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ARTICLE

Synthesis and characterization of NH_2-(AEEA)n-amphotericin B derivatives

Jinhua Zhang[1] · Jie Ma[2] · Yuanzhen Dong[2] · Wenjie Zhao[3] · Jun Feng[3,2]

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Abstract

To find novel amphotericin B (AmB) derivatives with high therapeutic potential, low toxicity, and water solubility, a series of nine N-substituted AmB derivatives were evaluated for their antifungal activity using the broth dilution method and for their hemolytic toxicity with sterile defibrinated sheep blood. Qualitative screening of the effect of the derivatives on two reference *Candida albicans* strains and of their solubility was performed based on the value of n (n is a positive integer), resulting in the identification of an optimal compound, NH_2-$(AEEA)_5$-AmB (DMR005; AEEA is 8-amino-3,6-dioxaoctanoic acid). Preliminary safety assessments of DMR005 were carried out via the MTT cell viability assay in vitro and acute toxicity assay in vivo. In general, DMR005 not only has higher water solubility and less toxicity than the parent polyene but also retains antifungal potency.

Introduction

Amphotericin B (AmB), a polyene macrolide antibiotic with activity against multidrug-resistant strains, has attracted attention since it was isolated from the metabolites of *Streptomyces* in 1953 [1]. Life-threatening opportunistic fungal infections are major causes of morbidity and mortality, especially in immunocompromised patients [2, 3]. Currently, AmB is the most effective drug for the treatment of systemic fungal infections [4–9]. However, it causes infusion-related and cumulative toxicity, particularly nephrotoxicity [10–12], resulting in a reduction in the routine use of deoxycholate micellar AmB formulations and the development of less-toxic and high-cost lipid AmB formulations [13, 14]. Although studies have shown that as drug carriers, liposomes can significantly reduce the toxicity of AmB, they still have two main disadvantages: high cost and instability. In addition, liposomes and other carrier designs cannot completely eliminate the toxicity of AmB.

Fortunately, there have been many reports on AmB modification, and many good results have been achieved. In 1997, Grzybowska et al. [15] obtained a more active compound by glycosylation of the amino group. In 2005, Hąc-Wydro et al. [16] obtained a more active compound by acylation of the amino group. In 2006, And et al. [17] obtained a more active compound by alkylation of the amino group. In addition to chemical modification of AmB, there are some reports on the biological modification of AmB. The biosynthetic pathway of AmB was changed by genetic engineering to achieve carbonylation at the C7 position, deoxycarbonylation at the C15 position, and modification of the amino group of the glucosamine moiety [18, 19], providing a group of excellent AmB derivatives.

It is believed that the low solubility of AmB could be related to its toxicity and side effects [20]. Considering the above description of AmB features and mature modification techniques, we aimed to find AmB derivatives with high therapeutic potential and water solubility. 8-Amino-3,6-dioxaoctanoic acid (AEEA) is a hydrophilic amino acid, which is closely related to its solubility. Therefore, the purpose of this study was to couple a series of AEEA groups to AmB. NH_2-(AEEA)n-OH oligomers were synthesized via solid-phase synthesis and characterized using

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✉ Jun Feng
fengj31@aliyun.com

[1] School of Pharmacy, Fudan University, Shanghai, China

[2] Shanghai Duomirui Biotechnology Ltd, Shanghai, China

[3] State Key Lab. of New Drug and Pharmaceutical Process, Shanghai Institute of Pharmaceutical Industry, China State Institute of Pharmaceutical Industry, Shanghai, China

RP-HPLC and ESI-MS. AmB conjugated to NH_2-(AEEA) n-OH was subsequently evaluated, including structure confirmation by ESI-MS/MS and assessments of antifungal activity, solubility in PBS, hemolysis, cytotoxicity in vitro, and acute toxicity in vivo.

Materials and methods

Materials

AmB and 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) were purchased from Thermo Fisher Scientific (Shanghai) Co., Ltd., China. (2-[2-(9*H*-Fluoren-9- ylmethoxycarbonylamino)ethoxy]ethoxy) acetic acid (Fmoc-AEEA-OH) was purchased from CS Biotechnology Co., Ltd, US. 2-Chlorotrityl chloride resin (2-CTC resin), *N*-hydroxysuccinimide (NHS), 1-hydroxybenzotriazole (HOBT), piperidine (PIP), and *N*,*N*-diisopropylethylamine (DIEA) were obtained from GL Biochemical (Shanghai) Ltd., China. *N*,*N*-Diisopropylcarbodiimide (DIC) was purchased from Shanghai Jingjing Biochemical Technology Co., Ltd., China. 2,2,2-Trifluoroethanol (TFE) was purchased from Saan Chemical Technology (Shanghai) Co., Ltd., China. Dichloromethane (DCM), *N*,*N*-dimethylformamide (DMF), and dimethyl sulfoxide (DMSO) were purchased from Sinopharm Chemical Reagent Co., Ltd., China. Dulbecco's minimal essential media (DMEM), HEPES, and fetal bovine serum (FBS) were obtained from Life Technologies Corporation, US. *Candida albicans* (ATCC10231, 90028) was obtained from American Type Culture Collection, Manassas, VA. Morpholine propanesulfonic acid (MOPS) and RPMI 1640 medium were purchased from Thermo Fisher Scientific, Mumbai, US. HEK293T cells were obtained from the Chinese Academy of Sciences Cell Bank. ICR mice (male, weighing 20–22 g) were purchased from Shanghai Xipuer-Beikai Experimental Animal Co., Ltd. All other reagents were of analytical or equivalent grade. Double distilled water was used throughout.

Synthesis, cleavage, and precipitation of Fmoc-(AEEA)n-OH oligomers

2-CTC resin and Fmoc-AEEA-OH were used as the carrier and the raw material, respectively. The Fmoc-(AEEA)n-OH oligomers were synthesized by a CS Bio solid-phase synthesizer (CS Biotechnology Co., Ltd, US).

After the synthesis of Fmoc-(AEEA)n-O-resin, TFE buffer [TFE: DCM = 1: 4 (V: V)] as a cleavage reagent was added at 10 ml per gram of resin and then stirred at room temperature for 1 h. The resin was removed by filtration, and the filtrate was removed by rotary evaporation. The product was subsequently washed with DCM 2–3 times, precipitated by diethyl ether, centrifuged, and vacuum-dried to obtain the desired oligomer.

Synthesis and purification of NH_2-(AEEA)n-AmB

As shown in Fig. 1, NH_2-(AEEA)n-OH (1) was coupled with AmB (3) via activation with a condensation reagent to obtain an intermediate product (4). By removing the 9-fluorenyl- methyloxycarbonyl (Fmoc) group, the target product NH_2-(AEEA)n-AmB (5) was obtained.

After the molecular weight of the crude compound was confirmed through ESI-MS, the target product was purified by HPLC and monitored by UV detection at 215 and 409 nm. The collection eluent was analyzed by HPLC and monitored by UV detection at 215, 383, and 409 nm. The target product with over 90% purity was collected, and by rotary steaming, as much acetonitrile as possible was removed in a short time under low-temperature conditions. The samples were lyophilized, and the structure of the final product was determined by ESI-MS-MS.

Antifungal susceptibility testing

The minimal inhibitory concentration (MIC) of each antifungal agent was determined by using the broth microdilution technique as described in the National Committee for Clinical Laboratory Standards [21]. MICs were determined in RPMI 1640 buffer (pH 7.0 adjusted by MOPS). The concentration of the initial inoculum was $\sim 1 \times 10^3$–5×10^3 CFU ml^{-1}. Solutions of NH_2-(AEEA)n-AmB and AmB were prepared at 1.28 mg ml^{-1} using DMSO. Incubation was performed in 96-well plates at 30 °C in a moist and dark chamber. MICs were recorded after 48 h. The MICs of NH_2-(AEEA) n-AmB and AmB were defined as the lowest concentration of drug that caused the total inhibition of visual fungal growth [22]. Samples were prepared in duplicate, and the determination was repeated.

Hemolysis assay

Sterile defibrinated sheep blood was collected and centrifuged at $1500 \times g$ for 10 min. The plasma was then removed, and red blood cells (RBCs) were washed three times with PBS (pH 7.4). After the final wash, the RBCs were suspended in PBS and diluted to an initial concentration of 2×10^8 RBCs per 100 µl. Solutions of NH_2-(AEEA)n-AmB were prepared at 1.28 mg ml^{-1} using PBS. AmB was dissolved in DMSO (10.24 mg ml^{-1}) and then diluted with borate buffer (pH 7.4) as a reference (204.8 µg ml^{-1}). Solutions of NH_2-(AEEA) n-AmB were prepared at concentrations ranging from 10 to 1280 µg ml^{-1}

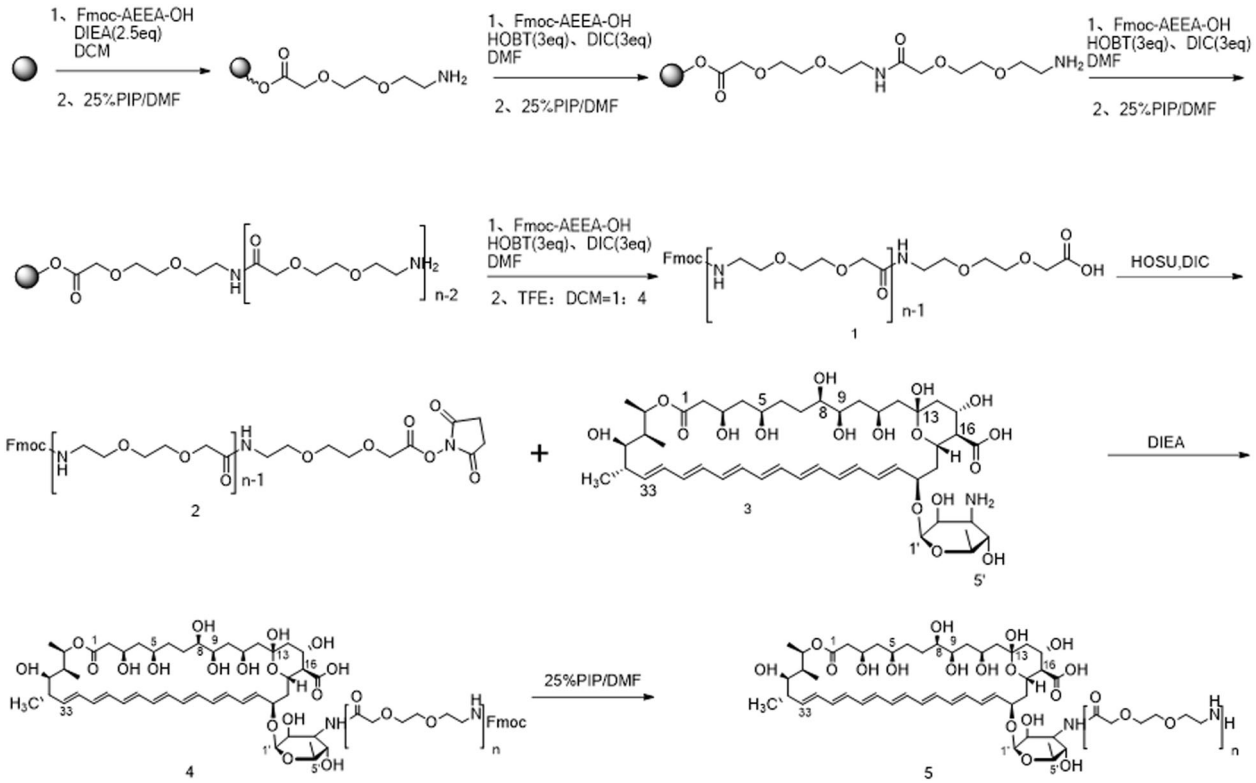


Fig. 1 The synthetic route for NH_2-(AEEA) n-AmB

in 96-well plates. AmB solutions were prepared at concentrations ranging from 1.6 to 204.8 μg ml^{-1} as a reference in 96-well plates. An RBC suspension (100 μl) was added to a 96-well culture plate and incubated for 1 h at 37 °C. The 96-well culture plate was then centrifuged for 5 min at 1500 × *g*, and the absorbance of the supernatant was measured at 540 nm using a microplate reader (SpectraMaxi3x, Molecular Devices, US). Distilled water and PBS were used as positive and negative controls, respectively. The percentage of hemolysis was calculated using the formula below [23]. In each experiment, samples were prepared in duplicate, and the experiments were repeated.

The error of the determination was less than 5%.

$$\text{Hemolysis}(\%) = (0.D_{540\,nm} - 0.D_{negativecontrol})/(0.D_{PositiveControl} - 0.D_{negativecontrol}) \times 100$$

Determination of equilibrium solubility of DMR005

The equilibrium solubility of DMR005 in PBS (pH 7.4) was measured by HPLC peak area normalization. After accurately weighed DMR005 was dissolved in PBS, we used HPLC to determine the linear relationship between the peak area and the concentration of DMR005. We performed HPLC using C18-reverse-phase silica gel column chromatography with a mobile phase consisting of (A) 0.05% trifluoroacetic acid in water and (B) 0.05% trifluoroacetic acid in acetonitrile and monitored by UV detection at 383 or 409 nm.

The shake-flask method proposed by Higuchi and Connors [24] is widely used in the determination of a drug's solubility. It determines the thermodynamic solubility of the drug after reaching equilibrium. First, 600.00 mg of DMR005 was weighed and added to 3 ml of PBS in a stoppered bottle. To facilitate dissolution, the solution was sonicated for 2 min, and the suspension was stirred for 24 h at 25 °C (±0.5 °C) without light. Finally, the suspension was centrifuged for 10 min at 25 °C [25].

Self-association of AmB and DMR005

The self-association of the compounds was monitored by UV-visible spectroscopy. Serial dilutions of the compounds were prepared with DMSO solution at a concentration 100-fold higher than the desired final concentration [26]. Then, 20 μl aliquots of each dilution were added to 2 ml of methanol or to 2 ml of PBS. Samples in PBS were incubated for 30 min at 30 °C. The UV-visible spectrum of each sample was recorded in the range of 300–430 nm (Beckman, model 3600 spectrophotometer) using 1.0 cm path length quartz cells.

Cytotoxicity assay

A cytotoxicity assay was performed to evaluate the in vitro toxicity of the DMR005 conjugate. DMR005 conjugate solution was prepared at 3.0 mg ml^{-1} using PBS. AmB was dissolved in DMSO and then diluted with borate buffer (pH 7.4) as a reference. HEK293T cells were cultured with 200 µl of DMEM containing 10% FBS and seeded at a density of 6×10^3 cells in a 96-well culture plate. The plate was incubated at 37 °C under 5% CO_2 overnight. After attaining 80% confluency, the cells were treated with different concentrations of AmB and DMR005 (11.7 to 1500 µg ml^{-1}) dissolved in borate buffer for 24 h. Cytotoxicity was determined using the MTT reagent and quantified spectrophotometrically at 590 nm using a microplate reader (SpectraMaxi3x, Molecular Devices, US). Cell viability was calculated against control groups treated with only DMEM. GraphPad Prism 5 was used to perform statistical analysis, and the results were expressed as the mean ± SEM. Multiple sets of data were compared using two-way ANOVAs. Values of $P < 0.05$ were considered statistically significant.

Acute toxicity assay in mice

Before the formal experiment, we conducted a preliminary experiment. A total of 27 mice were randomly divided into 15 groups. After tail vein injection, DMR005 and AmB were preincubated at 300 mg kg^{-1} and 25 mg kg^{-1}, respectively. Symptoms were observed, and the number of deaths was recorded. The range of doses causing 0 and 100% mortality was determined.

Based on the results of the pre-experiment, the intergroup dose group was set to 8 mice per group, and the immediate response of the animals after administration was observed and recorded. Body weight changes were monitored, and the state of the animals after the administration was recorded. At 7 days after administration, the experiment was terminated, all animals were sacrificed for gross dissection, and abnormalities were recorded. The LD_{50} was calculated using the Bliss method (basic positive rate: 0%).

Results

Characterization of NH_2-(AEEA)n-AmB

NH_2-(AEEA)n-AmB-oligomers were obtained by chemical modification of the amino group of the mycosamine moiety (Table 1).

Antifungal Activity and Red Blood Cell Toxicity

Candida albicans and sterile defibrinated sheep blood were chosen as representatives of pathogenic and host cells, respectively. The biological properties of AmB and NH_2-(AEEA)n-AmB are presented in Table 2. The MIC values found indicated that the antifungal activity of the parent AmB was well preserved in the studied derivatives. Among the tested compounds, DMR001–5 showed a relatively small (2–4-fold) decrease in activity.

RBCs are routinely used as a mammalian cell model in toxicity studies on polyene macrolide antibiotics. In this study, the toxicity of NH_2-(AEEA)n-AmB was monitored by hemolytic activity in sterile defibrinated sheep blood. When the hemolytic activity of DMR001–2 and AmB was examined, less than 5% hemolysis was observed in the presence of DMR001–2, even at concentrations of 320 and 640 µg ml^{-1}, corresponding to 12.8 µg ml^{-1} AmB. In addition, DMR003–8 and DMR010 do not cause hemolysis even at 640 µg ml^{-1} (Table 2).

Equilibrium solubility of DMR005

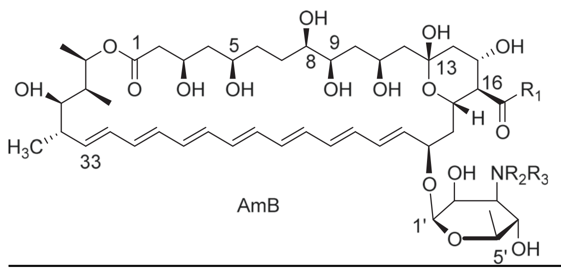
The calibration curve was determined in a linear range of 0.3–1.5 mg ml^{-1}. The regression equation was $Y = 13435 \times -2137.2$ (Y = peak area and X = concentration) with high accuracy ($R = 0.9998$).

To ensure the accuracy of the results, the measured concentration was in the middle of the standard curve. Before RP-HPLC analysis, the filtrates were diluted with PBS in a ratio varying from 1:150 to 1:300 and analyzed. The results showed that the measured concentration was within the linear range. The experiment was performed three times. The equilibrium solubility of DMR005 was 169 ± 3 mg ml^{-1}.

Spectroscopic properties and self-association

The absorption spectra of AmB and DMR005 are shown in Fig. 2. In polar organic solvents such as methanol, AmB exists as a monomer, and its UV-visible spectrum was characterized by sharp bands at 408, 383, 364, and 346 nm (Fig. 2a). The absorption spectrum of the methanol solution of DMR005 was similar to the spectrum of the monomeric form of AmB. No shifts in the typical bands were observed. The proportion of the bands was not related to concentration, and the band intensity was in accordance with Lambert-Beer's law. In contrast, the spectrum of the aqueous solution of AmB was dependent on concentration (Fig. 2b). The spectral line shape showed the coexistence of over two different species. The characteristic changes in the UV-visual spectrum of AmB reflecting its self-association revealed that the typical band of the monomeric form

Table 1 Structures of AmB and NH_2-(AEEA) n-AmB



Group	No	Compound	R_1	R_2	R_3	M [g/mol]
Reference	0	AmB		–H	–H	923
	1	DMR001		$[NH_2(CH_2CH_2O)_2CH_2CO]_1$-		1068
	2	DMR002		$[NH_2(CH_2CH_2O)_2CH_2CO]_2$-		1213
	3	DMR003		$[NH_2(CH_2CH_2O)_2CH_2CO]_3$-		1358
	4	DMR004	–OH	$[NH_2(CH_2CH_2O)_2CH_2CO]_4$-		1503
N-aminoacyl	5	DMR005		$[NH_2(CH_2CH_2O)_2CH_2CO]_5$-	–H	1648
	6	DMR006		$[NH_2(CH_2CH_2O)_2CH_2CO]_6$-		1793
	7	DMR007		$[NH_2(CH_2CH_2O)_2CH_2CO]_7$-		1938
	8	DMR008		$[NH_2(CH_2CH_2O)_2CH_2CO]_8$-		2084
	9	DMR010		$[NH_2(CH_2CH_2O)_2CH_2CO]_{10}$-		2374

Table 2 Antifungal effects of the examined compounds on *Candida albicans* (ATCC10231 and ATCC90028) and the hemolytic activities on sterile defibrinated sheep erythrocytes

Group	No	Compound	MIC (μg/mL)		Hemolytic activities (μg/mL)
			ATCC10231	ATCC90028	
Reference	0	AmB	1	1	12.8
	1	DMR001	2	2	320
	2	DMR002	4	4	640
	3	DMR003	4	4	n.hem.*
	4	DMR004	4	4	n.hem.*
	5	DMR005	4	4	n.hem.*
	6	DMR006	8	8	n.hem.*
	7	DMR007	8	8	n.hem.*
	8	DMR008	8	8	n.hem.*
	9	DMR010	16	16	n.hem.*

MIC minimal compound concentration that completely inhibits fungal growth, *n.hem.** hemolysis was not reached at concentrations up to 640 μg/ml

disappeared at 409 nm. The spectrum of DMR005 (Fig. 2b) in PBS was not subjected to similar concentration-dependent changes. AmB underwent self-aggregation at low or high concentrations. However, the proportions of the monomeric forms of DMR005 were high, and insoluble aggregates hardly existed (Fig. 2c).

HEK293T cell toxicity

DMR005 was much less toxic than AmB. At the lowest concentration tested (11.7 μg ml^{-1}), AmB killed more than 80% of HEK293T cells (Fig. 3), which was 100 times more powerful than DMR005 in terms of cell toxicity. DMR005 had very low or no toxicity to the cells even at 1500 μg ml^{-1}. Thus, compared to AmB, DMR005 had dramatically reduced toxicity to these cells.

Acute toxicity in mice

According to the deaths observed in each group, the mortality of the animals was calculated by the Bliss method (basic positive rate: 0%). The LD_{50} value of AmB was 7.2 mg kg^{-1}. After 4 days and 5 days of administration of AmB, the body weight of the surviving animals slightly increased or increased before decreasing, respectively, and after 7 days, the body weight of the living animals increased

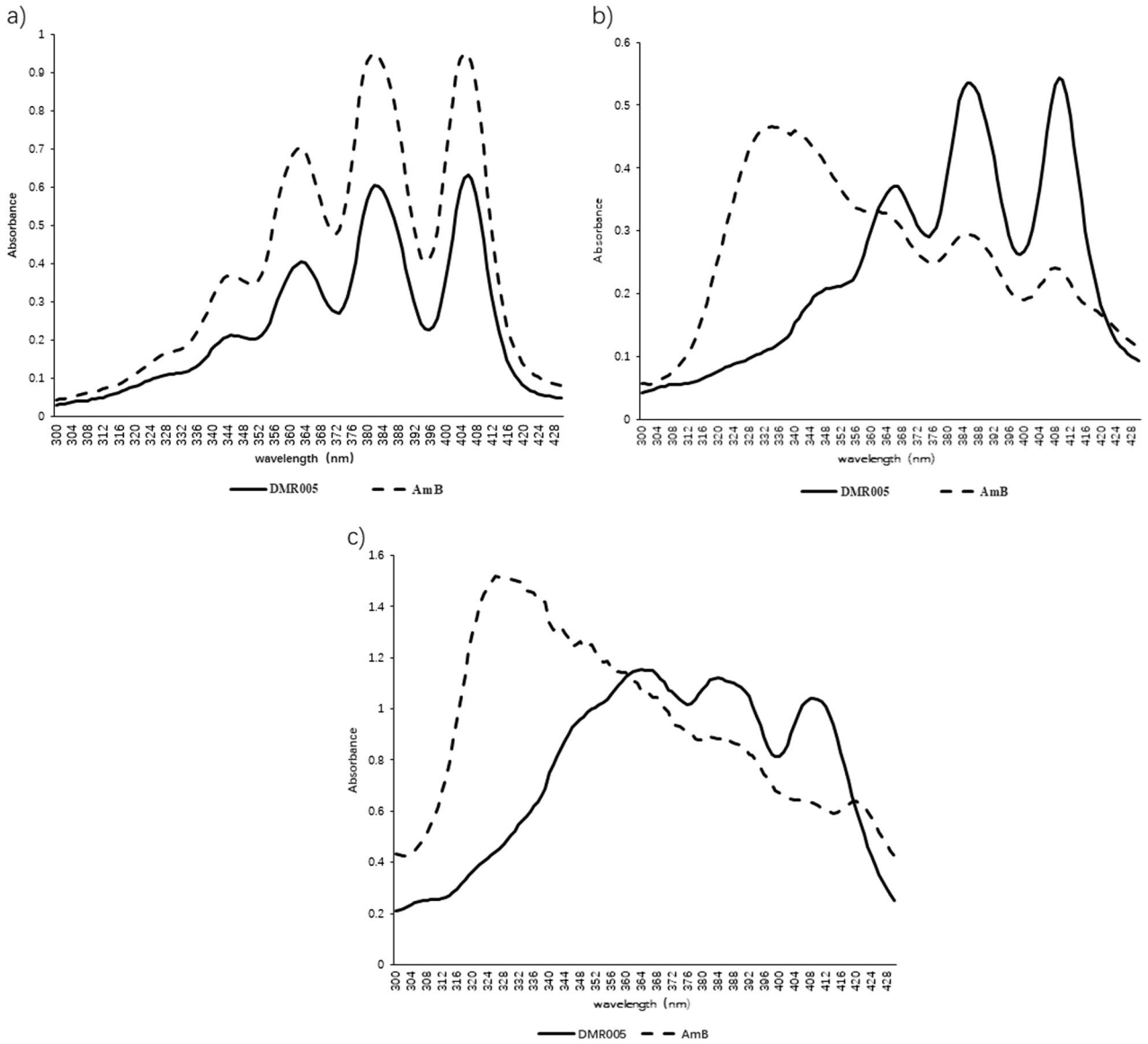


Fig. 2 Absorption spectra of 12.67 μg/ml AmB and DMR005 in **a** methanol, **b** PBS, and **c** 62.44 μg/ml AmB and DMR005 in PBS

slightly. After ~1 week of observation, all surviving animals were dissected, and no obvious abnormalities were found. The LD50 value of DMR005 was 57.9 mg kg^{-1}. The body weight of the surviving animals increased slightly after administration of DMR005, and the body weight of the living animals increased slightly after 7 days. After ~1 week of observation, all the surviving animals were dissected, and no obvious abnormalities were found. Thus, compared to AmB, DMR005 had reduced toxicity in mice.

Discussion

Since the discovery, structure confirmation, and approval of AmB for clinical use, the progress made in its modification and transformation has never stopped, from the initial transformations of chemical structures to modification with liposomes, nanoparticles, and organisms as prodrug carriers of macromolecules. AmB has been favored by scientists from all over the world. With the maturation and rapid development of solid-phase synthesis technology, this paper organically combines chemical synthesis with structural modification of AmB via solid-phase synthesis technology, resulting in the fundamental alteration of the side effects of AmB while retaining its antifungal activity. It is believed that the low solubility of AmB could be related to its toxicity and side effects [20]. Therefore, this study aimed to improve the solubility of AmB, and hydrophilic AEEA was selected as a raw material for solid-phase synthesis. Fmoc-(AEEA)n-OH was

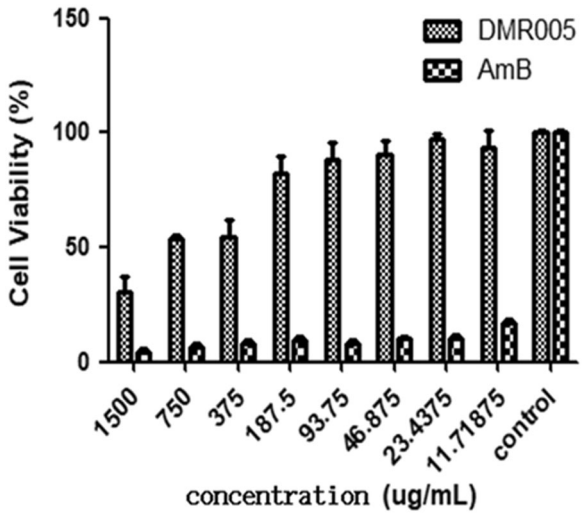


Fig. 3 Cell viability of HEK 293 cells treated with DMR005 and AmB using the MTT assay. Error bars indicated the mean ± SEM

activated using condensation reagents to generate an activated ester in the oligomers. The activated oligomers were reacted with AmB to give AmB-oligomers in amide form. Unfortunately, similar to AmB, the AmB-oligomers had three unstable sites in their structures: 1) the C13 hemiacetal group in the AmB-oligomers would be methylated in methanol solution; 2) at low pH, the glucosamine moiety was destroyed to produce *m/z* 801 impurities; and 3) the polyol fragment and conjugated heptaene bond were easily oxidized in aqueous solution under light at room temperature. However, it was found that AmB-oligomers coupled via an amide bond were very stable [27]. In this study, the main limitations associated with the use of AmB in antifungal therapy, such as poor aqueous solubility, toxicity, and hemolysis [28], were addressed by conjugating the drug to NH_2-$(AEEA)_5$-OH. DMR005 was found to exist in its monomeric form in PBS and was not aggregated. DMR005 was soluble in PBS, which is favorable for clinical use. DMR005 cannot cause hemolysis and showed potential antifungal activity against *Candida albicans* (ATCC10231, 90028). Furthermore, the nephrotoxicity and acute toxicity of DMR005 in vivo were significantly lower than those of AmB. In future studies, we will establish animal models for pharmacodynamic assessment in vivo. In conclusion, DMR005 is an excellent candidate for the treatment of fungal infections.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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