, we observed a different result. Specifically, after confirming binding and no binding of cholesterol for AmB and AmdeB, respectively,[8] we tested the cholesterol binding capacity of C2′deOAmB (Fig. 2B). In contrast to the results with ergosterol, *C2′deOAmB showed no evidence of binding cholesterol* in this experiment. Thus, the C2′ hydroxyl group of AmB plays a major role in binding cholesterol but not ergosterol.

We next tested the minimum inhibitory concentration (MIC) of AmB, AmdeB, and C2′deOAmB against two ergosterol-containing strains of yeast, *S. cerevisiae* and *C. albicans*. The latter represents the most common cause of fungal infections in humans. Consistent with our previous results[8] and the ergosterol binding data described above, we observed potent antifungal activity for AmB against both cell lines and no antifungal activity for AmdeB. Importantly, and consistent with the observation of retained ergosterol binding, when we tested C2′deOAmB in these same assays, we observed retention of potent antifungal activity against both strains of yeast (Fig. 3A).

Finally, we probed the activity of these same three compounds against human cells. Two of the most important toxic side effects associated with AmB are anemia and nephrotoxicity caused by damage to red blood cells and renal proximal tubule cells, respectively.[5a,6a,7h,17] AmB causes 90% hemolysis of human red blood cells at a concentration of 8.5 μM (Fig. 3A).[18] This is defined as the minimum hemolytic concentration (MHC). In stark contrast, we found that the corresponding MHCs for AmdeB and C2′deOAmB, both of which do not bind cholesterol in our ITC assay, to be >500 μM. Similarly, AmB causes 90% loss of cell viability of primary human renal proximal tubule epithelial cells[19] at a concentration of 2.4 μM [the minimum toxic concentration (MTC)]. Again, in stark contrast to AmB, both AmdeB and, most importantly, C2′deOAmB showed no evidence of toxicity up to their limits of solubility.[20] As shown in Fig. 3B, microscopy further revealed that human primary renal cells treated with just 2 μM AmB show severe abnormalities compared to DMSO

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treated controls. In contrast, cells treated C2′deOAmB show no visual evidence of toxicity even up to a concentration of 80 μM.[20]

These findings demonstrate that the leading structural model for AmB-sterol binding (Fig. 1)[10] will need to be revised. Two alternative models are suggested by our results. In the first, AmB interacts with these two sterols via distinct binding modes, and the C2′ hydroxyl group uniquely participates in a direct binding interaction with cholesterol. While we can not yet rule out this possibility, the structural similarity of the two sterols seems to make this scenario unlikely. We favor an alternative model in which the C2′ hydroxyl group stabilizes a conformer[21] of AmB that readily binds both ergosterol and cholesterol. Deletion of this hydroxyl group, we propose, favors a shift to a different conformer or set of conformers which retain the capacity to bind ergosterol but not cholesterol. Alternatively stated, this model predicts that deletion of the C2′ hydroxyl group of AmB causes a small molecule-based allosteric effect that results in ligand-selective binding.[22] Although further studies are required to test this hypothesis, we note that in the X-ray crystal structure of *N*-iodoacyl AmB[23] there is a prominent water-bridged hydrogen bond between the hydroxyl groups at C2′ and C13 that may serve to stabilize a particular conformation of the mycosamine appendage relative to the polyene macrolide core.

To our knowledge, no AmB derivatives with the demonstrated capacity to directly bind ergosterol but not cholesterol have previously been reported. We are also unaware of any reported derivatives with retained antifungal potency but no observable toxicity to human cells. These features, combined with a mechanistic model connecting sterol binding to cell killing,[8] suggest that C2′deOAmB will have a substantially increased therapeutic index in vivo. We further note that this derivative was generated via *removal of a single atom* from AmB. Thus, in contrast to the methyl ester of AmB and many other derivatives having modifications at the C41 and/or C3′ positions,[6] C2′deOAmB retains the amphoteric nature and many other potentially important features of the extensively clinically validated natural product. Combining all of these considerations with promising starting points for the development of a scalable synthesis (Schemes 1 & 2),[24] C2′deOAmB represents an exceptional candidate for further development as a potentially less-toxic clinical substitute for AmB. Preclinical studies to explore this potential are currently being targeted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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20. Due to the limited solubility of AmB and its derivatives in the media required for growing primary human renal cells (ATCC, PCS-400-030), 80 μM is the highest concentration achievable in this experiment.

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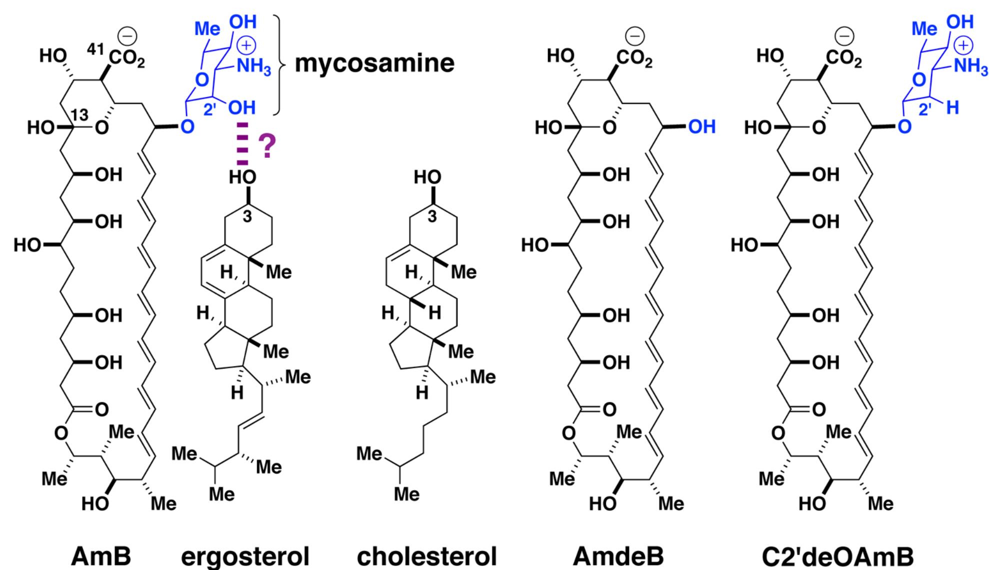

Figure 1.
The C2′ hydroxyl of AmB is predicted to play a critical role in binding both ergosterol and cholesterol. Structures of the synthetic derivatives of AmB designed to test this model.

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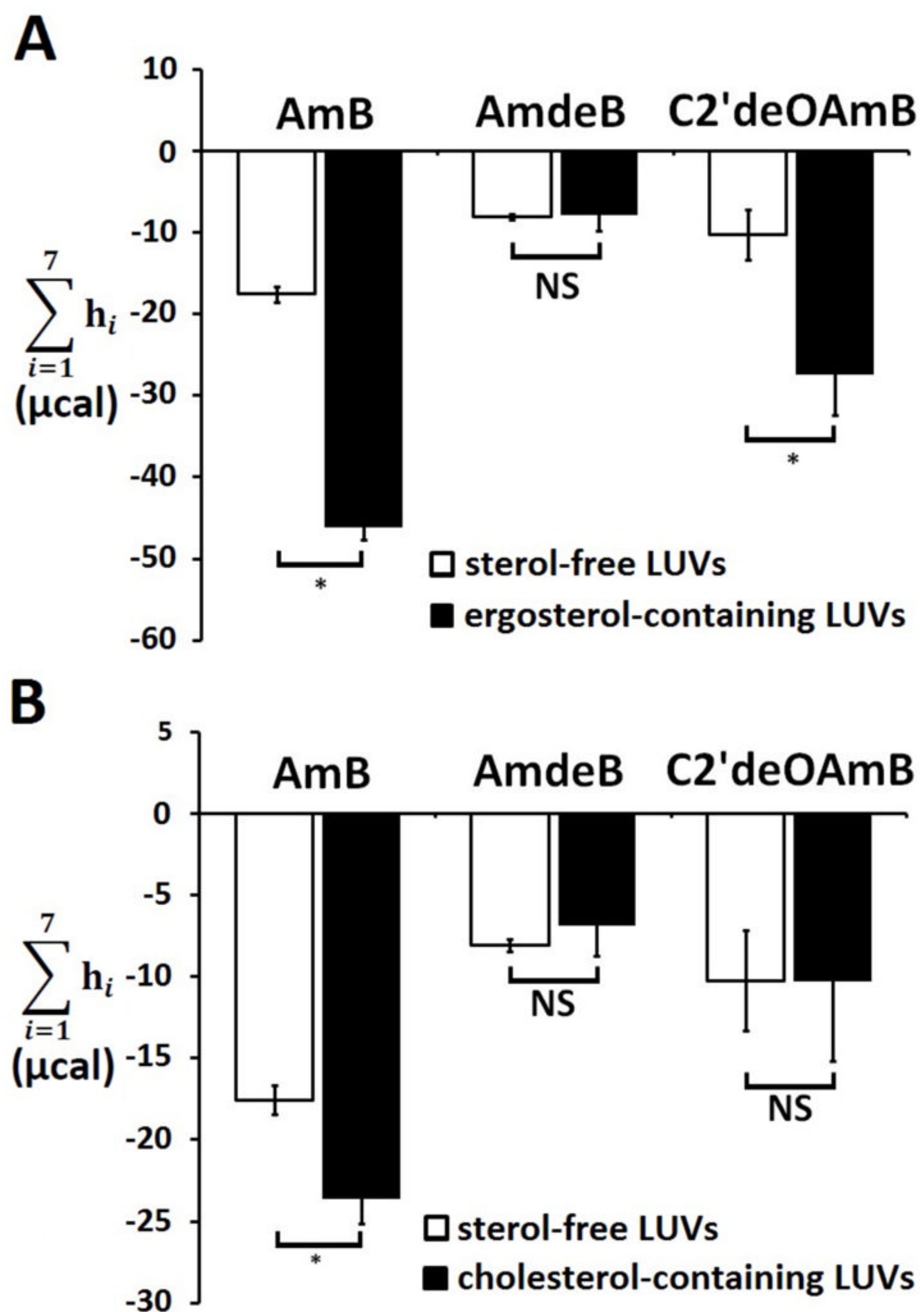

Figure 2.

Isothermal titration calorimetry to probe the binding of AmB, AmdeB, and C2′deOAmB to (A) ergosterol and (B) cholesterol. Values represent the mean of at least three experiments ± SD. * $p < 0.05$; NS, not significant.

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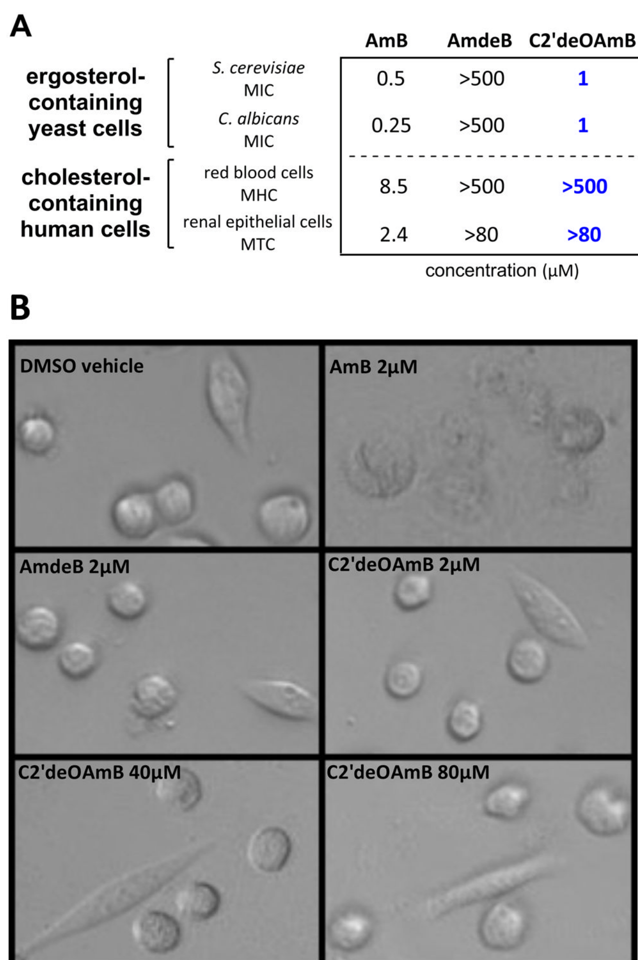
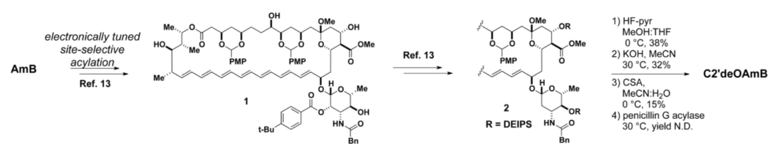


Figure 3.
(A) Minimum inhibitory concentrations (MIC) against ergosterol-containing fungal cells and minimum hemolytic concentrations (MHC) and minimum toxic concentrations (MTC) against cholesterol-containing human cells. (B) Microscopy images of primary human renal epithelial cells treated with AmB or its derivatives.



Scheme 1.

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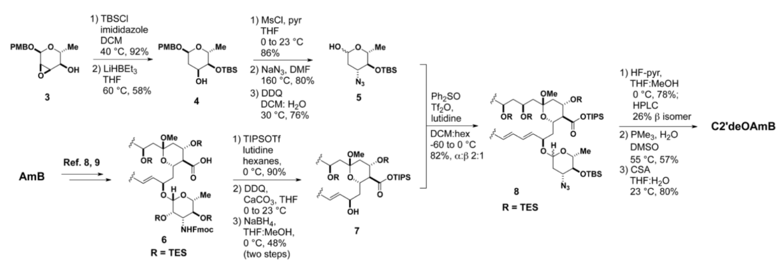

Scheme 2.