# Nontoxic antimicrobials that evade drug resistance

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Drugs that act more promiscuously provide fewer routes for the emergence of resistant mutants. This benefit, however, often comes at the cost of serious off-target and dose-limiting toxicities. The classic example is the antifungal amphotericin B (AmB), which has evaded resistance for more than half a century. We report markedly less toxic amphotericins that nevertheless evade resistance. They are scalably accessed in just three steps from the natural product, and they bind their target (the fungal sterol ergosterol) with far greater selectivity than AmB. Hence, they are less toxic and far more effective in a mouse model of systemic candidiasis. To our surprise, exhaustive efforts to select for mutants resistant to these more selective compounds revealed that they are just as impervious to resistance as AmB. Thus, highly selective cytocidal action and the evasion of resistance are not mutually exclusive, suggesting practical routes to the discovery of less toxic, resistance-evasive therapies.

ess selective pharmacological action is generally associated not only with decreased vulnerability to resistance but also with increased toxicity<sup>1,2</sup>. The classic example is AmB, an exceptionally resistance-evasive but also highly toxic antifungal agent that has remained the last line of defense in treating invasive fungal infections for over half a century<sup>3</sup>. An excess of 1.5 million people die from such infections each year, in large part because the extreme toxicity of AmB is dose limiting<sup>4</sup>. Extensive efforts to develop a clinically viable, less toxic amphotericin have been made but have not succeeded<sup>5</sup>. Moreover, it has remained unclear whether such a decrease in toxicity would come at the cost of an increase in vulnerability to pathogen resistance.

For decades, the pursuit of a less toxic amphotericin was guided by the widely accepted model in which AmB (Fig. 1a) kills cells via ion channel-mediated membrane permeabilization<sup>5,6</sup>. This model suggests that improving the therapeutic index of this drug requires the selective self-assembly of oligomeric ion channels in yeast versus human cells, a problem that has been very challenging to approach rationally. Contrary to this model, it was recently shown that AmB primarily exists as a large extramembranous aggregate that kills yeast by simply binding<sup>7</sup> and extracting<sup>8</sup> ergosterol and may kill human cells by similarly binding cholesterol<sup>9</sup>. Ergosterol is critical for many different aspects of yeast physiology<sup>10-14</sup>, and mutations that alter sterol biosynthesis in a manner that confers resistance abrogate fungal virulence<sup>15</sup>, explaining the failure of fungi to evolve AmB resistance in the clinic. This new sterol sponge model enabled efforts to improve the therapeutic index of AmB to focus on the simpler problem of selectively binding sterols, and this yielded the recent discovery of a new derivative, C2'deoxyAmB (C2'deOAmB) that binds ergosterol but not cholesterol and is toxic to yeast but not human cells9. Limited synthetic access to this derivative, however, has hindered its further development and the determination of whether this improvement in therapeutic index is coupled to a decreased capacity to evade resistance.

To rationalize the greater ergosterol-selective binding observed with C2'deOAmB, we took into consideration several new structural insights regarding these prototypical small molecule–small molecule interactions. First, the mycosamine appendage is critical for binding both ergosterol and cholesterol<sup>16</sup>. Recent solid-state NMR evidence also confirms direct contact between the A and B rings of ergosterol and the AmB polyene motif in the sterol sponge complex8. Previous work suggested a relationship between activities of AmB and rotational conformers of the mycosamine sugar<sup>17,18</sup>, and a recent crystal structure of an AmB derivative<sup>19</sup> (Fig. 1b) reveals a water-bridged hydrogen bond between the C2' and the C13 hydroxyl groups and also suggests an intramolecular salt bridge between what would correspond to C41 carboxylate and C3' ammonium ions in AmB (Fig. 1a). We propose that this pair of intramolecular polar interactions together stabilize the relative positions of the mycosamine appendage and the polyene motif in a ground state conformation of AmB that binds both ergosterol and cholesterol and that deletion of the C2' hydroxyl group disrupts this stabilization and thus favors a shift to an alternate conformer that selectively binds ergosterol. Alternatively stated, this model predicts that a ligand-selective allosteric effect underlies these small molecule-small molecule interactions, similar to that observed in a number of proteins<sup>20,21</sup>. Guided by this model, and further encouraged by previous reports of modest but promising improvements in therapeutic index<sup>5</sup>, we pursued more synthetically accessible disruptions of the putative intramolecular salt bridge between the C41 carboxylate and C3' ammonium ions.

#### RESULTS

#### Three-step synthesis of AmB ureas

For more than 50 years, a less toxic AmB derivative has been sought through semisynthesis and more recently via genetic manipulations of the producing organism<sup>5,22–25</sup>. The C16 carboxylate is easily manipulated, making this position an especially attractive target for derivatization. However, all of the previously reported derivatives have maintained a C16-C41 carbon-carbon bond. We discovered that treatment of a minimally protected variant of AmB (1) with diphenyl phosphoryl azide (DPPA) cleanly promotes a stereospecific Curtius rearrangement in which the C16-C41 bond is cleaved and the that resulting isocyanate is intramolecularly trapped by the

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**Figure 1 | Synthesis of AmB urea derivatives. (a)** Chemical structures of AmB and C2'deOAmB. (b) X-ray crystal structure of *N*-iodoacyIAmB showing an intramolecular water-bridged hydrogen bond between the C2' and C13 hydroxyl groups. (c) General scheme for synthesis of AmB ureas. (d) Synthesis of AmBMU, AmBAU and AmBCU from AmB in only three or four steps. Blue color highlights the differences from the natural product.

neighboring C15 alcohol<sup>26</sup> to form an oxazolidinone (2) (Fig. 1c), thus enabling us to explore a new chemotype. This particular oxazolidinone, in turn, is unexpectedly reactive to ring opening with primary amines under mild conditions, yielding a new class of urea containing amphotericins (3) with a nitrogen bond at C16. Notably, the parent heterocycle, 2-oxazolidinone, is unreactive under the same conditions.

We further found that 1 can be directly converted to 3 in a scalable one-pot operation involving serial addition of DPPA, an amine and aqueous acid. Starting with 1 g of fermented AmB and using methyl amine as the nucleophile, this overall three-step sequence yields 264 mg of AmB methyl urea (AmBMU, 4; 64% average yield per step, 25% overall yield; **Fig. 1d**). Using ethylene diamine produces 236 mg of AmB amino urea (AmBAU, 5; 61% average yield per step, 22% overall yield), and in a four-step variation, reaction with  $\beta$ -alanine allylester followed by deallylation yields 124 mg AmB carboxylatoethyl urea (AmBCU, 6; 58% average yield per step, 12% overall yield; full synthetic details and characterization available in the **Supplementary Note**). This chemistry thus provides rapid, efficient and scalable access to these new derivatives starting with a natural product that is already fermented on the metric ton scale.

#### AmB ureas are selectively toxic to yeast

With this new series of AmB derivatives in hand, we determined their sterol binding properties. We next asked whether the urea derivatives, like AmB<sup>8</sup>, act as a sterol sponge that extracts ergosterol from the membranes of yeast cells. Using an ultracentrifugation-based membrane isolation assay<sup>8</sup>, we quantified the amount of

ergosterol remaining in the membranes of *Saccharomyces cerevisiae* after treatment with AmB or the urea derivatives. As seen previously with AmB<sup>8</sup>, the majority of ergosterol was removed from yeast membranes upon treatment with each of the urea derivatives (**Fig. 2a**). We next probed sterol binding selectivity via isothermal titration calorimetry (ITC; **Fig. 2b**)<sup>9</sup>. AmB binds both ergosterol and cholesterol, but the aglycone, amphoteronolide B (AmdeB, 7), binds neither sterol<sup>16</sup>. Like C2'deOAmB<sup>9</sup>, all of the new C16 urea-containing derivatives retain the capacity to bind ergosterol but, within the limits of detection of this experiment, show no binding to cholesterol.

This sterol binding selectivity translated into a major improvement in therapeutic index in vitro (Table 1). Specifically, we determined the minimum inhibitory concentration (MIC) against S. cerevisiae and the minimum hemolytic concentration (MHC) against human red blood cells for AmB, a series of previously reported C41-and/or C3'-modified derivatives5 and the new AmB ureas. AmB is a potent antifungal (MIC  $0.5 \mu$ M) but is also highly toxic to human red blood cells (MHC 8.6 µM). AmdeB is nontoxic to both<sup>9,27</sup>. Previously reported modifications, including esterification to a methyl ester (AmBME, 8) (refs. 28,29), reduction of the carboxylic acid (C41MeAmB, 9) (ref. 27), conversion to a methyl amide (AmBMA, 10) (ref. 30) and double modification to form a triazoloethyl amide bis-aminopropyl derivative (AmBTABA, 11) (ref. 31), produce modest improvements in therapeutic index, with the best results obtained with AmBTABA (MIC 0.25  $\mu$ M, MHC 49  $\mu$ M). In contrast, all of the AmB urea derivatives retained antifungal activity but showed markedly reduced toxicity to human red blood cells.

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**Figure 2** | Sterol extraction and binding capacities of AmB ureas. (a) Percent ergosterol remaining in *S. cerevisiae* membranes after extraction by AmB or its derivatives. The percentage of ergosterol remaining was normalized to values from controls treated with DMSO only. Values represent the mean of at least three experiments  $\pm$ s.d. \**P* ≤ 0.05, \*\**P* ≤ 0.001; NS, not significant. (b) Total net isotherms from ITC showing AmB or derivatives binding to ergosterol (left) or cholesterol (right) containing large unilamellar vesicles (LUV).

The MHC of AmBMU and AmBAU exceeds the limits of solubility in this assay (500  $\mu$ M). Remarkably, the only structural difference between AmBMA and AmBMU is the insertion of a protonated nitrogen atom between the C16 and C41 carbons (**Fig. 1a,b**).

The activities of the AmB urea derivatives were further tested against a series of pathogenic yeast strains (**Table 2**) (ref. 32). Both AmBMU and AmBAU demonstrate potent antifungal activity against all strains tested, including invasive *Candida*, *Cryptococcus* and *Aspergillus*. Notably, *Cryptococcus neoformans* strains 89-610 and T1 are fluconazole resistant<sup>33</sup>, whereas *Aspergillus fumigatus* strains 11628 and 14532 have CYP51 mutations and are thus voriconazole

resistant<sup>34</sup>. AmBCU also retained activity but was generally somewhat less potent. The compounds were also tested for toxicity against human renal proximal tubule epithelial cells (RPTECs), the critical site of toxicity for AmB in patients. They all showed little or no toxicity to human telomerase reverse transcriptase 1 (hTERT1)-expressing RPTEC<sup>35</sup> and substantially reduced toxicity to the more sensitive primary RPTECs<sup>36</sup>.

#### AmB ureas are more efficacious in mice

On the basis of these results, we judged AmBMU and AmBAU to be especially promising and thus further evaluated both for efficacy and toxicity in vivo (Fig. 3a-d)<sup>37</sup>. In a mouse model of disseminated candidiasis, both AmBMU and AmBAU were substantially more effective than AmB at reducing fungal burden in the kidneys at all three tested doses (1 mg per kg body weight, 4 mg per kg body weight and 16 mg per kg body weight, intraperitoneal injection). The differences in efficacy were most pronounced at the 16 mg per kg body weight dose at 24 h after inoculation. Relative to AmB treatment, AmBMU reduced the fungal burden by 1.2 log units ( $P \le 0.0001$ ), and AmBAU reduced the fungal burden by nearly 3 log units ( $P \le 0.0001$ ). We speculate that an improved pharmacological profile, potentially due to a >20-fold increase in water solubility relative to AmB, may contribute to this unexpected marked improvement in antifungal activity for the new compounds in vivo.

Acute mouse toxicity was determined by single intravenous administration of AmB or its derivatives to healthy, uninfected mice and by monitoring for lethality (**Fig. 3d**). All of the mice in the 4 mg per kg body weight AmB dosage group died within seconds. AmBAU was substantially less toxic, with >50% lethality only observed at the 64 mg per kg body weight dosage group. Notably, all of the mice dosed with even 64 mg per kg body weight AmBMU survived with no observable toxicity.

#### AmB ureas still evade resistance

We next investigated the impacts of increased ergosterol selectivity on the development of resistance to these analogs. Owing to its unique mode of action<sup>7</sup>, AmB is not susceptible to most of the common mechanisms of resistance to other antimicrobials. Its lipid target is not as readily mutable as proteins or RNA, it is unaffected by efflux pumps, and its polyene macrolide structure is not

Table 1   Antifungal and human cell toxicity								
	Me, O, OH OH OH OH OH, OH HO, Me OH OH OH OH OH O, R Me OR							
Compound	R	R'	MIC in S. cerevisiae (μΜ)	MHC in red blood cells (µM)				
AmB	<sub>д</sub> г ОН	Mycosamine	0.5	8.55 ± 2.20				
AmdeB ( <b>7</b> )	P OH	ક <sub>ζ</sub> Η	>500	>500				
AmBME ( <b>8</b> )	ک <sup>ل</sup> OMe	Mycosamine	0.25	30.67±5.38				
C41MeAmB ( <b>9</b> )	ک <sup>خ</sup> Me	Mycosamine	0.5	$22.03 \pm 6.26$				
AmBMA ( <b>10</b> )	,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,	Mycosamine	0.25	15.32 ± 3.39				
AmBTABA ( <b>11</b> )		3 O Me OH NH2 H2N	0.25	48.5±8.7				
AmBMU	° ,≁N_H_N_Me	Mycosamine	0.5	>500				
AmBAU	$\overset{O}{_{_{\mathcal{F}}}} \overset{O}{_{_{\mathcal{H}}}} \overset{O}{_{_{\mathcal{H}}}} \overset{NH_2}{_{_{\mathcal{H}}}}$	Mycosamine	0.25	>500				
AmBCU	<sup>2<sup>ℓ</sup></sup> N H N OH	Mycosamine	3	323.8±30.2				

MIC values against S. cerevisiae and MHC values causing 90% hemolysis against human red blood cells  $\pm$  percent error standard deviation. Previously reported compounds were independently synthesized, and HPLC-purified and characterization data matched literature values.

## Table 2 | Antifungal and human cell toxicity against clinically relevant cell lines

	AmB	AmBMU	AmBAU	AmBCU
C. albicans K1	0.25	0.5	0.5	0.5
Candida glabrata 760	0.06	0.25	0.25	1
Candida tropicalis 5810	0.06	1	1	2
Candida parapsilosis 22019	0.06	0.25	0.25	1
C. neoformans H99	0.06	1	1	1
C. neoformans 89-610	0.125	1	1	1
C. neoformans T1	0.125	1	1	1
A. fumigatus 41	1	2	4	4
A. fumigatus 293	1	2	2	4
A. fumigatus 11628	0.5	0.5	1	2
A. fumigatus 14532	0.5	1	2	2
hTERT1 RPTEC	$6.4\pm1.3$	>80	$37.6\pm4.8$	>80
Primary RPTEC	$2.4 \pm 0.3$	$44.4 \pm 2.1$	11.3 ± 0.4	>80

MIC against yeast pathogens (C. albicans, C. glabrata, C. tropicalis, C. parapsilosis, C. neoformans and A. fumigatus) and minimum toxic concentration (MTC) values causing 90% toxicity against human hTERTI RPTECs and human primary RPTECs  $\pm$  percent error standard deviation. MIC values are given in µg ml<sup>-1</sup>. MTC values are given in µM.

a substrate for secretion via drug-detoxifying enzymes<sup>38</sup>. Moreover, ergosterol has a central role in many aspects of yeast physiology<sup>10-14</sup>. Mutations in genes involved in ergosterol biosynthesis can change sterol structures in ways that confer AmB resistance *in vitro*<sup>39</sup>; however, these mutations have enormous fitness costs *in vivo*, crippling fungal virulence<sup>15</sup>. As a consequence, resistance rarely, if ever, appears in the clinic<sup>40</sup>. We asked whether the improved sterol selectivity of AmBMU and AmBAU rendered them more vulnerable to the evolution of resistance.

We first determined the MIC of AmB, AmBMU and AmBAU against a panel of lab-generated strains carrying mutations in all seven of the nonessential *Candida albicans* late-stage ergosterol biosynthesis genes (**Fig. 4a**)<sup>15</sup>. To our surprise, AmBMU and AmBAU had *in vitro* resistance profiles that were very similar to that of AmB (**Fig. 4a**). As for AmB, only *erg2*, *erg6* or *erg3 erg11* mutants showed substantial resistance to AmBMU and AmBAU, and all of these mutants are known to be avirulent<sup>15</sup>. Thus, known ergosterol biosynthesis mutations do not appear to be a threat to the efficacy of AmBAU and AmBMU.

We next used exhaustive unbiased selections for survival in the presence of the compounds to ask whether any other mutations could confer resistance to AmBMU or AmBAU. One-step selections on plates containing 8× the MIC of AmB, AmBMU or AmBAU yielded no colonies with stable resistance to any of the drugs, even after ethyl methanesulfonate mutagenesis. We then used a gradual resistance-selection protocol in liquid culture, with serial two-fold increases in drug concentration. Most serial selections ended in extinction of the lineage. However, we did recover five to eight mutants for each drug that exhibited a greater than or equal to four-fold increase in MIC. Notably, all of these substantially resistant mutants were cross-resistant to all three compounds, suggesting no new mechanisms of resistance unique to AmBMU or AmBAU (Supplementary Results, Supplementary Table 1).

To identify the mutations responsible for resistance in these selections, we performed whole-genome sequencing of the wild-type (WT) strain and all 11 of the *in vitro*-evolved resistant mutants, including both strongly and weakly resistant isolates (**Supplementary Table 1**). Most mutants with strong resistance to AmB or the derivatives contained mutations in the *ERG2* or *ERG6* locus that subsequently underwent a loss of heterozygosity (**Supplementary Fig. 1**) (ref. 15). Unexpectedly, we also identified three independent mutants with low-level (approximately twofold) AmB resistance mediated through a recurrent substitution in ORF19.7285 (corresponding to a D216Y mutation), an uncharacterized WD40 repeat protein conserved across fungi. However, these mutants were no more resistant to AmBMU and AmBAU than to AmB (**Supplementary Table 1**).

We then asked whether any of the mutants with substantial resistance to AmBMU or AmBAU (greater than fourfold MIC increase) could elude the marked fitness defects previously demonstrated for AmB resistance. As previously reported<sup>15</sup>, all of the AmB-resistant mutants are extremely sensitive to oxidative stressors, which are continually encountered during the course of infection. In addition, they become highly dependent on the molecular chaperone Hsp90, which supports diverse fungal stress responses<sup>15</sup>. All of the mutants resistant to AmBMU or AmBAU were likewise severely sensitized to the oxidative stressor tert-butyl hydrogen peroxide and the Hsp90 inhibitor geldanamycin (Fig. 4b,c). Previous work has also demonstrated that AmB-resistance mutations disable filamentation, a key driver of virulence in Candida<sup>15</sup>. Again, in response to stimulation with fetal bovine serum at 37 °C, all mutants with strong resistance to AmBMU or AmBAU were unable to form the filaments observed in the WT (Fig. 4d).

We next asked whether resistance to AmBMU or AmBAU reduces competitive fitness *in vivo*. To do this, we infected mice with a pool of strains consisting of the WT parent (AmBMU and AmBAU sensitive) and 15 AmBMU or AmBAU-resistant mutants (with each strain comprising one-sixteenth of the total population). After allowing the infection to proceed for 4 d (in the absence of drug treatment),



**Figure 3 | Efficacy and toxicity of AmB ureas in mice. (a-c)** Quantification of the fungal burden in the kidneys of neutropenic mice infected with *C. albicans* 2 h, 6 h, 12 h and 24 h after a single intraperitoneal injection of AmB, AmBMU or AmBAU at dosages of 1 mg per kg body weight (**a**), 4 mg per kg body weight (**b**) or 16 mg per kg body weight (**c**). *P* values are relative to AmB at each indicated time point. \* $P \le 0.05$ , \* $P \le 0.001$ , \*\*\* $P \le 0.0001$ . (**d**) Dose response toxicity assessed via determination of lethality upon single intravenous injection of AmB, AmBMU and AmBAU to healthy mice at doses of ranging from 0.5 mg per kg body weight to 64 mg per kg body weight (five mice per dosage). Mice were monitored for survival up to 1 d.

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**Figure 4 | Characterization of mechanisms and costs of resistance to AmB ureas. (a)** Activity of AmB, AmBMU and AmBAU against *C. albicans* ergosterol biosynthesis mutants. Growth (as judged by OD<sub>600</sub> at 24 h) is shown relative to WT, with no compound added. Scale bar indicates relative growth ranging from bright green (equal to WT growth, 1.00) to black (zero growth, 0.00). (**b**,**c**) MIC of *tert*-butyl peroxide (**b**) and geldanamycin (**c**) compared to AmBMU for all of the resistant isolates selected; each point indicates one or more isolates. (**d**) Filamentation in response to serum at 37 °C. Representative images of WT and AmB-resistant (AmB-R), AmBMU-resistant (MU-R) and AmBAU-resistant (AU-R) mutants selected in AmB, AmBMU or AmBAU (all cross-resistant), stained with Calcofluor white. Scale bar, 10 μm. (**e**) Competitive infection of mice with 16 different strains (1 WT and 15 that are resistant to AmBMU and AmBAU). Fraction of pool sensitive or resistant to AmBMU and AmBAU, as determined before tail-vein injection and 4 d after isolation from kidneys. (**f**) Overall survival after tail-vein injection. Mice were injected with cells from either a pool of AmB-resistant strains, a pool of AmBAU-resistant strains, a parental WT strain (WT-low) or a pool of five passaged and mutagenized WT mutants (WT-mutagenized).

we killed the mice and tested the drug sensitivity of fungal colonies isolated from in the kidneys to determine the AmBMU and AmBAU-resistant fraction of the final population. Indeed, even over this short period of infection, the percentage of the surviving population resistant to AmBMU or AmBAU dropped substantially, and the drug-sensitive parent rapidly overtook the population (**Fig. 4e**).

Finally, we tested whether any of our AmBMU or AmBAUresistant mutants retained the capacity to cause lethal infection. To do so, we inoculated mice with pools of resistant mutants and compared their survival to mice infected with WT strains. At a low inoculum (to match that of an individual resistant strain), the WT strain killed all of the infected mice (**Fig. 4f**). WT strains subjected to the same mutagenesis and *in vitro* passaging used to generate the resistant strains also killed all of the mice. In stark contrast, all of the mice infected with pools of mutants selected for resistance to AmB, AmBMU or AmBAU survived the infection (**Fig. 4f**). Thus, AmBMU and AmBAU are no more vulnerable to resistance than AmB, which has evaded resistance despite its widespread clinical use for over half a century.

#### DISCUSSION

Our findings collectively reveal that selective antimicrobial action and evasion of resistance are not mutually exclusive. Here, compounds that bind with high selectivity to a pathogen-specific lipid evade the emergence of virulent resistant strains, suggesting that major costs in fitness are caused by even small changes in the structure of this lipid. This is likely because ergosterol is a central molecular node<sup>41</sup> in yeast physiology, being critical for the function of membrane proteins<sup>10</sup>, endocytosis<sup>11</sup>, vacuole fusion<sup>12</sup>, membrane compartmentalization<sup>13</sup> and pheromone signaling<sup>14</sup>.

Our results further suggest that AmBMU and AmBAU are exceptionally promising candidates for replacing AmB as a less

toxic treatment for invasive fungal infections. These new derivatives bind ergosterol but not cholesterol, maintain potent activity against a broad range of pathogenic fungi and are substantially more effective and less toxic than AmB *in vivo*, yet they still evade resistance. Moreover, these compounds are accessed in just three steps from AmB, which is already fermented and commercially available on the metric ton scale. All of the reagents used in their synthesis are already used on the process scale to prepare other pharmaceuticals, including diphenyl phosphoryl azide<sup>42</sup>.

Our results also support a new ligand-selective allosteric-effect model for guiding the rational development of other nontoxic amphotericins. This model predicts that disruption of intramolecular polar interactions between functional groups on the macrolide core and the mycosamine appendage cause a conformational shift in the molecule, moving from a conformation that binds both ergosterol and cholesterol to one that selectively binds ergosterol. Notably, the portion of AmB that contains all of these functional groups, i.e., the module comprising C13-C23, is completely conserved in every member of the mycosamine-bearing polyene macrolide family of natural products<sup>43</sup>. This includes a collection of 'aromatic polyenes' that are reported to be orders of magnitude more potent than AmB44 and effective against even Aspergillus infections that are very challenging to treat<sup>45</sup>. Thus, synthesizing the analogous urea derivatives of other polyene macrolide natural products could lead to ultrapotent and/or broader-spectrum yet still minimally toxic new antifungal agents.

More broadly, our results show that targeting pathogen-specific and polyfunctional lipids represents a promising blueprint for achieving the highly sought combination of low toxicity and evasion of resistance. Notably, nisin, which binds a bacterial polyfunctional lipid, lipid II, has similarly evaded resistance despite half a century of use as a food preservative<sup>46</sup>, and the recently discovered antibiotic teixobactin also binds lipid II and thus far has evaded resistance

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*in vitro*<sup>47</sup>. It was recently discovered that binding of the same lipid underlies the action of defensin peptides<sup>48</sup>, critical components of innate immunity that have retained antibiotic activity over more than two billion years of evolution. Other recent studies increasingly show that specific lipid-transmembrane protein interactions are critical for diverse cellular functions<sup>49</sup>. Thus, new microbespecific and polyfunctional lipids that are being discovered<sup>50</sup> present outstanding targets for the rational development of other nontoxic and resistance-evasive antimicrobials.

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#### **METHODS**

Methods and any associated references are available in the online version of the paper.

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### ARTICLE

 Han, X., Yang, K. & Gross, R.W. Multi-dimensional mass spectrometry-based shotgun lipidomics and novel strategies for lipidomic analyses. *Mass Spectrom. Rev.* 31, 134–178 (2012).

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#### Author contributions

S.A.D. and M.D.B. conceived the study and oversaw design of synthesis, biophysical and several biological experiments. B.M.V., L.W. and S.L. designed resistance studies.

D.R.A. designed mouse toxicity and efficacy studies. S.A.D. synthesized all of the compounds. B.M.V. executed all of the resistance studies. M.M.E. performed sterol binding and designed and performed cell toxicity assays. K.M. performed efficacy and toxicity studies in mice. S.A.D., B.M.V., S.L. and M.D.B. wrote the manuscript.

#### **Competing financial interests**

The authors declare competing financial interests: details accompany the online version of the paper.

#### Additional information

Supplementary information and chemical compound information is available in the online version of the paper. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html. Correspondence and requests for materials should be addressed to S.L. or M.D.B.



#### **ONLINE METHODS**

General reaction conditions. Owing to the light and air sensitivity of AmB, all reactions were performed in oven- or flame-dried glassware under an atmosphere of argon under low light conditions. Compounds were stored under an anaerobic atmosphere. All solvents were dispensed from a solvent purification as described in ref. 51 (THF,  $Et_2O$ : dry neutral alumina; DMSO, DMF, CH<sub>3</sub>OH: activated molecular sieves). Triethylamine and pyridine were freshly distilled under nitrogen from CaH<sub>2</sub>. Camphorsulfonic acid was recrystallized from ethanol. Water was obtained from a Millipore (Billerica, MA) MilliQ water purification system. Reactions were monitored by RP-HPLC using an Agilent 1200 Series HPLC system equipped with an Agilent Zorbax Eclipse C<sub>18</sub> 3.5- $\mu$ m, 4.6 × 75 mm column with UV detection at 383 nm at 1.2 ml/min or an Agilent 6230 ESI TOF LC/MS system equipped with an Agilent Zorbax Eclipse C<sub>18</sub> 1.8- $\mu$ m, 2.1 × 50 mm column with UV detection at 383 nm at 0.4 ml/min. Full experimental details and characterization for new compounds are in the **Supplementary Note**.

Extinction coefficient determination. Extinction coefficients (l mol<sup>-1</sup> cm<sup>-1</sup>) were determined as previously reported<sup>9</sup> and were as follows: AmB ( $\varepsilon_{406} = 164,000$ ), AmdeB ( $\varepsilon_{406} = 102,000$ ), AmBME ( $\varepsilon_{406} = 117,000$ ), C41MeAmB ( $\varepsilon_{406} = 102,000$ ), AmBMA ( $\varepsilon_{406} = 114,000$ ), AmBTABA ( $\varepsilon_{406} = 121,000$ ), AmBMU ( $\varepsilon_{406} = 87,000$ ), AmBAU ( $\varepsilon_{406} = 87,000$ ), AmBCU ( $\varepsilon_{406} = 58,000$ ).

*In vivo* sterol extraction studies and membrane isolation. This assay was performed in a manner similar to that previously described<sup>8</sup>. Specifically, 75-ml overnight cultures of *S. cerevisiae* were grown to stationary phase (OD ~1.7) in YPD media at 30 °C, with shaking. 49.5 ml of this culture was transferred to 50-ml Falcon centrifuge tubes.

Cells were treated with 500  $\mu$ l of DMSO, 500  $\mu$ M AmB, 500  $\mu$ M AmBAU, 500  $\mu$ M AmBMU or 500  $\mu$ M AmBCU (final compound concentration of 5  $\mu$ M). Falcon tubes were incubated in the shaking incubator at 30 °C for 2 h. Tubes were inverted at the 1 h time point for resuspension.

Yeast membranes were isolated using a modified version of Haas' spheroplasting and isosmotic cell lysis protocol and differential ultracentrifugation. After treatment, tubes were centrifuged for 5 min at 3,000g at 23 °C. The supernatant was decanted, and 5 ml of wash buffer (MilliQ H<sub>2</sub>O (89%), 1 M aq. DTT (1%) and 1 M aq. Tris buffer, pH 9.4 (10%)) was added. Tubes were vortexed for resuspension and incubated in a 30-°C water bath for 10 min. Tubes were then centrifuged for 5 min at 3,000g at 23 °C, and the supernatant was decanted.

1 ml of spheroplasting buffer (1 M aq. potassium phosphate buffer pH 7.5 (5%), 4 M aq. sorbitol (15%) and YPD medium (80%)) and 100  $\mu$ l of a 5 mg/ml aq. solution of lyticase from *Arthrobacter luteus* (L2524 Sigma-Aldrich) was added to each tube and vortexed for resuspension. Tubes were incubated in a 30°C shaking incubator for 30 min. After incubation, tubes were centrifuged for 10 min at 1,080g at 4 °C, and the supernatant was decanted.

1 ml of PBS buffer and 20  $\mu l$  of a solution of 0.4 mg/ml dextran in 8% Ficoll was added to each tube and mixed very gently to resuspend. This suspension was placed in an ice bath for 4 min and then transferred to a 30-°C water bath for 3 min.

The suspensions were transferred to 2-ml Eppendorf tubes, vortexed to ensure complete lysis and centrifuged at 15,000g at 4 °C to remove unlysed cells and cell debris. The resulting supernatants were transferred to thick-wall polycarbonate ultracentrifuge tubes (3.5 ml, 13 × 51 mm, 349622 Beckman Coulter). PBS buffer was added to the tubes to bring the volume up to ~3 ml. The tubes were centrifuged for 1 h at 100,000g at 4 °C in a Beckman Coulter TLA-100.3 fixed-angle rotor in a tabletop ultracentrifuge. The supernatant was poured off. The remaining membrane pellet was resuspended in 1 ml PBS buffer. 750 µl of the suspension was transferred to a 7-ml vial and stored at -80 °C until further analysis.

Gas chromatography quantification of sterols. The suspension was allowed to warm to room temperature, and 20  $\mu$ l of internal standard (4 mg/ml cholesterol in chloroform) was added. The suspension was dissolved in 3 ml 2.5% ethanolic KOH, which was vortexed gently, capped and heated in a heat block on a hot plate at 90 °C for 1 h. The vials were allowed to cool to room temperature. 1 ml of brine was added to the contents of each vial. Extraction was performed three times, each with 2 ml of hexane. Organic layers were combined, dried over MgSO<sub>4</sub>, filtered through Celite 545 and transferred to another 7-ml

vial. The contents of the vial were concentrated *in vacuo*. The lipid films were dried on high vac with  $P_2O_5$  for 30 min to remove residual water.

To the resulting lipid films, 100  $\mu$ l pyridine and 100  $\mu$ l N,O-bis(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (T6381-10AMP Sigma-Aldrich) was added and vortexed gently. This solution was heated at 60 °C for 1 h to produce TMS ethers. The vials were placed in an ice bath, and the solvent was evaporated off by nitrogen stream. Vials were kept at a low temperature to prevent evaporation of the sterol ethers along with the solvent. The resulting films were resuspended in 100  $\mu$ l of decane, filtered using a Supelco ISO-Disc PTFE Filter (4 mm  $\times$  0.2  $\mu$ m) and transferred to a GC vial insert for analysis.

Gas chromatography analysis was carried out on an Agilent 7890A gas chromatograph equipped with FID and a Agilent GC 7693 Autosampler. Samples were separated on a 30-m, 0.320-mm ID, 0.25- $\mu$ m film HP-5 capillary column (19091J-413 Agilent). Hydrogen was used as a carrier gas with a flow rate of 4 ml/min. Nitrogen make-up gas, hydrogen gas and compressed air were used for the FID. A split/splitless injector was used in a 20:1 split. The injector volume was 2  $\mu$ l. The column temperature was initially held at 250 °C for 0.5 min and then was ramped to 265 °C at a rate of 10 °C/min with a final hold time of 12.5 min. The injector and detector temperature were maintained at 270 °C and 290 °C, respectively.

ITC. ITC was performed as previously reported9.

Growth conditions for *S. cerevisiae*. *S. cerevisiae* were grown following known procedures<sup>9</sup>.

Growth conditions and MIC assay for *C. albicans, C. tropicalis, C. parapsilosis* and *C. glabrata.* The organisms were maintained, grown, subcultured and quantified on Sabouraud dextrose agar (SDA; Difco Laboratories, Detroit, MI) 24 h before the study, and the organisms were subcultured at 35 °C. MIC determinations were performed in duplicate on at least two occasions using the Clinical and Laboratory Standards Institute M27-A3 microbroth methodology<sup>52</sup>.

Growth conditions and MIC assay for *C. neoformans*. *C. neoformans* MIC was determined as previously reported after 48 h<sup>33</sup>.

Growth conditions and MIC assay for *C. fumigatus*. The organisms were maintained, grown, subcultured and quantified on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI). MIC determinations were performed in duplicate on at least two occasions using the Clinical and Laboratory Standards Institute M28-A2 microbroth methodology<sup>53</sup> at 48 h.

Hemolysis assays. Hemolysis experiments were performed following known procedures<sup>9</sup>.

WST-8 cell proliferation assays. *Preparation of primary RPTECs*. Primary human RPTECs were prepared following known procedures<sup>9</sup>.

Preparation of TERT1 RPTECs. TERT1 human RPTECs were purchased from ATCC (CRL-4031, Manassas, VA) and immediately cultured upon receipt. Complete growth medium was prepared using DMEM:F12 medium (ATCC, 30-2006), triiodo-L-thyronine (Sigma, T6397), recombinant human EGF (Life Technologies, PHG0311), ascorbic acid (Sigma, A4403), human transferrin (Sigma, T8158), insulin (Sigma I9278), prostaglandin E1 (Sigma, P7527), hydrocortisone (Sigma, H0888), sodium selenite (Sigma, S5261) and G418 (Sigma, A1720). Complete medium was stored at 4 °C and used within 28 d. TERT1 RPTECs were grown in a CO<sub>2</sub> incubator at 37 °C with an atmosphere of 95% air and 5% CO<sub>2</sub>.

WST-8 reagent preparation. WST-8 reagent was prepared and stored following known procedures<sup>9</sup>.

WST-8 assay. A suspension of primary or TERT1 RPTECs in complete growth medium was brought to a concentration of  $1\times10^5$  cells/ml. A 96-well plate was seeded with 99  $\mu$ l of the cell suspension and incubated at 37 °C with an atmosphere of 95% air and 5% CO<sub>2</sub> for 3 h. Positive and negative controls were prepared by seeding with 100  $\mu$ l of the cell suspension or 100  $\mu$ l of the complete medium. Compounds were prepared as 5-mM (AmB) and 8-mM (AmBAU, AmBMU, AmBCU and AmdeB) stock solutions in DMSO and serially diluted to the following concentrations with DMSO: 8,000  $\mu$ M, 6,000  $\mu$ M,

4,000  $\mu$ M, 3,000  $\mu$ M, 2,000  $\mu$ M, 1,500  $\mu$ M, 1,000  $\mu$ M, 800  $\mu$ M, 600  $\mu$ M, 400  $\mu$ M, 300  $\mu$ M, 200  $\mu$ M, 100  $\mu$ M, 50  $\mu$ M, 25  $\mu$ M, 10  $\mu$ M, 5  $\mu$ M, 2.5  $\mu$ M, 1  $\mu$ M, 0.5  $\mu$ M, 0.25  $\mu$ M and 0.1  $\mu$ M. 1- $\mu$ l aliquots of each solution were added to the 96-well plate in triplicate, with each column representing a different concentration of the test compound. The 96-well plate was incubated at 37 °C with an atmosphere of 95% air and 5% CO<sub>2</sub> for 24 h. After incubation, the medium was aspirated, and 100  $\mu$ L of serum-free medium and 10  $\mu$ l of the WST-8 reagent solution was added to each well. The 96-well plate was mixed in a shaking incubator at 200 r.p.m. for 1 min and incubated at 37 °C with an atmosphere of 95% air and 5% CO<sub>2</sub> for 2 h. Following incubation, the 96-well plate was mixed in a shaking incubator at 200 r.p.m. for 1 min, and absorbance values were read at 450 nm using a Biotek H1 Synergy Hybrid Reader (Wanooski, VT). Experiments were performed in triplicate, and the reported cytotoxicity represents an average of three experiments.

Data analysis. Percent hemolysis was determined according to the following equation (where "Abs" refers to absorbance at 450 nm):

% cell viability = 
$$\frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{neg}}}{\text{Abs}_{\text{pos}} - \text{Abs}_{\text{neg}}} \times 100\%$$

Concentration versus percentage hemolysis was plotted and fitted to 4-parameter logistic  $(4PL)^{54}$  dose-response fit using OriginPro 8.6. The MTC was defined as the concentration required to cause a 90% loss of cell viability.

Ethics statement. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin according to the guidelines of the Animal Welfare Act, the Institute of Laboratory Animal Resources Guide for the Care and Use of Laboratory Animals and the Public Health Service Policy.

In vivo mouse efficacy study. All of the studies were approved by the Animal Research Committee of the William S. Middleton Memorial VA Hospital (Madison, WI). Efficacy was assessed by CFU count in the kidneys of neutropenic mice with a disseminated fungal infection as described previously in refs. 40,55,56. A clinical isolate of C. albicans (K-1) was grown and quantified on SDA. For 24 h before infection, the organism was subcultured at 35 °C on SDA slants. A 106 CFU/ml inoculum was prepared by placing six fungal colonies into 5 ml of sterile, depyrogenated normal (0.9%) saline warmed to 35 °C. Sixweek-old ICR/Swiss specific-pathogen-free female mice were obtained from Harlan Sprague Dawley (Madison, WI). The mice were weighed (23-27 g) and given intraperitoneal injections of cyclophosphamide to render neutropenia (defined as <100 polymorphonuclear leukocytes per mm<sup>3</sup>). Each mouse was dosed with 150 mg per kg body weight of cyclophosphamide 4 d before infection and 100 mg per kg body weight 1 d before infection. Disseminated candidiasis was induced via tail vein injection of 100 µl of inoculum. AmB, AmBAU or AmBMU were reconstituted with 1.0 ml of 5% dextrose. Each animal in the treatment group was given a single 200-µl intraperitoneal (IP) injection of reconstituted AmB, AmBAU or AmBMU 2 h after infection. Doses were calculated in terms of mg of compound per kg of body weight. At each time point (6 h, 12 h and 24 h after infection), three animals per experimental condition were killed by CO<sub>2</sub> asphyxiation. The kidneys from each animal were removed and homogenized. The homogenate was diluted serially tenfold with 9% saline and plated on SDA. The plates were incubated for 24 h at 35 °C and inspected for CFU viable counts. The lower limit of detection for this technique is 100 CFU/ml. All of the results are expressed as the mean log<sub>10</sub> CFU per kidney for three animals.

*In vivo* mouse toxicity study. All studies were approved by the Animal Research Committee of the William S. Middleton Memorial VA Hospital (Madison, WI). Uninfected Swiss ICR mice were used for assessment of infusion toxicity. Groups of five mice were treated with single intravenous doses of AmB, AmBAU and AmBMU (reconstituted with 1.0 ml of 5% dextrose) or sterile pyrogen-free 0.85% NaCl administered via the lateral tail vein over 30 s. Dose levels studies included 0.5 mg per kg body weight, 1 mg per kg body weight, 2 mg per kg body weight, 32 mg per kg body weight and 64 mg per kg body weight. Following administration, mice were observed continuously for 1 h and then every 6 h up to 24 h for signs of distress or death.

**Resistance studies.** *MIC and growth assays.* Susceptibility of WT and resistant strains to AmB, AmBAU, AmBMU, *tert*-butyl peroxide (Sigma-Aldrich), geldanamycin and radicicol (A.G. Scientific) was determined in flat-bottom, 96-well microtiter plates (Costar) using a broth microdilution protocol adapted from CLSI M27-A3. Overnight cultures (14–20 h) were grown at 30 °C in YPD, and approximately  $5 \times 10^3$  cells were seeded per well. For AmB, AmBAU and AmBMU, MIC assays were performed at 37 °C in RPMI buffered with MOPS (0.165M) with 10% FBS (Sigma-Aldrich) added; for *tert*-butyl peroxide, geldanamycin and radicicol, MIC values were determined in YPD at 30 °C. MIC values were determined after 24-h incubation as the concentration of compound resulting in no visible growth in wells. For quantitative display of growth at drug dilutions, OD<sub>600</sub> was measured in a spectrophotometer (Tecan) and displayed as heat maps using Java TreeView 1.1.3 (http://jtreeview.sourceforge.net/).

Medium and growth conditions. C. albicans was generally grown and maintained as described previously<sup>15</sup>. Stocks were stored in 15% glycerol at -80 °C; strains were generally grown in YPD medium at 30 °C. Drugs were added directly to medium from DMSO stocks.

In vitro gradual selection of AmB, AmBAU or AmBMU resistance. Selection of resistance to AmB, AmBAU and AmBMU was performed as follows. 1 ml overnight (14-20 h) cultures of SC5314 (WT) were washed in PBS and then treated with 3% ethyl methanesulfonate (EMS) for 45 min. Cells were then washed 4× in YPD, resuspended in YPD and allowed to recover for 3 h. Cells were then inoculated to an OD<sub>600</sub> of approximately 0.025-0.05 in 100 ml YPD containing 0.25 µM AmB or AmB-AU or 0.375 µM AmB-MU. After 24-72 h, a 1-ml aliquot was removed from any culture that had grown to saturation and was subjected to another round of mutagenesis in the same manner as described above. After recovery, cells were then inoculated into a new YPD flask containing a 2× higher concentration of the same drug. Cultures that grew were subjected to one more round of EMS mutagenesis before inoculation into a twofold higher drug concentration (total of three rounds of EMS mutagenesis) and then passaged at twofold higher increments of drug concentration until reaching 2 µM AmB or AmB-AU or 3 µM AmB-MU. Cultures were passaged once more at 2  $\mu M$  AmB or AmBAU or 3  $\mu M$  AmBMU and then plated onto YPD medium and frozen in glycerol stocks before further evaluation.

Filamentation assay. Hyphal induction was performed by growing *C. albicans* overnight at 30 °C in YPD, washing in PBS and diluting 1:100 into RPMI plus 10% FBS (Sigma-Aldrich) at 37 °C in a culture tube on a rotating wheel. After 3 h, cultures were washed in PBS, resuspended in 250  $\mu$ g/ml Calcofluor white in a microcentrifuge tube and shaken at 30 °C for 10 min. Cells were then washed twice in PBS, concentrated tenfold, briefly sonicated in a water bath and mounted on slides for visualization under a DAPI filter set at 60× magnification.

Mouse model of systemic infection. All animal protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animals were maintained according to the guidelines of the MIT Committee on Animal Care (CAC). These studies were approved by the MIT CAC (protocol no. 0312-024-15). We used 7- to 12-weekold female Balb/c mice ordered from Taconic farms for all mouse virulence studies. All of the strains were prepared for inoculation by diluting overnight cultures (14-20 h) 1:100 into YPD, allowing them to grow into the log phase for 4-5 h and then washing them 3× in PBS before injection. Strains were injected into the lateral tail vein at a volume of 100  $\mu$ l. For mouse survival experiments, strains were grouped as follows: The WT mutagenized pool consisted of five SC5314 colonies subjected in parallel to mutagenesis and passaging (as described above) without drug exposure and injected at  $1.6 \times 10^5$  CFU per strain  $(8 \times 10^5 \text{ CFU total inoculum per mouse})$ . The WT low inoculum was the SC5314 parental strain injected at 1.6 ×105 CFU. AmB-resistant, AmBAU-resistant and AmBMU-resistant pools comprised strains isolated from each selection in the presence of the indicated drug, using strains that exhibited greater than fourfold MIC increase for the drug used. Individual resistant strains were present in the pools at  $1.6 \times 10^5$  CFU per mouse ( $8 \times 10^5$  total inoculum per mouse when pooled). Each strain or pool of strains was tested in at least two independent experiments, and data were pooled. Mice were weighed daily and monitored for signs of morbidity and killed when body weight decreased by 20% or when signs of extreme distress were apparent. For the competitive infection with quantification of kidney burden, a pool comprising 16 strains (1 SC5314 WT strain, 5 AmB-resistant strains, 5 AmBAU-resistant strains and 5 AmBMU-resistant strains, with each strain comprising one-sixteenth of the total) was used, with  $3 \times 10^4$  cells of each strain inoculated per mouse ( $4.8 \times 10^5$  total inoculum). Three mice were used per experiment in a total of two experiments. Mice were killed 4 d after infection, and kidneys were removed aseptically, homogenized and plated onto YPD plates. Pools of the inoculum immediately before injection were also plated. 184 colonies were randomly selected from the pre-infection plates, and colonies tested for growth in 96-well plates in the presence of 1  $\mu$ M AmBAU or 1.5  $\mu$ M AmBMU, allowing the fraction of wells from the pre- and post-infection pools exhibiting growth in either drug to be determined.

Whole-genome sequencing, alignment, mapping and variant calling. Wholegenome sequencing and analysis was performed as previously described<sup>15</sup>.

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