# Novel Water-Soluble Amphotericin B-PEG Conjugates with Low Toxicity and Potent *in Vivo* Efficacy

Assaf Halperin,[†,‡] Yana Shadkchan,[§] Evgeni Pisarevsky,[∥] Alex M. Szpilman,*[∥] Hani Sandovsky,[§] Nir Osherov,*[§] and Itai Benhar*[†,‡]

[†]Department of Molecular Microbiology and Biotechnology, The George S. Wise Faculty of Life Sciences; [‡]Center for Nanoscience and Nanotechnology; and [§]Department of Clinical Microbiology and Immunology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv 69978, Israel

[∥]Schulich Faculty of Chemistry, Israel Institute of Technology, 3200008 Technion-City, Haifa, Israel

Ⓢ *Supporting Information*
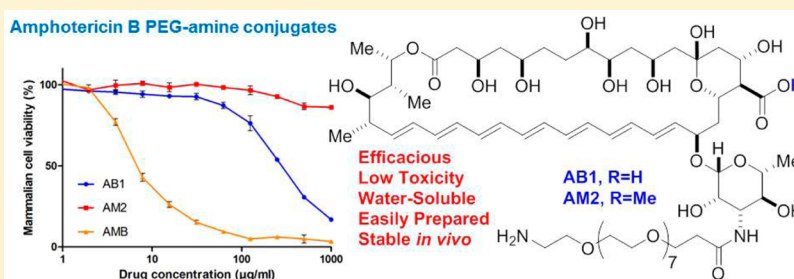
ABSTRACT: Systemic fungal infections are an increasingly prevalent health problem, especially among immunocompromised patients. Antifungal drug development lags far behind in comparison to other types of antimicrobial drugs. Current commercially available antifungals are limited by their insufficient potency, side effects, drug–drug interactions, developing drug-resistance, and narrow formulation options. Here, we report the preparation and evaluation of two novel PEG amide conjugates of amphotericin B (AMB (**1**)): AB1 (**4**) and AM2 (**5**). These compounds are nonlabile, they are prepared in only two and three synthetic steps, respectively, and they show antifungal activity against a wide range of clinical fungal isolates. Their toxicity is significantly lower, and their water solubility is up to 5000-fold higher than that of AMB (**1**). *In vivo* efficacy studies in a mouse model of systemic candidiasis showed that AM2 (**5**) successfully cured all the mice at concentrations above 3.5 mg/kg body weight. In conclusion, these properties make AB1 (**4**) and AM2 (**5**) promising candidates for clinical use.

■ INTRODUCTION

During recent decades, systemic fungal infections have emerged as an increasingly prevalent problem. Infections are rising in frequency among immunocompromised patients, including individuals suffering from HIV/AIDS or diabetes mellitus, or following organ transplantations and immunosuppressive chemotherapy during cancer treatment. The most clinically important invasive opportunistic fungal pathogens belong to one of the four groups: *Aspergillus, Candida, Cryptococcus,* and *Pneumocystis.* They are responsible for the majority of morbidity and 90% of lethal fungal-related cases. For example, 30% to 50% of patients suffering from invasive aspergillosis die due to late diagnosis, drug resistance, and brain dissemination that is poorly treated by current drugs.[1,2]

Antifungal drug development lags far behind in comparison to other types of antimicrobial drugs, and current commercially available agents are limited by their insufficient potency, side effects, drug–drug interactions, developing drug-resistance, and narrow formulation. Since 2001, no new classes of antifungals have been approved. All this emphasizes the urgent and important need for developing a novel generation of antifungal

therapeutics characterized by high potency, selectivity, and improved pharmacokinetic properties.[1,2]

Currently used antifungals for systemic infections include three major classes of drugs with different mechanisms of action: polyenes (disrupt fungal membranes), azoles (inhibit ergosterol biosynthesis), and echinocandins (inhibit synthesis of cell wall β-glucan), but most suffer from serious drawbacks, including low specificity (toxicity), narrow spectrum, drug–drug interactions, and high cost.[3]

Amphotericin B (**1**, AMB) (Figure 1), a polyene macrolide antibiotic first isolated from the soil bacterium *Streptomyces nodosus,* has been used for the last 60 years as the "gold standard" antifungal drug for the treatment of a wide array of systemic mycotic infections and parasitic-derived leishmanial diseases.[4] Leishmania is considered a "neglected disease". The market for antileishmania drugs is estimated at less than $200 million/year.[5,6] AMB (**1**) is a veteran drug that has preserved its privileged status due to incremental improvements in formulation such as innovative liposomal delivery systems that
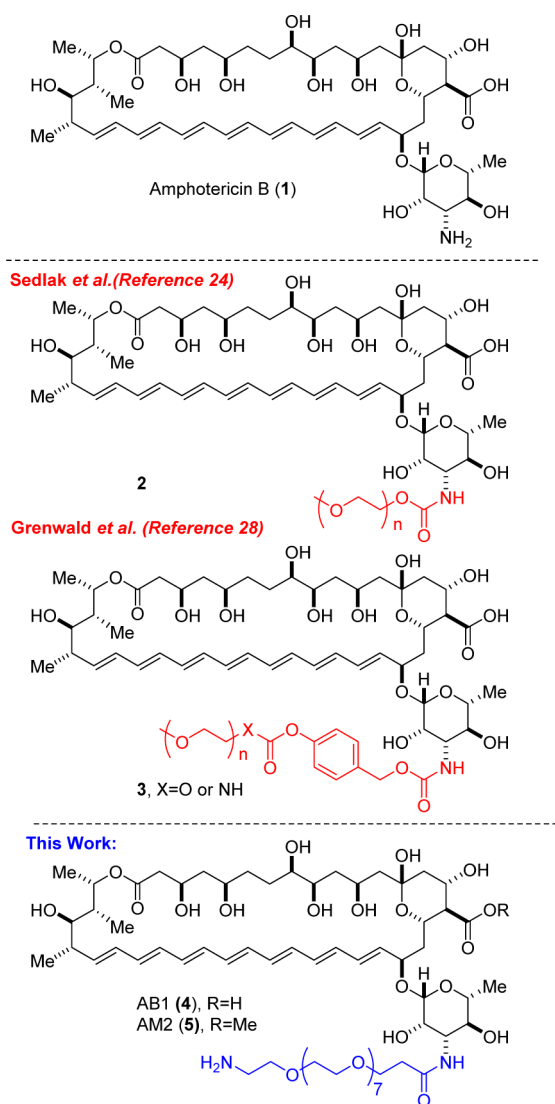
Figure 1. Structure of AMB (**1**), PEG derivatives **2** and **3**, and the novel ethanol-amine-PEG-AMB conjugates **4** and **5** reported herein.

reduce its toxicity. Studies have shown that AMB (**1**) is active against select viral, parasitic, and possibly prion infections.[7] However, because of the often dose-limiting toxicity of this natural product, mortality rates for systemic fungal infections persist near 50%.[8] The development of new antifungal drugs with an improved therapeutic index is therefore an urgent and major challenge for antifungal drug developers.

Although the mode of action of AMB (**1**) remains unclear, it is generally believed that it recognizes and possibly binds to the fungal membrane ergosterol, forming trans-membrane channels leading to leakage of minor cell constituents, cell membrane disruption, and finally fungal death. An alternative proposal is that AMB creates a "sponge" that extracts ergosterol out of the membrane.[9,10] AMB (**1**) is an attractive structural platform for the development of new drug candidate derivatives due to its very wide spectrum of activity and fungicidal action and the fact that minimal development of resistance has been reported so far. On the other hand, AMB (**1**) is notorious for its severe and potentially lethal side effects in systemic treatment. This is presumably due to poor differentiation between mammalian cell membranes cholesterol and fungal cell ergosterol, leading to mammalian cell toxicity. Side effects associated with AMB

(**1**) treatment include fever, nausea, vomiting, headache, low blood pressure, allergic symptoms, nephro- and hepatotoxicity. Therefore, it is often relegated to the role of the last line of defense in the treatment of severe fungal infections. The amphiphilic nature of AMB (**1**) makes it poorly soluble in most organic solvents and almost insoluble in water under physiological conditions of pH = 6−7. AMB (**1**) is usually administrated intravenously (IV) as a complex with sodium deoxycholate, which serves as a dispersion agent, forming water-soluble micellar colloidal complexes with AMB (**1**).[11] During the 1990s, novel lipid-associated formulations of AMB (**1**) were developed in order to increase its therapeutic index, including a lipid complex, a liposomal form and a colloidal dispersion.[12] Comparative clinical studies demonstrated that lipid formulations preserve efficacy and have reduced nephrotoxicity compared to conventional AMB (**1**). However, major disadvantages such as higher dose requirements and high cost, have limited their clinical use.[11,13]

While these formulations are used successfully in the clinic, their associated limitations have guaranteed the continued interest in developing superior formulations[14] and semi-synthetic derivatives[9] of AMB (**1**). To this end various conjugates of AMB (**1**) to biomolecules and water-soluble polymers have been reported.[9] One approach has been to attach carbohydrates such as β-arabinogalactan to the mycos-amine moiety of AMB (**1**) (Figure 1).[15−23] Another strategy is the attachment of a water-soluble polymer such as polyethylene glycol (PEG) to the mycosamine moiety AMB (**1**). Sedlak et al. reported the attachment of PEG to AMB (**1**) through the amine function of the mycosamine using a carbamate linker (**2** in Figure 1).[24−26] This PEG−AMB conjugate is believed to serve as a prodrug that releases free AMB (**1**) upon hydrolysis of the carbamate function in vivo.[24−26] Greenwald reported a similar type of compound in which an additional hydroxyl-(methyl)-phenol spacer is located between the PEG and the carbamate function (**3** in Figure 1). Enzymatic hydrolysis of the labile ester function leads to rapid release of free AMB (**1**) through a cascade reaction.[27,28]

In contrast, the study of AMB conjugates where the water-solubilizing entity, PEG, forms a permanent part of the active molecule has not been reported. Here, we report the preparation and biological profiling of two such PEG amine derivatives of AMB where a PEG linker has been linked to mycosamine via a relatively stable amide bond; AB1 (**4**) and AM2 (**5**) (Figure 1). These compounds are readily prepared through a short, three or four-step synthesis process and in comparison to AMB (**1**), have greatly reduced toxicity, significantly improved water solubility and preserve activity against a wide range of clinical fungal isolates.

■ **RESULTS**

Chemistry. The synthesis of the novel AMB derivatives AB1 (**4**) and AM2 (**5**) is shown in Scheme 1. The reaction of AMB (**1**) with commercially available **6** (1.2 equiv) in the presence of pyridine (1.2 equiv) in dry DMF gave the corresponding adduct **7** with 47% yield. Hydrolysis of the labile Fmoc protection group by the action of piperidine then led to the free amine **4** in 42% yield (after 2 steps). Alternatively, treatment of **7** with TMS-diazomethane followed by Fmoc deprotection afforded the methyl ester derivative **5** in 10% yield (after 3 steps). Conjugates **4** and **5** were fully characterized by NMR, FTIR, and HRMS (SI).

Scheme 1. Preparation of Conjugates AB1 (4) and AM2 (5)[a]
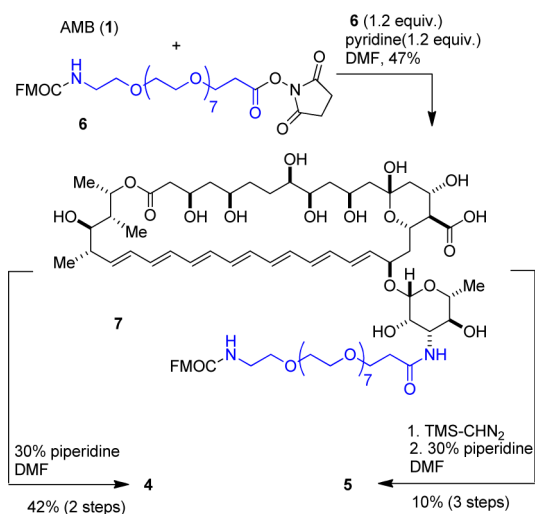
[a]See Figure 1 for structures.

We also synthesized AMB-PEG compounds conjugated to longer PEG chains. However, we found that while the water solubility improved with increasing length of the PEG conjugate, the potency was undermined (not shown). We decided to focus our efforts on the two compounds described herein, AB1 (4) and AM2 (5). These two compounds differ by the modification of the carboxyl group of AMB (1) (Figure 1, compounds 4 and 5, respectively).

Solubility and stability of AB1 and AM2. The solubilities of AB1 (4) and AM2 (5) in aqueous solution and of unmodified AMB (1) are described in Table 1. As shown,

Table 1. In Vitro Solubility and Stability Profile of PEG-Conjugated AMB Formulations

Compound	Solubility in saline (mg/mL/mM)	% hydrolysis in PBS after 4 h	% hydrolysis in PBS after 24 h
AB1 (4)	5.5/4.080	Not observed	0.5
AM2 (5)	0.7/0.514	Not observed	0.8
AMB (1)	<0.001/<0.001	Not applicable	Not applicable

AB1 (4) was >5000 more soluble than AMB (1), and AM2 (5) was >700 more soluble than AMB (1). We suggest that the methyl-ester group that replaced the carboxyl group of AMB

(1) in AM2 (5) results in the lower water solubility compared to AB1 (4).

AB1 (4) and AM2 (5) were stable in PBS, and no hydrolysis could be observed after 4 h in the buffer. Prolonged incubation for 24 h resulted in hydrolysis of 0.5% and 0.8%, respectively as determined by HPLC (SI). Further support of stability was provided by the pharmacokinetics experiments described below. The AM2 (5) that was extracted from mouse serum (after as much as 24 h post injection), and analyzed by HPLC showed only an intact compound peak, suggesting negligible hydrolysis *in vivo* over 24 h (SI).

***In-vitro* efficacy.** Efficacy (potency of antifungal activity) was evaluated by measuring minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) values for 17 fungal isolates. As shown in Table 2, AB1 (4) and AM2 (5) were 2−16-fold less potent than AMB (1) but retained its broad-spectrum antifungal activity. The kinetics of killing by AM2 (5) were compared to that of AMB (1) by time-kill analysis. As shown in Figure 2, the molecules had a similar behavior in the assay, achieving 100% fungicidal activity after 2 h of incubation.
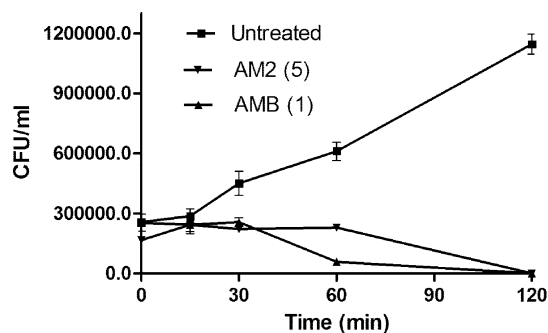

Figure 2. Time-kill analysis of AM2 (5) in comparison to AMB (1). *C. albicans* CBS 562 was incubated in liquid YPD medium in the presence of 2 μg/mL AM2 and 0.5 μg/mL for AMB (1) (2 × MIC). At different time-points, aliquots were plated to determine the number of remaining viable cells (CFU/mL). Results are presented as mean ± standard deviations of three independent experiments.

Nonspecific toxicity. The nonspecific toxicity of compounds 1, 4, and 5 was evaluated by the following two assays. The first assay was lysis of fresh human erythrocytes (hemolysis) in the presence of drug. As shown in Figure 3, AMB (1) was very toxic to hRBCs, AB1 (4) was less toxic

Table 2. In Vitro Efficacy (MICs and MFC in mg/mL) of the AMB−PEG Compounds AB1, AM2, and Unmodified AMB against Different Fungal Strains[a]

| | | AMB-PEG conjugates | | | | | |
| | | AB1 (4) | | AM2 (5) | | AMB (1) | |
Strain	No. of Isolates*	MIC	MFC	MIC	MFC	MIC	MFC
Candida albicans	2	1−2	4	1	2	0.25	0.5
Candida krusei	3	4−8	8	2	4	0.5	1−2
Aspergillus fumigatus	4	4−16	4−16	1−8	2−8	0.25−1	0.5−4
Aspergillus niger	3	1−4	1−4	2	2	0.5	0.5−2
Aspergillus flavus	3	4−8	4−8	2−4	2−4	0.5−1	0.5−1
Aspergillus terreus	1	32	32	16	16	2	4
Rhizopus oryzae	1	8	8	4	4	0.25	0.25

[a]Minimal inhibitory concentrations (MICs), and minimal fungicidal concentrations (MFCs) were determined after 48 h of incubation. *Number of isolates of a given species independently isolated from distinct patients.

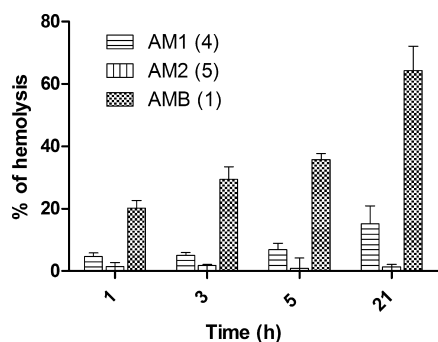(about 4-fold less), and AM2 (**5**) had negligible toxicity under the assay conditions.

Figure 3. Hemolytic activity toward hRBCs (human red blood cells). Fresh hRBCs were suspended in PBS and were incubated in the presence of 6 μg/mL drug. Dilution of hRBCs in sterile double distilled water served as a control for 100% hemolysis. After 1, 3, 5, and 21 h, the optical density of each sample was measured at 425 nm.

The second assay was measuring the viability of mouse embryonic fibroblasts (MEFs) by an MTT assay (Figure 4).
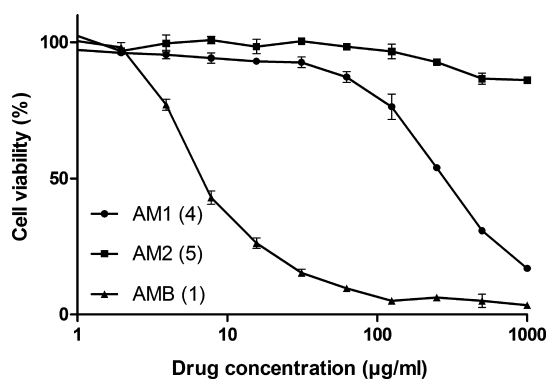

Figure 4. MTT-viability assay of mouse embryonic fibroblasts (MEFs) in the presence of drugs. Mouse embryonic fibroblasts (MEFs) were seeded in 96-well plates and incubated in 100 μL culture medium containing serial dilutions of AMB (**1**), AB1 (**4**), or AM2 (**5**) or left untreated. After 24 h, the media was replaced by fresh media (100 μL per well) containing 1 mg/mL MTT reagent and the cells were incubated for an additional 3 h at 37 °C. MTT-formazan crystals were dissolved by the addition of 100 μL per well of extraction solution, and the absorbance was recorded at 570 nm. The results were expressed as the percentage of living cells relative to the untreated control. The inhibition concentration (IC_{50}) value is the concentration of the compound which inhibited cell growth by 50%.

AMB (**1**) had an IC_{50} of 6.6 μg/mL in this assay. AB1 (**4**) had an IC_{50} of 280 μg/mL (40 times less toxic than AMB (**1**)). AM2 (**5**) did not affect cell viability at concentrations as high as 1000 μg/mL, suggesting it is at least 600 times less toxic than AMB (**1**).

***In-vivo* toxicity.** Toxicity to mice was evaluated by a single IV injection in ICR mice. As shown in Table 3, AMB (**1**) had an LD_{50} of 1.1 mg/kg, which is in agreement with mouse toxicity values reported in the literature.[29] AB1 (**4**) and AM2 (**5**) were far less toxic, reaching maximal injected doses of 22 and 42 mg/kg, respectively without any apparent toxicity. The maximal injected doses were limited by the maximal injection volume (200 μL) and solubility in saline of the compounds. For AM2 (**5**), the maximal water solubility (0.7 mg/mL) in 200 μL

Table 3. *In Vivo* Acute Toxicity of AMB (**1**), AB1 (**4**), and AM2 (**5**) in Mice

Compound	Maximal reached injected dose (mg/kg body weight)	LD_{50} (mg/kg body weight)
AB1 (**4**)	22	Not reached
AM2 (**5**)	42	Not reached
AMB (**1**)	1.1	1.2

yields 140 μg, which for a 25 gr mouse achieves a dose of 5.6 mg/kg. We could reach 42 mg/kg by making a stock solution of AM2 (**5**) in 100% DMSO further diluting it into saline for injection (at a final concentration of 2% DMSO).

At the highest injectable doses, mice injected with AB1 (**4**) or AM2 (**5**) did not show any signs of toxicity. Mice injected with AMB (**1**) at twice the LD_{50} died instantaneously. We can conclude that the mouse LD_{50} values of AB1 (**4**) and AM2 (**5**) are at least 20−40-fold higher, respectively, than AMB.

Considering the lower toxicity and higher potency of AM2 (**5**) compared to AB1 (**4**), we focused in the *in vivo* pharmacokinetics and efficacy experiments primarily on AM2 (**5**).

Pharmacokinetics (PK). A single IV dose blood PK study was carried out in ICR mice. AMB (**1**) and AM2 (**5**) were evaluated in the study. The PK graphs are shown in Figure 5,
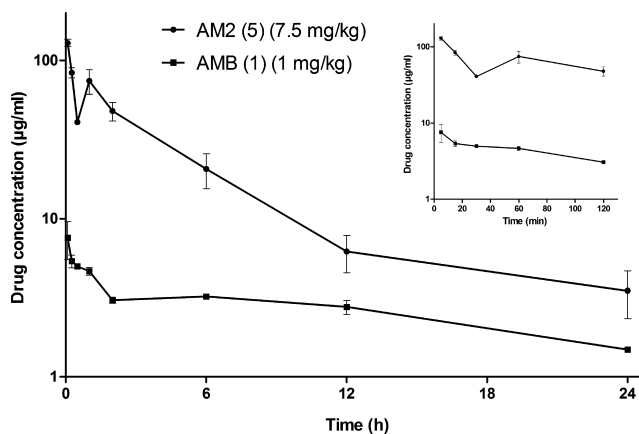

Figure 5. AMB (**1**) and AM2 (**5**) concentrations in serum following a single IV injection. Female ICR mice were injected IV with 7.5 mg/kg (body weight) of AM2 (**5**) or with 1 mg/kg (body weight) of AMB (**1**). Drug concentrations in sera were measured by extracting the drugs with methanol and analyzing the methanol fractions by HPLC at 407 nm at different time intervals (5, 15, 30 min and 1, 2, 6, 12, 48 h) after administration. After 48 h, AM2 (**5**) and AMB (**1**) were no longer detectable. The serum drug concentrations during the first 120 min of the PK study are shown in the inset. Values for three mice are shown ± SD.

and the calculated PK values are shown in Table 4. An interesting observation is that AM2 (**5**) had a faster rate of elimination and a shorter serum half-life than AMB (**1**) (The serum half-life of AMB (**1**) is 15.96 h, while that of AM2 (**5**) is 3.153 h). Of note, following AM2 (**5**) administration, no free AMB (**1**) could be detected in the serum samples suggesting the PEG moiety was not hydrolyzed during 24 h in the mice, further supporting the stability of the compound.

Considering that PEGylation is in many cases applied to extend the half-life of molecules that are conjugated to PEG, the shorter half-life of AM2 (**5**) may seem unusual. However, it is well documented that AMB (**1**) binds to serum proteins, in

Table 4. Pharmacokinetics of AMB (1) and AM2 (5) Administered IV to ICR Mice[a]

AMB preparations	Parameters					
	K_e (h)[b]	$t_{1/2}$ (h)[c]	V_d (L)[d]	CL (ml/h)[e]	AUC_{0-24} ($\mu g \cdot h/mL$)[f]	MRT_{0-24} (h)[g]
AMB (1) (1 mg/kg)	0.043	15.964	0.006	0.250	63.983	9.664
AM2 (5) (7.5 mg/kg)	0.220	3.153	0.002	0.508	421.234	5.431

[a]Pharmacokinetic parameters were calculated by the one compartment, first-order elimination model of the mean serum drug concentration−time data following bolus IV administration of AMB (1) and AM2 (5) to ICR mice. [b]k_e = elimination rate constant $(LnC_1 - LnC_2/t_2 - t_1)$. [c]$t_{1/2}$ = elimination half-life $(0.693/k_e)$. [d]V_d = volume of distribution $(Dose/C_0)$. [e]CL = clearance $(k_e \cdot V_d)$. [f]AUC = area under the serum drug concentration−time curve. [g]MRT = mean residence time.

particular to plasma lipoproteins, to albumin, and to α1-acid glycoprotein, which results in the extension of its half-life.[30,31] To study if binding to serum proteins can be an explanation for our observation, we carried out an ultrafiltration experiment where AMB (1) and AM2 (5) were incubated with serum and centrifuged using a 30 kDa cutoff centrifugal ultrafiltration device. To calculate the % of the compound bound to serum proteins, we subtracted the free drug quantity in the filtrate from the total drug. We found that while only 11% of AMB (1) could be found in the filtrate (suggesting that 89% was retained in the retentate), 90% of AM2 (5) could be found in the filtrate (suggesting that only 10% was retained in the retentate). This result suggests that, indeed, AMB (1) binds to serum proteins more than does AM2 (5), providing an explanation for their different PK behavior. A similar PK behavior has been reported for other PEG conjugated AMB compared to free AMB (1).[28]

In-vivo **efficacy.** Therapeutic efficacy was studied using a systemic mouse model of candidiasis. The results are shown in Figure 6. AMB (1) at 1 mg/kg (nearly the MTD) rescued 10 of the 12 mice in the group. AM2 (5) required a higher dose, 3.5 mg/kg, to achieve the same level of efficacy, and at 7 mg/kg (far below the MTD), it rescued all the mice. At necropsy, fungal burden in mouse tissues was evaluated. As shown in Figure 7, fungal hyphae were abundant in the kidneys of untreated mice (panel a) and barely detectable in treated mice.
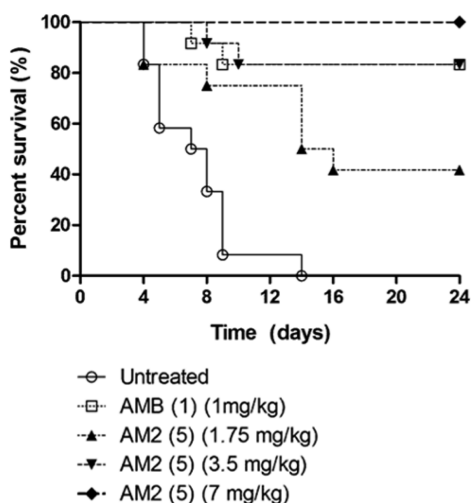

Figure 6. *In vivo* efficacy of AM2 (5) and AMB (1): Kaplan−Meyer survival curve. Naïve ICR female mice (n = 12 per group) were infected with *Candida albicans* CBS562 (5×10^4 spores/mouse) and monitored for survival for 24 days. The mice that were treated with AMB (1) at 1 mg/mg body weight and the mice that were treated with AM2 (5) in doses starting from 1.75 mg/kg body weight showed a significant increase in their survival rates as compared to the untreated group (p value = 0.009 as calculated by GraphPad Prism 5).

The fungal burden values in kidneys and spleens shown in Figure 8 are in agreement with the efficacy data.

■ **DISCUSSION**

The administration of many currently available antifungals, including notably AMB, is limited by their negligible water solubility, severe side effects, drug−drug interactions, and narrow formulation options. We have designed and prepared two new active AMB derivatives that are more water-soluble than AMB (1). These compounds show high potency, comparable to AMB, and have much lower toxicity than AMB (1). This was achieved by selective conjugation of a water-soluble entity ($PEG-NH_2$) to the single amine group in the side-chain moiety of AMB (1).

In order to circumvent the conflicting issues of chemoselectivity, retain biological activity through a free amine function, and attach a difficult-to-remove water solubilizing PEG group, we designed AMB conjugates AB1 (4) and AM2 (5) (See Figure 1). These compounds combine a PEG group attached to the mycosamine function through a relatively stable amide bond with an ethanolamine cap that serves as the basic amine function. Conjugate AB1 (4) was prepared by conjugation of the Fmoc protected ethanolamine-functionalized PEG to AMB (1) through a classical *N*-hydroxysuccinimide strategy (Scheme 1). Subsequent deprotection afforded conjugate AB1 (4) (Figure 1), which retains a free carboxylic acid function. Alternatively we carried out methyl esterification of the carboxylic group, obtaining the corresponding ethanol-amine capped-PEG-AMB conjugate AM2 (5) (see Figure 1).

In contrast to using large and size-heterogeneous biopolymers or large-size PEGs (5−40 kDa), we decided to utilize short and size-defined PEG molecules. This approach creates a well-defined medium-molecular-weight product that can be easily separated and characterized by NMR and other common techniques (as shown in the SI). The short and specific synthesis and well-defined structural nature of the antifungal conjugate is expected to be an advantage in clinical approval as well. Proof of concept is that the covalent attachment of a short, eight unit PEG-amine molecule to insoluble AMB (1) led to a significant increase in water solubility, from 0.001 mg/mL of parental AMB (1) up to 5 mg/mL for AB1 (4), an enhancement of ∼5000-fold (see Table 1). The low water solubility of AMB (1) leads to poor absorption and restricts administration to the IV route only. Overcoming this barrier may enable oral administration, which may be much more convenient for some patients. Interestingly, we found that the solubility of short to medium size PEG chains to AMB (1) correlated with the length of the PEG chain. Conjugation to a four unit PEG-amine resulted in a conjugate much more water-soluble than AMB (1) (0.3 mg/mL of the conjugate compared to <0.001 mg/mL for AMB (1)) but less water-soluble than the
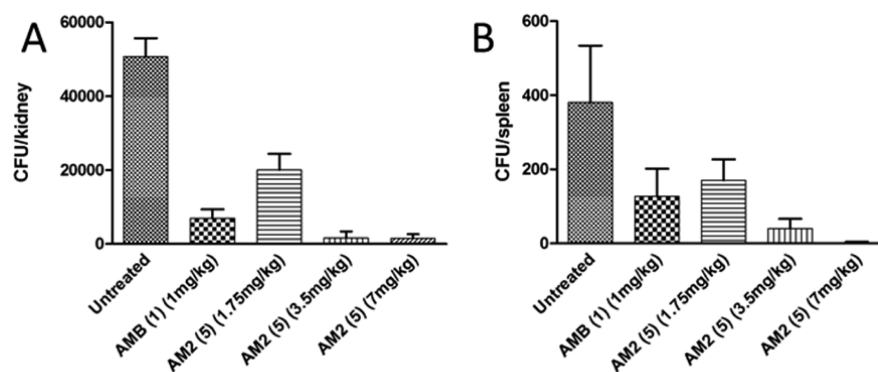
Figure 7. Fungal burden: (A) Kidney tissue fungal burden of mice infected with *C. albicans* and treated either with AMB (**1**) at 1 mg/kg or with AM2 (**5**) at 1.75, 3.5, or 7 mg/kg body weight compared to untreated controls. (B) Spleen tissue fungal burden for the same groups of mice.

eight unit PEG-amine of AB1 (**4**) (5.5 mg/mL) and AM2 (**5**) (0.8 mg/mL). Conjugation to a 44 unit PEG-amine resulted in a conjugate much more water-soluble (10 mg/mL) than the eight unit PEG-amine of AB1 (**4**) and AM2 (**5**). However, this conjugate was 30-fold reduced in efficacy compared to AMB (**1**) (data not shown). We chose to further study the eight-unit PEG amine conjugates, as they presented the best combination of improvement in water solubility with a minimal loss of potency.

Previously reported AMB−PEG conjugations, where large sized PEGs were conjugated to the mycosamine ring of AMB (**1**) via labile carbamate bonding or conjugated by an imine bond, resulted in pH-dependent cleavable bonding.[24−26] In contrast, in our approach, we created a stable amide AMB−PEG bond that is not cleaved by serum components. The previously described labile AMB−PEG conjugations rapidly release free AMB (**1**) directly into the blood, resulting in AMB (**1**) accumulation in the kidneys, leading to toxicity. Our amide derivatives remain intact, as shown by HPLC (SI), and retain their antifungal activity as a conjugate. We could postulate that the reduced general toxicity of AB1 (**4**) and AM2 (**5**) (see Figures 3 and 4 and Table 3) is due to the fact that our compounds are active in the PEG conjugated state.

In contrast to the cleavable bond of AMB−PEG derivatives previously described, our AMB (**1**) PEG conjugates can be potentially used for conjugation to drug delivery carriers of choice in the future (for example, targeted nanoparticles, dendrimers, etc.). For this purpose we created a universal PEG linker conjugated to AMB (**1**), that (1) will provide solubility for AMB (**1**); (2) introduce a spacer between the two conjugated molecules (drug and carrier) to avoid possible steric hindrance; and (3) bear a terminal amine as a chemically active group that can easily undergo conjugation chemistry. Using a short eight-unit scaffold PEG-amine provided the ideal solution to our demands, especially the ability to site-specifically protect it by Fmoc group attachment during the synthesis steps and to deprotect it when necessary. Since AB1 (**4**) has amine and carboxylic groups on the same molecule, application of conjugation chemistry may result in the formation of an amide bond between these two groups on the same or neighboring molecules and result in unwanted dimers—or even oligomers, reducing the yield of the desired product. To overcome the creation of such byproducts, we altered the chemically reactive carboxylic group to an inactive methyl ester (in compound **5**) and thereby abolished the possibility of amide bond formation between two molecules or self-reactivity.

Of note, the methyl-ester derivate of AMB underwent development in the late 1970s. This modification resulted in AMB derivatives that in a salt form had improved water solubility and reduced toxicity in comparison to AMB (**1**).[32] The clinical development of AMB methyl esters was abandoned when during experiments in dogs, and in clinical trials it showed nephrotoxicity and CNS toxicity.[33−35] Compound AM2 (**5**) differs from such compounds in that it has an addition of a covalently attached PEG chain on the mycosamine. It is therefore expected that the PD and TOX profile of AM2 (**5**) will differ from other methyl-ester AMB derivatives, which should be carefully evaluated in the future. Of note, AM2 (**5**) is less water-soluble than AB1 (**4**) (see Table 1), probably due to the larger contribution of the charged carboxyl group of AB1 (**4**) to the overall solubility of the compound compared to the methyl group of AM2 (**5**).

Most previously described chemical alterations of AMB (**1**) were done on one of two active groups existing on AMB (**1**): the C-16 carboxylic group (red in upper panel of Figure 1) and the amine group of the mycosamine ring (blue in upper panel of Figure 1). Previous studies showed that even slight chemical modifications on these groups in AMB (**1**) lead to a significant loss of antifungal activity.[9,36] Therefore, to overcome these known limitations, we took into account three important empirically observed general principles: (1) the presence of a positively charged nitrogen atom (protonable or bearing a fixed charge) is crucial for biological activity; (2) the carboxylic group does not contribute to activity and can be modified; (3) other parts of the AMB (**1**) molecule (the ring system, polyene moiety) have to stay unmodified to preserve the potency.[17] Guided by this approach, our synthesized compounds and, in particular, the methyl ester form (AM2 (**5**)) preserved their antifungal activity, resulting in a mild decrease in MICs compared to native AMB (**1**).

Whereas native AMB (**1**) administration is limited by its maximum tolerated dose (of about 1 mg/kg body weight per day for a mouse), our derivatives showed significantly reduced toxicity profiles (see Table 3). This enabled us to use larger doses of drug when necessary without reaching the acute toxicity limits. Our work describes a novel, simple, and low-cost chemical approach to synthesize novel AMB-PEG-amine derivatives. These molecules showed improved profiles: high stability in serum, increased solubility in aqueous media, reduced *in vivo* toxicity, and high *in vivo* efficacy. All these properties significantly increase the therapeutic index of these compounds, making them promising candidates for further investigation and potential clinical use.

■ CONCLUSIONS

Amphotericin B (AMB (**1**)) is commonly used to treat systemic fungal infections. However, AMB (**1**) has many drawbacks, associated primarily with its high toxicity and its poor water solubility that limit its usefulness. Here we report the preparation and evaluation of two novel PEG(8)-amine conjugates of AMB (**1**), AB1 (**4**), and AM2 (**5**). These compounds are prepared in only two and three synthetic steps, respectively (see Scheme 1) and show antifungal activity against a wide range of clinical fungal isolates (see Table 2). AB1 (**4**) and AM2 (**5**) are different from AMB (**1**) conjugates that have been described previously in that the bond linking the PEG-amine chain to AMB (**1**) is only negligibly hydrolyzable; thus, it is not cleaved in buffer or in serum and the compounds are active in the conjugated state. The systemic toxicity of AB1 (**4**) and AM2 (**5**) is significantly lower, and their water solubility is up to 5000-fold higher than AMB (**1**) while the loss in potency is minor. Combined, these properties make AB1 (**4**) and AM2 (**5**) much safer and well-tolerated drug candidates compared to AMB (**1**). *In vivo* efficacy studies in a mouse model of systemic candidiasis showed that AM2 (**5**) successfully cured all the mice at concentrations above 3.5 mg/kg body weight. In combination, these properties make AB1 (**4**) and AM2 (**5**) promising candidates for further clinical development.

■ EXPERIMENTAL SECTION

Chemicals. AMB (**1**) was purchased from Apollo Scientific (UK), Fmoc-NH-PEG(8)-NHS from Iris Biotech GmbH (Germany), all other reagents and solvents were purchased from Sigma-Aldrich (Israel) and Acros Organics/Fisher Scientific (Holland Moran, Israel). Unless stated otherwise, they were used without further purification. All solvents were ACS grade. All chemical reactions were carried out in oven-dried glassware under a positive pressure of dry nitrogen or argon.

Aqueous solutions were prepared with fresh deionized water (18.0 MΩ cm specific resistance) obtained with a Direct-Q3 Ultrapure Water Systems (Merck Millipore). Pyridine was distilled from KOH. Thin layer chromatography (TLC) was performed using Merck Silica Gel 60 F254 TLC glass plates and visualized by UV light. TLC plates were stained using ceric ammonium molybdate (CAM) or potassium permanganate stain. Chromatographic purification of products was performed on E. Merck Silica Gel 60 (230−400 mesh) using a forced flow of eluant at 0.3−0.5 bar pressure. Concentration under reduced pressure was performed by rotary evaporation at 35 °C at the appropriate pressure. NMR spectra were measured on Bruker instruments operating at 400 and 100 MHz for ^{1}H and ^{13}C acquisitions, respectively. IR was done on Bruker FTIR Model Alpha/Diamond ATR accessory. Analytical HPLC was used for the characterization of the different substances. UltiMate3000 system (Dionex) was used equipped with 3000 pump, VWD-3000 UV−vis detector and Chromeleon 6.80 software. The column used was LiChroCART 250 × 4.6 mm Purospher STAR (5 μm) C-18 RP (reverse phase). Chromatographic conditions: flow: 1.0 mL/min, linear water (buffer A)/acetonitrile (ACN) (buffer B) gradient (buffer A − 100% water, 0.1% TFA; buffer B − 100% ACN, 0.1% TFA).

Liquid Chromatography Mass Spectroscopy (LC-MS): The spectra were acquired using liquid chromatography (LC) (Acquity-UPLC, Waters Inc., USA) coupled with an UV detector (Acquity-TUV detector, Waters Inc., USA) and mass

spectrometer (SYNAPT - High Definition Mass Spectrometry (Waters Inc., USA)).

Growth media. YPD medium for the growth of fungi is composed of 1% (w/v) yeast extract (Difco), 2% (w/v) peptone (Difco), and 2% (w/v) dextrose (Merck). For YPD agar plates, 2% (w/v) agar was added. YAG medium: 0.5% (w/v) yeast extract, 1% (w/v) glucose, 10 mM MgCl$_2$, supplemented with 0.1% (v/v) trace elements solution, and 0.2% (v/v) vitamin mix. YAG plates are made of YAG medium +2% agar. RPMI-MOPS medium is composed of RPMI 1640 medium (Biological Industries, Beit Haemek, Israel) containing 0.165 M morpholinepropanesulfonic acid MOPS buffer at pH 7.0

Strains and inoculum preparation. *Animals.* Female ICR mice, 6 weeks old, weighing 20−24 gr, were used in all experiments. Animals were kept under conventional conditions and were given food and water *ad libitum*. The Ethics Committee of the Faculty of Medicine of Tel-Aviv University granted permission for the animal experiments described in this study.

Fungi. Strains of *Aspergillus fumigatus, Aspergillus flavus, Aspergillus terreus* and *Aspergillus niger, Rhizopus oryzae, Candida albicans* and *Candida krusei* were isolated from patients in Israeli hospitals and used in the *in vitro* efficacy studies. *C. albicans* strain CBS 562. ATCC 18804 was used in the *in vivo* studies.

Chemistry. *Compound AB1 (**4**).* AMB (**1**, see Scheme 1) (50 mg, 1 equiv) was reacted with NHS−PEG(8)−Fmoc (49.5 mg, 1.2 equiv) in 2 mL dry DMF in the presence of pyridine (1.2 equiv) with stirring under inert atmosphere (argon) and exclusion of light for 23 h. The reaction mixture was poured into excess cold diethyl ether. The solid residue was washed twice with ether and dried under vacuum. The product was purified on silica gel column (CHCl$_3$/MeOH/H$_2$O 10:4:0.3, R$_f$ = 0.64) to give (**7**) as a pale yellow powder (40 mg, 47% yield). The Fmoc protecting group was then removed using 3 mL of 30% piperidine in DMF for 30 min at room temperature. The final product was recovered by precipitation with cold diethyl ether, washed twice and centrifuged (CHCl$_3$/MeOH/H$_2$O 10:4:0.3, R$_f$ = 0.5). The obtained yellow product (**4**) (Scheme 1) (28 mg, 82% yield) was lyophilized and stored at −20 °C under argon protected from light in a dark vial. The purity of the final compound was determined by RP-HPLC and was ≥95%.

*Compound AM2 (**5**).* Product (**7**) (50 mg, 1 equiv) was dissolved in 2 mL dry DMF, cooled on ice and (trimethylsilyl)-diazomethane solution (4 equiv, 2.0 M in hexane) was added and brought to room temperature, followed by stirring for 3h. The reaction mixture was poured into excess cold diethyl ether. The solid residue was washed twice with ether and dried under vacuum. The product was purified on a silica gel column (CHCl$_3$/MeOH/H$_2$O 10:1.7:0.05, R$_f$ = 0.43) to give (**3**) as a pale yellow powder (13.61 mg, 27% yield). The Fmoc protecting group was removed as described in the previous section. The final product was purified by flash chromatography (CHCl$_3$/MeOH/H$_2$O 10:4:0.3, Rf = 0.59) to give AM2 (**5**) as an orange powder (4.16 mg, 36% yield). HPLC RT 16.18 min, (AMB, 16.70 min). The purity of the final compound was determined by RP-HPLC and was ≥95%.

Solubility and stability studies. The solubility of the conjugates was determined by dissolution in saline (0.9% NaCl). Briefly, a known amount of compound was added to 3 mL of saline in a 20 mL glass scintillation vial. The mixture was vortexed for 5 min, sonicated for 2 min in an ultrasonic bath

(MRC) and centrifuged for 10 min at 14,000 rpm. Than the supernatant was filtered through a 0.45 μm hydrophilic filter membrane, and the filtrate was lyophilized to dryness and weighed. Hydrolysis rates of (dissociation of PEG conjugate) of the PEG-AMB derivatives AB1 (**4**) and AM2 (**5**) were studied in phosphate-buffered saline (PBS, pH 7.4) and in fresh human plasma and determined by HPLC and read by the UV detector at 407 nm.

In vitro efficacy (MICs). The sensitivity test was performed according to the CLSI method. (CLSI. *Reference method for broth dilution antifungal susceptibility testing of yeasts; Approved Standard - Third edition.* CLSI document M27-A3. Wayne, PA: Clinical and Laboratory Standards Institute; 2008). RPMI MOPS media was used throughout the experiment as growth and dilution media. Spores and yeast cells were harvested in 0.05% Tween from 24 to 48 h cultures grown in rich YAG medium. The spores and yeast cells were counted and diluted to a final concentration of 2.5×10^4 /mL. A stock solution of 5 mg/mL AMB (**1**) was prepared in dimethyl sulfoxide. AB1 (**4**) and AM2 (**5**) solutions were prepared in sterile saline. 2-fold dilutions of each drug (32 μg/well to 0.06 μg/well) were prepared in 100 μL of RPMI−MOPS in 96 well plates. 2500 spores or yeast cells were added to each well. Results were recorded 48 h post incubation at 37 °C. The MIC was defined as the lowest drug concentration that resulted in complete inhibition of visible growth. To obtain the MFC, 10 μL of each serial dilution was taken from each well and spread on Sabouraud dextrose agar. Plates were incubated at 37 °C for 48 h. The MFC was defined as the lowest drug concentration that yielded three or fewer colonies (i.e., 99% of the inoculum was killed).[37]

Time kill studies. For time kill studies, *C. albicans* strain CBS 562 was grown overnight from a single colony in 50 mL of liquid YPD at 30 °C. Next day, the starter was diluted 1:100 and grown to $OD_{600 \, nm}$ = 0.5−0.6. Then, cells were counted and diluted to 10^6 cells/mL in YPD. AM2 (**5**) and AMB (**1**) were added at concentrations 2-fold above the MIC determined for this strain (2 and 0.5 μg/mL, respectively) and shaken at 30 °C. At different time points (0, 15 min, 30 min, 1 h, 2 h) samples were removed, diluted and plated on YPD agar plates. Colonies were counted after incubation for 24 h at 30 °C. Control measurements included untreated *C. albicans* cells. All time kill curve studies were conducted in duplicate and in three independent experiments.

Hemolysis of human red blood cells (hRBCs). The assay was carried out essentially as described in.[38] Fresh hRBCs were diluted in PBS to a ratio of 1:20, and incubated in Eppendorf tubes under slow rotation in the presence of 6 μg/mL PEG-amphotericin B conjugate (AB1 or AM2). Amphotericin B was used as positive control. Dilution of hRBCs in sterile double distilled water served as a control for 100% hemolysis. After 1, 3, 5, and 21 h, 20 μL of each test tube were removed and diluted in 180 μL PBS. Samples were centrifuged 10 min at 1200 rpm and the optical density of the supernatant was measured at 425 nm.

In vitro toxicity study on mammalian cells (MTT). One ×10^4 mouse embryonic fibroblasts (MEFs) per well were seeded in 96-well plates and incubated at 37 °C. After 24 h the media was replaced with 100 μL culture medium containing serial dilutions of AMB (**1**), AB1 (**4**), or AM2 (**5**) or left untreated. After 24 h, the media was replaced by fresh media (100 μL per well) containing 1 mg/mL MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), re-

agent and the cells were incubated for an additional 3 h at 37 °C. MTT-formazan crystals were dissolved by the addition of 100 μL per well of extraction solution (20% SDS, 50% N,N-dimethylformamide (DMF), pH = 4.7) and incubated for 16 h at 37 °C. Absorbance at 570 nm was recorded on a Biotek Synergy HT plate reader. The results were expressed as the percentage of living cells relative to the untreated control. The inhibition concentration (IC_{50}) value is the concentration of the compound which inhibited cell growth by 50%.

In vivo toxicity (acute toxicity in mice). The experiment was performed on 6-week-old female ICR mice (n = 6). Different concentrations of each compound were injected intravenously through the tail vein, 0.2 mL per mouse, until death was observed. Stock solutions of AMB (**1**) and AM2 (**5**) were prepared in DMSO and further diluted into sterile saline containing up to 2.5% DMSO. The solutions were filtered through 0.45-μm cellulose acetate sterile filters prior to injection.

In vivo efficacy. The experiment was performed on naïve 6 week old female ICR mice (n = 20). Experimental systemic murine candidiasis was produced as described in,[39] by intravenous inoculation of *C. albicans* CBS 562 into the tail vein using an inoculum of 5×10^4 cells in 0.2 mL saline per mouse (fungal count determined by microscopic counts on a hemocytometer). Treatment began 24 h after inoculation with *C. albicans* and consisted of four consecutive daily intra-peritoneal injections of AM2 (**5**) at a range of 1.75−8.75 mg/kg/day body weight. Control groups included infected untreated mice and mice treated with standard AMB (**1**) (1 mg/kg/day body weight). Survival and mouse body weight were monitored for 24 days.

Measuring fungal burden. Female ICR mice were inoculated with *C. albicans* in groups of 3 as described above and were killed 48 h later for assessment of fungal burden. One kidney and the spleen were removed aseptically from each animal and homogenized in 1 mL of sterile saline. Serial 10-fold dilutions of the homogenates were spread on YAG plates, and colony-forming units (CFU)/organ was determined from the colony count after 24 h incubation at 37 °C. All mouse experiments were performed in duplicates.

Pharmacokinetics in mice. AMB (**1**) and AM2 (**5**) solution in saline was administrated IV at a dose of 1 and 5 mg/kg body weight, respectively. The mice received an IV bolus via the tail vein. Mice were bled over a 48 h period at the following time points: 0, 5, 15, 30 min and 1, 2, 6, 12, 24, and 48 h postdosing (3 mice per time point). Bleeding was conducted using the facial vein technique into sterile Eppendorf tubes. Whole blood samples of 150 μL were collected at each time point, stored 30 min at RT for clotting and centrifuged 10,000 rpm for 10 min until serum separation. The serum samples were stored at −20 °C.

The concentration of AMB (**1**) and of AM2 (**5**) in the serum was measured by HPLC using a modification of the method of Echevarria;[40] 200 μL of methanol was added to 100 μL of serum sample and vortexed for 20 s, stored for 30 min at room temperature (22 °C), then centrifuged at 14000 rpm for 10 min. The supernatant was filtered through 0.22 μm filter and aliquots of 100 μL were analyzed by HPLC. AMB (**1**) and AM2 (**5**) were detected at a wavelength of 407 nm.

Ultrafiltration experiment. This experiment was carried out essentially as described[30] with a few modifications. 75 μg of AMB (**1**) and AM2 (**5**) were mixed with 2.5 mL of human serum (pool from unanimous healthy volunteers) and

incubated for 1 h at 37 °C. The samples were applied into a centrifugal ultrafiltration device with 30000 cutoff (Vivaspin 6, Sartorius, Germany) that was placed in a tabletop centrifuge and spun for 25 min at 6000 g 16 °C. Samples from the total, retentate and filtrate were extracted with methanol, dried by SpeedVac, suspended in 250 μL of methanol and analyzed by HPLC to quantify the drug as described above for the blood samples.

ASSOCIATED CONTENT

Ⓢ Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b01862.

> Analytical data that was obtained during the synthesis and evaluation of compounds AB1 (**4**) and AM2 (**5**): tabulated ^{1}H and ^{13}C NMR, FTIR, and HRMS data, copies of ^{1}H NMR spectra as well as RP-HPLC chromatograms of compound (**5**) (PDF)
> Table of water solubilities (CSV)

AUTHOR INFORMATION

Corresponding Authors
*E-mail: nosherov@post.tau.ac.il. Phone: +972 3 640 9599. Fax: +972 3 640 9160.
*E-mail: benhar@post.tau.ac.il. Phone: +972 36407511. Fax: +972 36405829.
*E-mail: amszpilman@gmail.com. Phone +972 4 829 5953.

Notes
The authors declare no competing financial interest.

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ABBREVIATIONS USED

PEG, polyethylene glycol; HIV, human immunodeficiency virus; AB1, PEG(8)-amine conjugate of Amphotericin B; ABCC, Amphotericin B lipid complex; ABCD, Amphotericin B colloidal dispersion; AM2, PEG(8)-amine conjugate of Amphotericin B methyl ester; AMB, amphotericin B; ACN, acetonitrile; AIDS, acquired immune deficiency syndrome; CAM, ceric ammonium molybdate; *C. albicans*, *Candida albicans*; CFU, colony-forming unit; CNS, central nervous system; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; Fmoc, fluorenylmethyloxycarbonyl; L-AMB, liposomal amphotericin B; FTIR, Fourier transform infrared spectroscopy; HPLC, high-performance liquid chromatography; hRBC, human red blood cells; HRMS, high resolution mass spectrometry; IR, infrared; IV, intravenous injection; LC, liquid chromatography; LC-MS, liquid chromatography mass spectroscopy; MEF, mouse embryonic fibroblasts; MTD, maximum tolerated dose; NMR, nuclear magnetic resonance; MIC, minimal inhibitory concentration; MFC, minimal fungicidal concentration; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); nm, nanometer; OD, optical density; PBS, phosphate-buffered saline; PK, pharmacokinetics; RT, retention time; SD, standard deviation; SI, Supporting Information; TFA, trifluoroacetic acid; TLC, thin layer chromatography; TMS, trimethylsilane; UPLC, ultraperformance liquid chromatography; UV, ultraviolet; YAG, yeast extract-agar-glucose; YPD, yeast extract peptone dextrose medium

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