Our Path to Less Toxic Amphotericins

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Abstract We launched our research program with the search for small molecule replacements for missing proteins that underlie currently incurable human diseases. This pursuit drew us to amphotericin B, a remarkable natural product that is the archetype for ion-channel-forming small molecules. We quickly realized, however, that there was a second very important reason to study this natural product, as it represents the most powerful, broad-spectrum, and resistance-evasive treatment for invasive fungal infections, which still kill more than 1.5 million people each year—more than malaria or tuberculosis. The problem with amphotericin B is that it is highly toxic to humans, which limits the dose that can be administered. Through an extensive series of synthesis-enabled studies, we came to understand that, in contrast to the longstanding mechanistic model, amphotericin B kills yeast by simply binding ergosterol—channel formation is not required. This allowed us to focus squarely on the actionable problem of selectively binding ergosterol over cholesterol en route to an improved therapeutic index. This journey has yielded new types of amphotericin derivatives that bind ergosterol but not (detectably) cholesterol, and kill yeast but are substantially less toxic than amphotericin B in vitro and in vivo. This advanced mechanistic understanding has also brightened the prospect of developing small molecules that replace missing protein ion channels, thereby operating as prostheses on the molecular scale.

Key words small molecules, protein-like functions, resistance-evasive, antimicrobials, N-methylimidodiacetic acid boronates

I am very grateful to Victor Snieckus for encouraging me and my students to couple the IUPAC Award in Organic Synthesis with a personalized account of our discovery of less toxic amphotericins.

We certainly did not start with this objective. Our program began (and continues!) with the pursuit of small molecules that can replace the missing proteins that underlie many currently incurable human diseases, thereby operating as prostheses on the molecular scale. When I was a medical student at Harvard in 1998, I encountered a courageous 22-year-old college student with cystic fibrosis who was taking 17 different medicines on a daily basis, but none of these were actually addressing the molecular defect underlying her illness. I recall sharing with her all the things I had recently learned about the protein ion channel she was missing, and at some point, she stopped me, and asked, “If we know exactly what the problem is, why can’t we fix it?”

I was deeply moved by this question. As a medical student, I became fascinated by the capacity for small molecules to bind and inhibit proteins and thereby treat diseases caused by an excess of protein function. However, as was the case with this young cystic fibrosis patient, for many diseases caused by a deficiency of protein function there is no protein target to inhibit. Knowing how effective small molecules can be as medicines, I became very excited about another question...might it be possible to find small molecules that can autonomously replicate the functions of missing proteins, thereby operating as prostheses on the molecular scale?

I began hunting for an answer. The next year, as a new graduate student in Stuart Schreiber’s lab in the Department of Chemistry and Chemical Biology, I began learning how to make molecules, and I became fascinated by the tremendous power coupled to this process. I was also taking Andy Myers’ Chem 115 Advanced Organic Synthesis course, and he drew on the chalkboard the chemical structure of the beautifully complex polyene macrolide natural product amphotericin B (Figure 1). Andy also mentioned that this small molecule was known to form ion channels in cells,
and my imagination started running wild. I was fascinated by the prospect that, as the product of extensive evolution, this natural product might represent a billion-year head start for discovering a small molecule replacement for the missing ion channel that causes cystic fibrosis. More broadly, the existence of this natural product was, for me, Mother Nature’s way of screaming at the top of her lungs that there was much more that small molecules could do to improve human health beyond binding to proteins and turning them off. I became committed to finding out if this was in fact the case.

I thus began a deep dive into 50 years of literature on amphotericin B (AmB). It was a daunting task, as there were thousands of published papers, but I quickly learned that there was a second, really important reason to study this natural product. It was already a clinically approved drug that serves as the powerful and fungicidal last line of defense in treating life-threatening invasive fungal infections that occur in millions of people worldwide each year.1 Perhaps most remarkably, despite half a century of clinical utilization, there has been little or no emergence of pathogen resistance to AmB. This stands in stark contrast to the clinical history of all other antimicrobials.1 There is also, however, a major problem with amphotericin – it is highly toxic to humans, particularly to the kidneys, and this toxicity all too often precludes the administration of this drug at the doses and/or durations required to treat effectively an invasive fungal infection. This toxicity has earned this drug the name ‘amphoterribile’ among patients and doctors alike. Because other treatments are less effective and/or increasingly vulnerable to the emergence of pathogen resistance, failure to treat with amphotericin B too often leaves no good options, and nearly half of all the patients with an invasive fungal infection succumb to their diseases. This accounts for more than 1.5 million deaths per year, which is more than malaria or tuberculosis.2 These staggering statistics made it clear that a less toxic amphotericin could have an extraordinary impact on human health.

Diving into the literature also made it apparent that the pursuit of both AmB-based ion channels for replacing missing proteins and less toxic antifungal agents would require a better understanding of how AmB kills yeast and human cells. The leading and widely accepted model, which appears in many textbooks (including the one used in my pharmacology class that I took at MIT the next year),3 states that amphotericin kills cells by permeabilizing their membranes. This mechanism had daunting negative implications for both goals. If the AmB ion channels were, in fact, inherently highly toxic to human cells, this would make it very difficult to utilize those channels to alternatively promote physiology in the setting of a missing protein. Moreover, this mechanism suggested that improving the thera-

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**Biographical Sketches**

**Matthew M. Endo** received his B.S. degree in chemistry from Seattle University in 2008. In 2009, he began his Ph.D. work under Professor Martin Burke focusing on understanding the mechanism of action and developing therapeutic derivatives of amphotericin B.

**Alexander G. Cioffi** received his B.S. degree in chemistry from Saint Louis University in 2011. That same year he began his Ph.D. work under Professor Martin Burke focusing on the prospect of replacing missing proteins with small molecule mimics.

**Martin D. Burke** received his undergraduate degree at Johns Hopkins in 1998, a Ph.D. from Harvard in 2003, and an M.D. from Harvard Medical School in 2005. He is currently a Professor at UIUC and an Early Career Scientist of the Howard Hughes Medical Institute. His lab is focused on the synthesis and study of small molecules with protein-like functions.
peutic index of AmB would require selective self-assembly of structurally enigmatic small-molecule-based channels in yeast but not human cells. Many decades of effort by leading laboratories worldwide to achieve this had not yielded a clinically viable, less toxic amphotericin derivative. Fortunately, there were encouraging hints in the literature that membrane permeabilization might not actually be the primary mechanism by which amphotericin kills cells, and I came to see the illumination of this mechanism as a critical first step in pursuit of both of these functional objectives.

Amphotericin B was discovered in January of 1953, by William Gold and his colleagues, at the Squibb Institute for Medical Research, as an isolate from a soil bacterium, Strep-tomyces nodosus, found on the banks of the Orinoco River region of Argentina. Prior to this, the prognosis for patients with fungal infections was extremely grim, with mortality rates near 100%, and new treatments were desperately needed. This highly potent and broad-spectrum antifungal was quickly introduced into the clinic and dramatically changed the outlook on invasive mycoses.

Just a few years later in 1958, David Gottlieb and Herbert Carter at the University of Illinois at Urbana-Champaign discovered that the antifungal activity of another polyene macrolide could be attenuated by adding extracts from carrots to the yeast growth medium. A series of extractions led to the identification of sterols as the key promoters of this protection, and that co-administration of the same sterols could inhibit the antifungal activity of AmB. This led the authors to suggest that polyene macrolides may inhibit sterol biosynthesis, and that this effect was rescued via the addition of exogenous sterols. In retrospect, my students and I alternatively view this result as the first hint of the mechanism that was recently uncovered by our synthesis-enabled studies of this natural product: amphotericin primarily kills yeast by simply binding ergosterol.

Soon after David Gottlieb’s initial report, Stephen Kin-sky at Washington University in St. Louis found that AmB caused a decrease in the dry weight of the mycelial mats of the red bread mold, Neurospora crassa, and an increase of cytoplasmic constituents in the growth medium. This led to the suggestion in 1961 that AmB primarily kills cells by permeabilizing membranes. This mechanistic model quickly came to dominate the thinking in this area, and continued to do so for the next half a century.

Three specific mechanisms of membrane permeabilization were considered: gross membrane disruption, carrier-type ion transport, and discrete ion channel formation. In 1968, Thomas Andreoli at Duke University reported that the electrical resistance of planar lipid bilayers decreased but the physical integrity of the membrane was maintained in the presence of AmB. This argued against gross membrane disruption. A few years later, Alan Finkelstein at the Albert Einstein College of Medicine compared the electro-physiological properties of AmB with the known ion carrier, valinomycin. Valinomycin-mediated conductance increased linearly with increasing concentration, while AmB-mediated conductance alternatively showed an exponential relationship. Moreover, the conductance due to valinomycin addition increased with increasing temperature, while AmB-promoted conductance decreased with increasing temperature. These observations argued against AmB acting as an ion carrier and supported the proposal of self-assembly into multimeric ion channels. Remarkably, similar to their protein counterparts, these ion channels showed discrete conductances, as well as gating and ion selectivity properties, making AmB the first small molecule known to perform autonomously protein ion channel-like functions.

Figure 2 (A) Bird’s eye view of the barrel-stave model of the AmB ion channel. Polar interaction between AmB and (B) neighboring AmB molecules, (C) membranous phospholipids, and (D) membrane-embedded ergosterol that were predicted to be critical in stabilizing the AmB ion channel and the biological activity of AmB.
This series of observations had a progressive solidifying effect on the membrane permeabilization model for the cytocidal action of AmB. It became widely accepted, and these AmB ion channels were extensively studied. From 1973–1974, Thomas Andreoli, Alan Finkelstein and Ronald Holz, and Ben de Kruijff and Rudy Demel all proposed what is now the classic barrel-stave model of the AmB ion channel structure (Figure 2A). In this model, eight AmB molecules self-assemble to form a transmembrane pore where the hydrophobic polyene units point outward and interact with membrane components, and the polyl units point inward, collectively creating a hydrophilic pore that enables ion conductance. Intermolecular salt-bridges between C41 carboxylate and C3′ ammonium ions on the neighboring AmB molecules were predicted to play a critical role in stabilizing the self-assembled structure (Figure 2B).

There were, however, competing hypotheses for the roles played by these conspicuous oppositely charged functional groups that make AmB amphoteric. One was that these groups form polar interactions with phospholipid head groups and thereby anchor AmB to the membrane and/or facilitate channel assembly (Figure 2C).17 and another hypothesis predicted that one or both of these functional groups are involved in direct binding interactions with membrane-embedded sterols (Figure 2D).17,18 Driving the latter proposal, it had been observed early on that only membranes that contained sterols were sensitive to permeabilization by AmB, and one explanation for this was that a direct binding interaction between these two small molecules was required for ion channel self-assembly. However, it had proven very difficult to obtain definitive evidence for or against this putative intramembranous small molecule–small molecule binding interaction, and several studies had in fact concluded that AmB does not bind cholesterol, the primary sterol in human cells.19 Thus, an alternative model proposed that indirect effects on global membrane properties, rather than direct binding interactions, were responsible for the sterol dependence of AmB-mediated membrane permeabilization.20

A wide range of experiments had been performed to test all of these hypotheses, including many readily accessible covalent modifications of the C41 carboxylate and/or C3′ amine functional groups.18a–f,21,22 However, small molecule self-assembly can be exceptionally sensitive to steric effects that result from even minor covalent modifications,23 and it was thus difficult with such experiments to determine if observed changes in activity were caused by loss of polar interactions and/or gain of steric interactions. Moreover, some covalent modifications retained the theoretical capacity for polar interactions. For example, the methyl ester of AmB retains the potential capacity to form polar interactions with a neighboring ammonium ion, which was used to rationalize the retained biological activity of this semisynthetic derivative.18c In other studies, covalent linking of two molecules of AmB,21 AmB to a phospholipid,22 or AmB to a molecule of sterol were performed.18d But these modifications yielded derivatives with little or no antifungal activity, and it was unclear if this was due to lack of solubility and/or lack of flexibility of the linking elements.

An extensive range of biophysical techniques had also been employed to try to probe the existence and importance of all of these putative intermolecular interactions, including UV/Vis,16,18a,24–26 circular dichroism (CD),18a,24–26 fluorescence,16,25 Raman,4 solution NMR,25 and solid-state NMR (SSNMR) spectroscopy,27 as well as differential scanning calorimetry,24–26 atomic force microscopy,29 transmission electron microscopy (TEM),28 and surface plasmon resonance.29 However, the results were collectively inconclusive and/or conflicting. For example, results from UV/Vis and CD spectroscopic studies have been interpreted as evidence of direct AmB–cholesterol and AmB–ergosterol interactions,24 but the same observations have alternatively been interpreted as reflecting changes in AmB aggregation.30 In another study, SSNMR with deuterium-labeled AmB and cholesterol19,31 suggested that there was little to no interaction between these small molecules. The same experiments with ergosterol suggested similar mobility of AmB and ergosterol, which was interpreted as supporting a direct binding interaction.19 In other SSNMR experiments utilizing 13C-labeled AmB and 6-fluoroergosterol, direct intermolecular interactions were not observed. Thus, despite extensive investigations by many different labs and techniques, the molecular underpinnings of AmB-mediated cell killing remained unclear.

This extensive literature survey also supported an alternative and exciting path forward. There are twelve protic functional groups appended to the cyclic core of AmB, and most of these functional groups had been predicted to play some type of specific role in AmB function. For example, as mentioned above, the C41 carboxylate and C3′ amine were predicted to promote intermolecular AmB–AmB, AmB–lipid, and/or AmB–sterol interactions.18a–f,21,22 The series of hydroxy groups stretching from C3 to C13 were predicted to line collectively a hydrophilic central pore that conducts ions.14b,15,16 The C35 hydroxy group was predicted to be critical for forming transmembrane ion channels. Finally, in alternative models for sterol binding, the C2′ hydroxy group on the mycosamine appendage was predicted to hydrogen-bond with the 3β-hydroxy group on ergosterol and cholesterol and thereby stabilize both of these putative small molecule–small molecule complexes.18f–h,33 Given all these specific predictions, I became excited about a project designed to comprehensively test all of them by systematically deleting each of the protic functional groups appended to AmB and determining the biophysical and biological consequences. Analogous to an alanine scan with a protein, this comprehensive data set could help substantially illuminate the mechanism(s) of action of AmB.
It became immediately clear, however, that the process of making such derivatives of this highly complex natural product was going to be the slow step. This bothered me a lot, and I began trying to figure out a way to release this synthesis bottleneck. In this vein, I was inspired by an encounter with my good friend, Rahul Kohli, who was my classmate in the M.D./Ph.D. program at Harvard and pursuing his thesis studies with Chris Walsh. Rahul and I regularly met up for drinks on Thursday nights at ‘The Cellar’, a pub just outside of Harvard Square. While sharing stories one night about our adventures in the lab, I was struck by the tremendous efficiency with which Rahul was synthesizing new derivatives of the peptide tyrocidine A, using an automated peptide synthesizer in his lab. This allowed him to spend the majority of his time studying the function of these peptides, leading to a flood of high-impacting breakthroughs. I was envious of this tremendous pace of discovery, and began to wonder whether it might be possible to make derivatives of AmB using an analogous process. Specifically, could we use one reaction iteratively to assemble a collection of building blocks, having all of the required functional groups, oxidation states, and stereochemistry pre-installed? More broadly, could the same approach be used to make many different types of small molecules? And could such an approach even be automated? I grew increasingly excited about the potential of such a platform to help shift the slow step in small molecule science from synthesis to function.

With all of these questions racing through my head, I finished my Ph.D. studies and returned to Massachusetts General Hospital to finish my clinical rotations. During this time period, I wrote a set of research proposals targeting synthesis-enabled pursuit of ‘molecular prosthetics’ and sent them to many schools asking for a job. I was thrilled to get an interview at the University of Illinois at Urbana-Champaign, and then an offer on the spot, and I jumped at the chance to start my research program at this historic powerhouse for synthetic organic chemistry.

Starting in the summer of 2005, two of my first graduate students, Dan Palacios and Tom Anderson, focused on understanding the role of the C41 carboxylate and C19 mycosamine sugar by synthetically deleting each of these functional groups, one at a time. Without a building-block-based synthesis platform yet in place, we thought that the fastest way we might get our hands on these two specific derivatives was via selective degradations of the natural product.

We quickly learned first-hand the difficulties of synthesizing derivatives of AmB, as this complex polyene macrolide is sensitive to light and oxygen, and is minimally soluble in most organic solvents and water. After substantial optimization, however, we were ultimately able to achieve the semi-synthesis of these two targets. MeAmB (Figure 3A) was prepared by globally protecting AmB to enhance its solubility in common organic solvents and then selectively activating the C41 carboxylate for reduction into the primary alcohol via formation of a thioester intermediate. Iodination, reduction, and global deprotections led us to achieve the synthesis of MeAmB. We were also able to optimize the oxidative deglycosylation and diastereoselective ketone reduction methodology, previously described by the Nicolaou and Masamune groups, to achieve a very efficient synthesis of AmdeB (Figure 3A).

With these derivatives in hand, we first questioned whether these functional group deletions caused changes to the conformation of the polyene macrolide skeleton, which would complicate the analysis of the biophysical properties. We thus performed extensive NOESY and phase-sensitive COSY NMR experiments combined with amplitude-constrained multiplet evaluation to determine the set of ground state conformations of these derivatives. We found that in both cases the macrolide structure was unaltered compared to AmB (Figure 3B). This greatly simplified our further analysis, as we could attribute any differences in activity solely to the functional group deletions.

We were thus in an exciting position to investigate the three different predictions for the roles of these two functional groups in the mechanism of action of AmB. In preliminary studies we found that the derivative lacking the mycosamine sugar (AmdeB) was completely devoid of antifungal activity, whereas the derivative lacking oxidation at C41 (MeAmB) was surprisingly equipotent to the natural product (Figure 3A). This revealed a critical role for the mycosamine appendage, and also suggested that the predicted intermolecular polar interactions between the C41 carboxylate and C3’ ammonium ion were not required for channel formation and/or channel formation was not re-
required for antifungal activity. It also remained unclear at this point if one or both of these groups was required for binding phospholipids and/or sterols, and how these putative binding events related to channel forming and antifungal activities.

My students, Ian Dailey and Brandon Wilcock, joined Dan Palacios to test explicitly the hypothesis that the AmB ion channel was stabilized by a network of intermolecular polar interactions between the C41 carboxylate and C3′ ammonium ions (Figure 2B), first by utilizing a potassium efflux assay with \textit{S. cerevisiae} cells (Figure 4A top). We found that, like AmB, MeAmB produced a robust efflux of potassium ions, whereas AmdeB showed no membrane permeabilization. To test whether such permeabilization was caused by direct effects on lipid bilayers, we turned to a simplified model membrane system using egg phosphatidylcholine large unilamellar vesicles (LUVs) containing 10% ergosterol (Figure 4A bottom). Paralleling the yeast results, we observed that, like AmB, MeAmB produced a robust efflux of potassium ions, whereas AmdeB showed no membrane permeabilization. To further clarify whether the observed efflux was due to discrete ion channels or just gross membrane disruption, we also built a voltage clamp electrophysiology rig and measured single ion channel recordings in planar lipid bilayers (Figure 4B). We observed discrete single ion channels with MeAmB and AmB, whereas ion channel formation with AmdeB was never observed. Collectively, these experiments established that: (1) the predicted ring of intermolecular polar interactions is not required for ion channel activity, and (2) the mycosamine appendage plays a key role in both forming ion channels and killing yeast cells.

The second proposed role for the C41 carboxylate and C3′ ammonium ion was binding to a phospholipid and thereby anchoring AmB to the membrane (Figure 2C). We tested this hypothesis by evaluating the capacity of our probes to bind to yeast cells in a centrifugation-based binding assay. The percent binding of AmB, MeAmB, and AmdeB to yeast cells was found to be similar. We further tested, in an LUV system, the ability of these derivatives to bind to lipid membranes. As seen in live yeast cells, we observed no difference in the binding to LUVs by AmB, MeAmB, and AmdeB. Thus, inconsistent with the phospholipid anchoring model, both the C41 carboxylate and the C19 mycosamine were not necessary to bind to lipid membranes (Figure 4C).

We next tested the predictions that the C41 carboxylate and/or mycosamine appendages of AmB are critical for binding ergosterol (Figure 2D). To test this model, we adapted and extensively optimized an isothermal titration calorimetry (ITC) based assay. When we titrated a suspension of sterol-deficient LUVs into a solution of AmB, only a small exotherm was observed (Figure 4D). However, when we repeated the experiment by titrating with 10% ergosterol-containing LUVs, we observed a substantial increase in heat evolved (Figure 4D). We then probed whether this observed increase in net exotherm was due to AmB directly binding ergosterol or to the capacity of ergosterol to modulate global membrane properties. Fortunately, it had been previous-
ly demonstrated that lanosterol modifies global membrane properties similarly to ergosterol, so we could differentiate between both models utilizing 10% lanosterol LUVs in our ITC assay. When we titrated the lanosterol-containing LUVs into the AmB solution, we observed no increase in net exotherm relative to that observed with the sterol-free LUVs. These results collectively revealed that AmB directly binds ergosterol.

With an assay in hand to measure directly binding to membrane-embedded ergosterol, we next tested our functional-group-deficient derivatives. MeAmB produced an increase in net exotherm similar to that of AmB, demonstrating that oxidation at C41 is not necessary for AmB to bind ergosterol (Figure 4E). However, when we repeated the same set of experiments with AmdeB, we observed strikingly different results. Titration of 10% ergosterol LUVs in solutions of AmdeB produced no significant exotherm relative to sterol-free LUVs, thus revealing that the mycosamine appendage plays a critical role in promoting the AmB–ergosterol binding interaction, and that this binding interaction is critical for antifungal activity (Figure 4E). Thus, AmB is a rare, if not unique, example of a drug that exerts its effects via a small molecule–small molecule interaction.

These studies collectively clarified the role of the two oppositely charged functional groups appended to AmB, but there remained two possible explanations for how AmB kills yeast. In the first and long-standing leading model, AmB binds to ergosterol and then forms transmembrane ion channels, and the resulting membrane permeabilization causes cell death. In an alternative model that we considered, the simple binding of ergosterol is sufficient for killing yeast, and channel formation represents a second complementary mode of action.

Albeit contrary to the leading model, several pieces of evidence from our recent results and continued deep dive into the literature on polyene macrolide antifungals led us to hypothesize that the latter dual mechanism may be correct. First, there were a few reports of dissociation between membrane-permeabilizing and cell-killing activities in yeast. It had been proposed that such observations may be attributable to redox activity of the AmB polyene motif causing oxidative damage to the membrane. However, our finding that AmdeB, which retains this polyene motif and still binds to membranes and yeast cells but has zero antifungal activity, strongly argued against this mechanism. Thus, it seemed that there must be a non-channel-mediated, but as of yet unidentified, mechanism of cell killing at play. Second, a smaller mycosamine-containing polyene macrolide antifungal agent had recently been shown to bind ergosterol but not form ion channels. Third, increasing evidence supported the conclusion that ergosterol plays many diverse roles in yeast cell physiology, including promoting the proper function of many membrane-spanning proteins, membrane compartmentalization, endocytosis, pheromone signaling, and vacuole fusion. Therefore, it seemed plausible that the simple binding and sequestration of this central molecular node in yeast cell physiology could be sufficient to kill yeast. Finally, it had been observed that mutations in sterol biosynthesis genes that lead to changes in membrane sterol composition and in vitro resistance to AmB were associated with a substantial loss of pathogenicity in vivo. Thus, the sterol-binding mechanism alone might explain why resistance to AmB is so rare in the clinic.

To make the critical distinction between these two mechanistic models, we sought a derivative of AmB in which the capacity to form ion channels was eliminated but the capacity to bind ergosterol was preserved. If the classic membrane permeabilization model was correct, such a compound should have little or no antifungal activity. If, alternatively, sterol binding alone was necessary and sufficient for cell killing, such a derivative should retain fungicidal activity. An interesting series of molecular modeling studies predicted that the C35 hydroxy group on AmB was critical for ion channel formation, whether AmB formed a single-barrel or double-barrel-type of pore (Figure 5). This suggested that a derivative of AmB missing this single hydroxy group (C35deOAmB) would not form ion channels, but would maintain the ability to bind sterols. We thus be-
came intensely interested in making this single-atom-deficient derivative of AmB as a key probe to move this program forward.

Scheme 1 Analogous to peptide coupling, an iterative cross-coupling strategy for the general synthesis of small molecules is enabled by the N-methyliminodiacetic acid (MIDA) ligand.

This is where we ran head-on into the synthesis bottleneck we had anticipated, as the use of conventional strategies and methods to synthesize this single-atom-modified variant of such a highly complex natural product represented a Herculean challenge. As mentioned above, we had been inspired by the recognition that other classes of similarly very complex biomolecules, such as peptides, oligonucleotides, and increasingly oligosaccharides, could now be produced on-demand via generalized building-block-based synthesis platforms, and asked whether a similar approach could enable us to better gain access to derivatives of AmB.

Each of these advances had been enabled by the development of a standardized synthesis strategy that involved the iterative assembly of prefabricated bifunctional building blocks. In contrast, the synthesis of natural products has traditionally focused on the development of customized approaches to each target, which has caused this process to remain slow and specialist-dependent. We were encouraged by the recognition that, as with these other biopolymers, most small molecule natural products are biosynthesized via the iterative assembly of a small set of building blocks: polyterpenes from isopentenyl and dimethylallyl pyrophosphate, polyketides from malonyl coenzyme A (CoA) and methylmalonyl CoA, polyphenylpropanoids from phenylpyruvic acid, fatty acids from malonyl CoA, and amino acid derivatives. This suggested to us that small molecules should similarly possess inherent modularity that stands to enable their more generalized building-block-based construction.

Because carbon–carbon bonds primarily comprise the backbones of most natural products, we decided to build such a platform around the increasingly powerful, general, and functional group tolerant metal-mediated cross-coupling chemistry. Specifically, analogous to amino acids, we sought to embed both the halogen and boronic acid components of a Suzuki–Miyaura reaction into bifunctional building blocks that we called ‘haloboronic acids’. The problem was that when placed under conditions conducive with coupling, such bifunctional building blocks will indiscriminately oligomerize. In order to precisely control their assembly, my graduate student, Eric Gillis, sought a way to reversibly attenuate one end of these bifunctional building

Scheme 2 (A) Linear-to-cyclized strategy utilizing iterative cross-coupling to assemble the linear precursors that can be cyclized to give the desired macrocycle. (B) Synthesis of C35deOAmB via iterative cross-coupling.
blocks similar to the fluorenylmethoxycarbonyl (Fmoc) protecting group for an amino acid. Specifically, we sought a ligand that could reversibly attenuate the reactivity of a boronic acid, thereby permitting the iterative cross-coupling shown in Scheme 1.45

The logic guiding our search was simple. The transmetallation event in a Suzuki–Miyaura coupling was thought to require an empty p orbital on boron.46 We thus looked at trivalent heteroatomic ligands that would complex with a boronic acid and thereby transform an sp²-hybridized boron atom into an sp³-hybridized center that lacks a p orbital. This led us to the discovery that N-methyliminodiacetic acid (MIDA) boronates47 are unreactive toward cross-coupling.48 Further making them excellent building blocks for synthesis, MIDA boronates are also air-stable, non-hygroscopic, free-flowing, crystalline solids that are compatible with silica gel chromatography. They are also unreactive to many standard reagents, including oxidants, reductants, acids, bases, nucleophiles, and electrophiles, thus enabling the synthesis of complex boron-containing building blocks from simple MIDA boronate starting materials.49 The MIDA ligand can be easily removed in the presence of mild aqueous, free-flowing, crystalline solids that are compatible with silica gel chromatography. They are also unreactive to many standard reagents, including oxidants, reductants, acids, bases, nucleophiles, and electrophiles, thus enabling the synthesis of complex boron-containing building blocks from simple MIDA boronate starting materials.49 The MIDA ligand can be easily removed in the presence of mild aqueous base, which is critical for applications to complex molecules. Moreover, my student, David Knapp, found that the rate of this hydrolysis can be controlled by the choice of the base utilized, and this was found to enable the efficient cross-coupling of even highly unstable boronic acids via a slow-release strategy.50

With the goal of maximizing the generality of this platform for small molecule synthesis, we have further sought methods for the synthesis of Csp³-rich targets. Toward this goal, my student, Junqi Li, developed chiral versions of MIDA, which have enabled the asymmetric synthesis of chiral non-racemic Csp³ building blocks.51 Junqi Li, Andrea Palazzolo, and Seiko Fujii in the group also developed a linear-tocyclized strategy that enables iterative building-block-based assembly of linear precursors to be coupled to biomimetic intramolecular cyclizations, and thereby achieve building-block-based syntheses of even very complex and Csp³-rich macrocyclic and polycyclic natural products (Scheme 2A).52

These advances have collectively led to the utilization of iterative cross-coupling to synthesize a number of natural products, in each case using just one reaction iteratively to assemble a collection of pre-fabricated building blocks. These include ratahine,48,52 retinal,52,53 parinaric acid,52,53 crocacin C,50,52 peridinin,54 synechoxanthin,55 asnipyrone B,56 physarigenin A,56 neurosporaxanthin B⁻D-glucopyranoside,56 citreofuran,52 oblongolide,52 and the polycene cores of AmB53 and vacidin A.57 Moreover, my graduate students, Eric Woerly and Jahnabi Roy, found that 75% of all polycene natural product motifs can be synthesized from just 12 bi-functional MIDA boronate building blocks and one coupling reaction,58 a result that suggests many natural products might be accessible from a practically accessible number of building blocks. Building on this momentum, Eric Gillis and Steve Ballmer created a small molecule synthesis machine which was employed by Junqi Li, Seiko Fujii, Michael Schmidt, Andrea Palazzolo, Jonathan Lehmann, and Greg Morehouse (as well as several students from University Laboratory High School!)58 to prepare many different types of small molecules, such as pharmaceuticals, materials components, and complex natural products, including highly complex macrocyclic and polycyclic structures, via the same fully automated iterative building block assembly process.52

While this MIDA boronate platform was in early development, we returned to the challenging problem that had become a major bottleneck for our research program: the synthesis of a derivative of AmB lacking a hydroxy group at C35. Through the lens of iterative cross-coupling, my students, Kaitlyn Gray, Dan Palacios, and Ian Dailey, envisioned synthesizing this target in a straightforward manner by the iterative linking of building blocks BB₁, BB₂, and BB₃ (Scheme 2B). Building blocks BB₁ and BB₂ could be easily accessed from our previous work developed by my post-doc, Suk Joong Lee,53,57 and Matthew Endo, Brice Uno, and Brandon Wilcock came onboard to assist with the synthesis of BB₁. We were able to obtain building block BB₁ by the selective degradation of the readily available natural product through global protection, ozone-mediated excision of the polycene, cleavage of the western fragment, and finally installation of the MIDA boronate. This first building block was coupled with the iodotrienyl MIDA boronate BB₂ to produce the pentaenyl MIDA boronate intermediate. The final iterative coupling with BB₃ yielded the full linear carbon skeleton which, following macrolactonization and global deprotection, enabled access to C35deOAmB (Scheme 2B).

With our desired probe in hand, we first determined its ability to bind membrane-embedded ergosterol utilizing the aforementioned ITC-based assay. Similarly to AmB, a substantial increase in net exotherm occurred when titrating C35deOAmB with ergosterol-containing POPC LUVs compared to sterol-deficient POPC LUVs. Thus, as with AmB, C35deOAmB retained the capacity to bind ergosterol (Figure 6A). Utilizing the same LUV system, we tested the capacity of C35deOAmB to permeabilize lipid membranes in a potassium ion efflux assay. While AmB causes rapid efflux of potassium ions from the ergosterol-containing POPC LUVs at just 1 μM, C35deOAmB showed no permeabilization activity even up to concentrations of 30 μM (Figure 6B). More importantly, C35deOAmB also did not permeabilize yeast cells (Figure 6C).

At this point, the stage was set for the key experiment to determine if sterol binding alone was enough to be toxic to yeast cells. In the event, we observed that C35deOAmB, which binds ergosterol but does not form ion channels, maintained potent (Figure 6D) and fungicidal (Figure 6E) activity against yeast. Collectively, these results strongly
support the conclusion that AmB primarily kills yeast by simply binding ergosterol.59

To more deeply probe this novel mechanism, we wanted to further understand, at the structural and biophysical level, how AmB binds ergosterol and how this binding leads to killing of yeast cells. Thus, we teamed up with Chad Rienstra’s group at the University of Illinois at Urbana-Champaign, and my students Tom Anderson, Alexander Cioffi, and Katrina Diaz joined Rienstra students, Mary Clay, Grant Hisao, Marcus Tuttle, Andrew Nieuwkoop, and Gemma Comellas to consider three possible models for the location of ergosterol in the presence or absence of AmB: the ion channel model (Figure 7A), the surface adsorption model (Figure 7B), and the novel sterol sponge model (Figure 7C).

We specifically adapted an experiment based on the NMR paramagnetic relaxation enhancement (PRE) of $^{13}$C nuclei caused by proximal lipid-appended spin labels to probe the position of AmB relative to lipid bilayers.60 After confirming the position of the spin labels near the head groups or tails of the lipids, we conducted this PRE experiment in the presence of uniformly labeled $^{13}$C-AmB (Figure 7D). Strikingly, no PRE values were observed with either spin label to any $^{13}$C resonance with AmB (Figure 7E). These results are consistent with the majority of AmB being at least 20 Å away from the spin labels. An extensive series of additional experiments further supported the conclusion that AmB primarily exists as a large extramembranous aggregate.

We then collaborated with Tamir Gonen at the HHMI Janelia Research Campus to conduct TEM studies of ergosterol-containing liposomes in the presence of AmB, which allowed us to observe visually such aggregates on the surface of liposomes (Figure 8A).

Rienstra and I further sought to understand how this large extramembranous aggregate kills yeast. Due to the essential role of sterols in eukaryotic cell physiology, we hypothesized that these AmB aggregates might cause cytotoxicity by extracting ergosterol from yeast membranes, thus acting as a sterol sponge. To test this hypothesis, we again collaborated to perform PRE experiments in which liposomes containing $^{13}$C-ergosterol were analyzed in the presence of increasing concentrations of AmB. As the concentration of AmB increased, we observed a progressive decrease in the ergosterol PRE, consistent with AmB extracting ergosterol from lipid bilayers (Figure 8B). Finally, we performed a series of $[^1H]^{13}$C-$[^1H]^{13}$C experiments that revealed direct evidence of intermolecular interactions between these two small molecules, thus confirming the existence of this archetypical small molecule–small molecule interaction (Figure 8C).

To test the validity of this sterol sponge model in vivo, my students, Katrina Diaz and Alex Cioffi, first incubated S. cerevisiae cells with AmB and then harvested their membranes via an adapted ultracentrifugation method.61 Compared to a blank, AmB extracted ergosterol from yeast cells in a time-dependent fashion (Figure 8D) that also paralleled its cell-killing effects (Figure 8E).

Finally, this new sterol sponge model would predict that presaturating the AmB aggregate with ergosterol should diminish its capacity to extract sterol from membranes and thus diminish toxicity. Enabling us to directly test this hypothesis, we developed a protocol for preforming a stable
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AmB–ergosterol complex. This complex was unable to extract ergosterol or kill yeast cells. We speculate that a similar phenomenon may have been responsible for the attenuating effects of carrot extracts observed by Gottlieb and Carter.\(^8\) All of this data is consistent with the conclusion that AmB primarily kills yeast by acting as a sterol sponge.

This advanced mechanistic understanding had important implications for the functional goals that we were pursuing. Rather than trying to self-assemble selectively structurally enigmatic ion channels in yeast versus human cells, it enabled our efforts to improve the therapeutic index to focus squarely on the more actionable goal of simply binding ergosterol but not cholesterol. This mechanism also suggested that it should be possible to separate the channel activity of AmB from its cell-killing effects, thereby enabling this natural product to serve as a starting point for the pursuit of small molecule replacements for missing proteins.

To enable more rational pursuit of both of these objectives, we next sought to understand the AmB–ergosterol and AmB–cholesterol binding interactions at an atomistic level. The leading structural models predicted that the axial C2′ hydroxy group was critical in forming a key hydrogen bond with the 3β hydroxy of both sterols.\(^{18f-h, 33}\) Several prior studies designed to probe this putative interaction had yielded conflicting results. Conformationally constrained derivatives of AmB have suggested that the C2′ hydroxy is necessary for binding both sterols.\(^{18f}\) On the other hand, recent molecular dynamics studies propose that this interaction is important for binding ergosterol but not cholesterol.\(^{18h}\) A doubly modified AmB derivative where the C2′ hydroxy is epimerized and the C41 carboxylate was methyl esterified maintained antifungal and membrane permeabilization activity.\(^{62}\) However, an additional modification involving methyl etherification at C2′ led to substantial loss in both activities.

To clarify its role in binding both sterols, Brandon Wilcock and Brice Uno in my group synthesized a derivative of AmB in which the C2′ hydroxy group is simply deleted (C2′deOAmB), and Matthew Endo directly determined the capacity of this compound to bind both ergosterol and cholesterol via ITC (Figure 9A). As described above, the leading model predicted that C2′deOAmB should no longer be able to bind either sterol. We were thus surprised to find that

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**Figure 7** Three models for the activity of AmB: (A) the classic ion channel model, (B) the surface adsorption model, and (C) the new sterol sponge model. Paramagnetic relaxation enhancement (PRE) values that correlate inversely to distance from the spin label for (D) POPC and (E) AmB.\(^{9d}\)
C2’deOAmB maintained the ability to bind ergosterol. Even more surprising, with this same derivative we observed no detectable binding with cholesterol (Figure 9B).

These binding data suggested that this derivative should kill yeast but not human cells. We tested C2’deOAmB in microbroth dilution assays with *S. cerevisiae* and *C. albicans* and found that this ergosterol-binding derivative maintained antifungal activity against both yeast strains. We then moved to analyzing our probes against both human red blood cells and primary renal cells as they represent two of the most important targets of toxicity: hemolysis and nephrotoxicity. At just 8.5 μM, AmB caused 90% hemolysis of human red blood cells, while the inactive AmdeB caused no hemolytic activity, even up to 500 μM. Similar to AmdeB, C2’deOAmB caused little or no hemolysis, even at 500 μM. We observed similar results in primary renal proximal tubule epithelial cells (RPTECs), in which just 2.4 μM of AmB resulted in 90% loss of viability in these cells, while up to the limits of solubility in this assay (80 μM), we observed no loss in viability with both AmdeB and C2’deOAmB (Figure 9C).

Collectively, these results demonstrated that the leading structural model for the binding between AmB and sterols was incorrect, as the C2’ hydroxy is not necessary for binding ergosterol, but is required for binding cholesterol. One potential explanation is that AmB binds ergosterol and cholesterol in two distinct binding modes. However, the similarities in structure between both ergosterol and cholesterol seem to make that possibility unlikely. Instead, we favored a different reasoning based on parallel observations in proteins in which allosteric modifications can lead to selective modifications in binding to different ligands. Specifically, we postulated that the C2’ hydroxy helps stabilize a conformation of AmB that binds both ergosterol and cholesterol. Upon deleting this hydroxy group, we reasoned that a shift occurs to favor a conformation that selectively binds ergosterol over cholesterol. With this model in mind, we noted that a crystal structure of N-iodoacetyl AmB shows a prominent water-bridged hydrogen bond between the C2’ hydroxy and the C13 hemiketal (Figure 10). We thus reasoned that this crystal structure may represent the ground-state conformation of AmB, and that deletion of the C2’ hydroxy group disrupts this key stabilizing element and shifts the conformation of the sugar relative to the polyene macrolide core, resulting in ergosterol-selective binding.
C2′deOAmB thus revealed that a modification of AmB could lead to binding of ergosterol but not cholesterol, demonstrated that such selective binding translates to a dramatically improved therapeutic index in vitro, and suggested a potentially predictable structural model for how such selectivity was achieved. However, several key questions remained unanswered. Foremost, it was unclear if these in vitro results would translate to an improved therapeutic index in vivo. Moreover, it was unclear if an improved therapeutic index would come at the cost of a decreased capacity to evade resistance. As described above, one of the most remarkable features of AmB is that despite half a century of utilization, there has been no significant emergence of pathogen resistance in the clinic. It has long been suspected that the exceptional toxicity of AmB and its capacity to evade resistance were linked. In fact, less selective drugs are generally associated with increased capacity to evade resistance, but also with increased toxicity. Thus, it was unclear if a less toxic AmB derivative would be more vulnerable to pathogen resistance.

Answering both of these questions required access to larger quantities of a non-toxic AmB to support a wide range of in vitro and in vivo studies. Furthermore, the global yearly clinical supply of AmB is on the order of multiple metric tons. Thus, in order for an AmB derivative to have a chance to be advanced for clinical development, it must be accessible in an exceptionally efficient manner, and the lack of scalable access to C2′deOAmB limited its further development. We thus sought a different AmB derivative that would demonstrate a similar improvement in therapeutic index but would be potentially accessible on the metric-ton scale.

The ligand-selective allosteric model and the aforementioned crystal structure suggested a potential path forward. Specifically, in addition to the water-bridged hydrogen bonding interaction between the C2′ hydroxy and C13 hemiketal groups, the X-ray crystal structure of N-iodoacetyl AmB suggested to us that an intramolecular salt bridge between the C41 carboxylate and what would be a C3′ ammonium ion may also help stabilize the ground-state conformation of AmB predicted to bind both ergosterol and cholesterol. We reasoned that perturbing this putative interaction may represent an alternative way to cause a similar shift in the position of the sugar relative to the polyene macrolide core that would lead to the same selective stero-binding that had been achieved with C2′deOAmB. Importantly, many different types of modifications to alter this putative intramolecular salt bridge could be accessible in just a few steps from the already ton-scale fermented natural product (Figure 10).

Due to its ease of modification, the C41 carboxylate has been a common site for derivatization, and has previously yielded derivatives of AmB with encouraging but modest improvements in its therapeutic index. However, we no-
ticed that all of these derivatives had retained the C16–C41 carbon–carbon bond. As part of contemporaneous studies, Stephen Davis, my graduate student, discovered that diphenyl phosphoryl azide promotes an efficient Curtius rearrangement on a minimally protected AmB, cleaving the C16–C41 bond to form an isocyanate intermediate (Scheme 3). The isocyanate intermediate is immediately trapped by the neighboring C15 hydroxy forming an isolable oxazolidinone intermediate. Surprisingly, this oxazolidinone is quite easily opened under mild conditions with amines leading to a new class of AmB derivatives, the AmB ureas, which can be accessed in just three steps from the natural product. Guided by the ligand-selective allosteric effects model described above, we questioned whether, like C2’demAmB, these AmB ureas might selectively bind ergosterol over cholesterol.

Scheme 3 Synthesis of AmB ureas

With the aforementioned structural model for ligand-selective allosteric effects having been illuminated by our mechanistic studies of AmB cytotoxicity, we reasoned that these new derivatives might perturb the putative intramolecular salt bridge interaction and thereby favor ergosterol over cholesterol binding. Furthermore, we found that these ureas are easily accessed in just three steps (and just one pot) from the minimally protected natural product, which is available from fermentation on the metric-ton scale. We thus decided to test whether these new derivatives might demonstrate a similarly improved therapeutic index and thereby represent a production-scale accessible less toxic AmB.

Matthew Endo tested the sterol binding of the urea derivatives via ITC and found that they retain the capacity to bind ergosterol (Figure 11A). However, within the limits of detection, we observed no binding to cholesterol (Figure 11B). We then evaluated these urea derivatives in antifungal and hemolysis assays and compared them to many previously reported modifications of the C41 carboxylate, including methyl esterification (AmBME), reduction to a methyl group (MeAmB), and conversion into a methyl amide (AmBMA). We also resynthesized a doubly modified derivative (AmBTABA) that represented one of the largest previously reported improvements in in vitro therapeutic index. These derivatives all demonstrated modest improvements in therapeutic index compared to AmB. However, in stark contrast, the AmB ureas showed a remarkable decrease in toxicity to human red blood cells with both the methyl urea (AmBMU) and aminoethyl urea (AmBAU) failing to show complete hemolysis even up to 500 μM. Interestingly, the only structural difference between AmBMU and AmBMA is a protonated nitrogen atom between the C16 and C41 carbons.

Figure 11 Total exotherm measured by ITC to determine (A) ergosterol-, and (B) cholesterol-binding capacity of the AmB urea derivatives

We further probed the activity of these AmB ureas against a panel of pathogenic Candida, Cryptococcus, and Aspergillus strains, including strains that are resistant to theazole class of antifungals. In all of the strains, the AmB ureas maintained potent antifungal activity. Final in vitro testing looked at the renal toxicity of the urea compounds against primary RPTECs and an immortalized human renal cell line (iHERT1 RPTECs). In both types of renal cells, we saw diminished toxicity compared to AmB. This promising series of in vitro assays led us to seek out a collaboration with David Andes at the University of Wisconsin at Madison, who has developed the most widely employed mouse model of invasive candidiasis.
Enabled by our efficient three-step/one-pot synthesis of the AmB ureas, we were able to generate substantial quantities of these derivatives for in vivo testing in collaboration with Andes and his colleague, Karen Marchillo. Neutropenic mice infected with a clinical isolate of C. albicans were treated with AmB, AmBMU, or AmBAU at 1, 4, or 16 mg per kg (Figure 12A–C). We observed a pronounced reduction in fungal burden by both AmBMU and AmBAU compared to AmB, especially at the 16 mg per kg dosage at 24 hours post-treatment. To study in vivo toxicity, we monitored lethality from intravenous injections of AmB or the urea derivatives to healthy and uninfected mice (Figure 12D). At just 4 mg per kg, AmB killed all of the mice within seconds. It was not until 64 mg per kg administration that lethality exceeded 50% from AmBAU, and even at 64 mg per kg, no lethality was observed for AmBMU.

Finally, we teamed up with Susan Lindquist and her colleagues, Ben Vincent and Luke Whitesell, at the Massachusetts Institute of Technology to determine whether the improved therapeutic index observed for these AmB ureas came at the cost of increased vulnerability to resistance. AmBMU and AmBAU were tested against a panel of lab-generated C. albicans strains with mutations to various ergosterol biosynthesis genes. Similar to AmB, only the erg2, erg6, and erg3erg11 mutants were resistant to the urea derivatives and these mutants are known to be avirulent. We sought out other potential mutations that may incur resistance by gradual resistance-selection with AmB and both urea derivatives. We then sequenced the full genome of each of the rare resistant mutants that we were able to generate against AmB, AmBMU, and AmBAU. For all three compounds, resistance mutations were primarily found in the ERG2 or ERG6 locus, and no unique resistance mutations were observed with AmBMU or AmBAU relative to AmB.

Moreover, like AmB-resistant mutants, the AmBMU- and AmBAU-resistant mutants were sensitive to an oxidative stressor (tert-butyl peroxide) and were highly dependent on stress response chaperone Hsp90 (inhibition by geldanamycin). Furthermore, the ability to filament is a key factor in virulence, and it was previously shown that AmB-resistant mutants lose the capacity to filament. Similarly, mutants resistant to the urea derivatives were substantially deficient in the capacity for filamentation (Figure 13A).
nally, we infected mice with our AmBMU- and AmBAU-resistant mutants and determined whether these mutants were capable of causing lethal infection. While the wild-type C. albicans were lethal over the course of the two-week observation, the AmB-, AmBMU-, and AmBAU-resistant strains were unable to induce lethality (Figure 13B). Thus, the AmB ureas are no more vulnerable to resistance than AmB, which has evaded resistance in the clinic for half a century. These AmB ureas have now been licensed to REVOLUTION Medicines, a new biotech company committed to the development of optimal drug candidates and the pursuit of clinical studies.

Throughout the course of this exciting journey to less toxic amphotericins, we have not lost sight of our original goal of replacing missing protein ion channels with small molecule surrogates — the objective that first pulled our attention to this archetypal ion-channel-forming natural product. In fact, as mentioned above, our finding that ergosterol binding, rather than membrane permeabilization, underlies the fungicidal activity of amphotericin suggests that the ion-channel-forming and cell-killing activities of AmB might be separable. My students, Alexander Cioffi, Jennifer Hou, Anthony Grillo, and Katrina Diaz have recently achieved this separation in yeast, which enabled us to make the exciting discovery that AmB can serve as a functional surrogate for missing potassium-transporting proteins and thereby restore vigorous and sustainable yeast cell growth. And thus our journey continues.

References


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