Structure-Antifungal Activity Relationships of Polyene Antibiotics of the Amphotericin B Group

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ABSTRACT:

A comprehensive comparative analysis of the structure – antifungal activity relationships for the series of biosynthetically engineered nystatin analogues, their novel semisynthetic derivatives, as well as amphotericin B (AMB) and its semisynthetic derivatives was performed. The data obtained revealed the significant influence of the structure of the C7 – C10 polyol region on the antifungal activity of these polyene antibiotics. Comparison of positions of hydroxyl groups in the antibiotics and in vitro antifungal activity data showed that the most active are the compounds in which hydroxyl groups are in the positions C8 and C9 or C7 and...
C10. Antibiotics with OH groups at both C7 and C9 positions had the lowest activity. The replacement of the C16 carboxyl with methyl group did not significantly affect the in vitro antifungal activity of antibiotics without modifications at the amino group of mycosamine. In contrast, the activity of the N-modified derivatives was modulated both by the presence of CH₃ or COOH group in the position C16, and the structure of the modifying substituent.

The most active compounds were tested in vivo to determine maximum tolerated doses (MTD) and antifungal activity on the model of candidosis sepsis in leucopenic mice (cyclophosphamide-induced). Study of our library of semisynthetic polyene antibiotics led to the discovery of compounds, namely, N-(L-lysyl)-BSG005 (3n) and, especially, L-glutamate of 2-(N,N-dimethylamino)ethyl amide of S44HP (2j) with high antifungal activity that are comparable in the in vitro and in vivo tests to AMB, and have better toxicological properties.

INTRODUCTION:

Despite the relatively recent introduction of new antifungal drug such as next generation azoles and echinocandins, polyene macrolides continue to be the most potent broad-spectrum antifungals available for the clinical use. Amphotericin B (AMB) (1) (Fig. 1) is the drug of choice for the treatment of mycotic infections caused by a wide range of fungi (1). Clinical use of this drug mostly covers life-threatening fungal infections, particularly in patients who have undergone organ transplantation, received aggressive chemotherapy, and patients with AIDS. However, AMB therapy is limited by considerable toxicity (nephrotoxicity, central nervous system and liver damage side effects) and very poor aqueous solubility. Reduction of AMB toxicity would minimize adverse side effects in patients and also allow high-dose treatment of infections caused by emerging fungal pathogens that are sensitive only to high concentrations of the drug. Until recently, the search for less toxic polyene antibiotics was carried out in two main directions: chemical modification of the natural antibiotics and investigations of lipid and liposomal formulations of polyenes.
Chemical modifications of the C16 carboxyl or/and mycosamine 3’ amino groups of AMB were extensively studied and several semisynthetic derivatives of AMB with improved characteristics have been described (2-5). However, despite some promising initial results obtained for such derivatives, no new AMB-based antifungal agent has appeared on the market, except for the lipid and liposomal formulations (1, 6).

Over the last years, a novel promising concept for the search of less toxic and highly active polyenes based on genetic engineering of antibiotic-producing microorganisms has been developed (7,8). The application of this new approach made available polyenes with unique structures, which can hardly be obtained by chemical modifications of natural antibiotics. For example, the strain of *Streptomyces noursei* producing nystatin A1 (NYT) was modified by biosynthetic engineering to produce a range of novel polyene macrolides which have differences in the position C16 and in polyhydroxylated (polyol) region C7 – C10: S44HP (2), BSG005 (3), BSG022 (4), BSG019 (5), BSG003 (6), and BSG018 (7) (Fig. 1) (9,10). Some of these novel polyene macrolides obtained by biosynthetic engineering were as active as AMB in the *in vitro* tests. Moreover, antibiotics S44HP (2) and BSG005 (3) (Fig 1) demonstrated advantages over AMB in the *in vivo* tests (11, 12).

**Figure 1.**

The important role of the hydroxyl groups in the C7 – C10 region for the antifungal activity of the antibiotics 1–7, demonstrated in previous investigations, revealed that the most active were compounds (1–5) (minimum inhibitory concentrations, MICs, against *Candida albicans* were 0.11 – 0.20 μg/ml) (9-12). For compounds 6 and 7 the shift of hydroxyl group from C10 to C9 led to the dramatic decrease of antifungal activity (MICs against *C. albicans* were 12 and 9 μg/ml, correspondingly) (10).

The new bioengineered polyene macrolides (2–7) represent a special interest as scaffolds for chemical modifications at the amino and/or carboxyl groups. Series of mono- and di-
substituted derivatives of S44HP (2) were recently obtained by chemical modifications of the exocyclic C16 carboxyl and/or the amino group of mycosamine moiety (13, 14). It has been demonstrated that some of these novel derivatives are superior to AMB in terms of both toxicity and therapeutic efficacy in mice models. Thus, combination of genetic engineering techniques and chemical modification methods appears to be a very promising approach to the search for a new polyene drug candidate. It is also useful for the investigations of the structure – activity relationships and the mechanism of action of antifungal antibiotics of this type, which is still insufficiently understood.

The aim of the present work was the analysis of the structure – antifungal activity relationships for the series of novel semisynthetic derivatives of genetically engineered antibiotics S44HP, BSG005, BSG003 and BSG022 in comparison with AMB, its semisynthetic derivatives and bioengineered antibiotics BSG018 (7) and BSG019 (5). The data obtained revealed the influence of the particular positions of the hydroxyl groups in the region C7 – C10 and a substituent in position C16 and 3’-N mycosamine on the antifungal activity of polyene antibiotics.

MATERIALS AND METHODS

Polyene antibiotics. Sigma-Aldrich Co. (St. Louis, MO) supplied AMB (1). SINTEF (Trondheim, Norway) supplied biosynthetically engineered nystatin analogues 2 – 7. Commercially available Fungizon was used in the in vivo experiments for comparison.

Synthesis of polyene analogs. A series of semisynthetic derivatives of AMB (1a – 1i), BSG005 (3k – 3n), BSG022 (4j) and BSG003 (6j) and were synthesized by the methods which have been described earlier for the synthesis of the corresponding S44HP derivatives (2a – 2n) (13,14). Purification of the semisynthetic derivatives was carried out using column chromatography on silica gel Merck (Whitehouse Station, NJ, USA) or on Sephadex G25.
The purity of the obtained compounds was confirmed by the TLC and HPLC methods. HPLC was carried out on a Shimadzu HPLC instrument (Kyoto, Japan) of the LC 10 series on a Kromasil 100-C18 column (4×250 mm, particle size 6µm) at an injection volume of 20µL and a wavelength 408 nm with flow rate 1.0 ml/min. System A: 0.2% ammonium formate (HCOONH₄) (pH 4.5) and acetonitrile (CH₃CN), the proportion of CH₃CN varied from 30 to 70 % for 30 min; system B: 0.2% ammonium formate (HCOONH₄) (pH 4.5) and acetonitrile (CH₃CN), the proportion of CH₃CN varied from 25 to 65% for 40 min; system C: 0.01M orthophosphoric acid (H₃PO₄) (pH 2.6) and acetonitrile (CH₃CN), the proportion of CH₃CN varied from 30 to 70 % for 30 min. The structure of the obtained compounds was confirmed by physicochemical and spectral methods (Table 1). Mass-spectra data were obtained on MALDI TOF Bruker BIFLEX III instrument.

L-Glutamate salt of 2j. Water soluble form of 2j (L-glutamate of 2j) was obtained as it has been described previously (14).

**Biology**

*In vitro antifungal activity.* Antifungal activity *in vitro* was tested against yeast strains *Candida albicans* ATCC 14053, *Cryptococcus humicolus* ATCC 9949 and fungous strains *Aspergillus niger* ATCC 16404 (all received from were received from American Type Culture Collection (ATCC)) and *Fusarium oxysporum* VKM F-140 (All-Russian Collection of Microorganisms; VKM - Department of the G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms RAS at the Pushchino Biological Research Center), using the broth microdilution method (2-fold dilution) as described in CLSI documents M27-A and M38-A (15-16). The medium RPMI 1640 with L-glutamine and phenol red, without sodium bicarbonate, supplemented with 0,2% glucose (ICN Biomedicals Inc., Ohio, USA), buffered with 0.165 M morpholinepropanesulfonic acid (MOPS; ACROS ORGANICS, New Jersey, USA), pH
7.0, was used in study. There were two series (batches) of liquid medium RPMI 1640 used in
study. The compounds tested were initially solubilized in dimethyl sulfoxide (DMSO) at a
starting concentration of 1600 μg/ml. Series of dilutions (1600 to 3.13 μg/ml) were prepared
from the stock solutions in the same solvent and then diluted 50-fold in the test medium and
twice when inoculated, that reduced the final solvent concentration to 1%.

MICs are measured as the lowest concentrations of agents that prevent any visible growth.
Experiments were made three times for every agent and repeated twice in four-week interval.
The results of the experiments were definitely reproducible. In cases of full coincidence of the
data obtained the MIC is represented as a single number. In other cases MICs obtained are
represented in the table as the ranges of numbers. The results of MICs obtained in vitro with two
series of broth media RPMI 1640 are presented in the Tables 2 – 5.

**In vivo Antifungal Activity**

**Animals.** Male mice of hybrids of first generation (C57Bl/6xDBA/2)F1 B6D2F1, weight
20-22g., received from the Central farm "Kryukovo" of Russian Academy of Medical Science
(RAMS) were used. Animals were maintained in the vivarium in plastic cages (with hardwood
bedding in environmentally controlled conditions: 24 ± 1°C, 12/12 hours light/dark cycle) on a
standard diet of bricketed forages with easy access to drinking water (ad libitum). After 2-week
quarantine period, healthy animals were used in experimental work. The animal experiments
were performed in compliance with the EU and Russian Guidelines for Animal Experiments and
Welfare authorized by the Russian Ministry of Health (1045-73 and 52-F3).

**Antifungal agents.** Solutions of the studied compounds were prepared “ex temporae” and
were kept in dark glass vials to avoid ingress of light. The solutions were prepared as following:
dry antibiotic substances (5 mg) were mixed with dry sodium deoxycholate (4.1 mg) in a sterile
glass vial. Phosphate buffer (10 ml) (NaH₂PO₄ – 1.59 g; Na₂HPO₄ – 0.96 g; H₂O – to 100 ml)
was added to the mixture and the suspension was immediately subjected to vigorous shaking for 10 minutes until homogeneous solutions were formed. The obtained solutions (2 ml) were placed into the new sterile glass vials, 6 ml of 5% neutral sterile glucose solution was added, and the resulting solutions (0.125 mg/ml) were used for intravenous administration. Preparation of the sample of AMB was also carried out in the same way.

Maximum tolerated dose (MTD) of compounds 3n and 5 and L-glutamate of 2j, as well as AMB (1) were determined. The antibiotic preparations were singly injected into the mice’s tail vein within 1-1.5 hours after the preparation of solutions. The speed of injection did not exceed 0.5 ml per minute. Each antibiotic was used in a range of doses resulting in 0% to 100% lethality and a minimum of 3 intermediate doses. Animals were randomized into groups, each containing 6 mice. Toxicity-characterizing doses MTD were calculated with the method of “probit analysis” according to Litchfield – Wilcoxon by statistical analysis program “StatPlus-3.5.0. – 2005”. The results are shown in Table 6.

Study of specific activity of polyene antibiotics on the model of the candidosis sepsis in leucopenic mice.

Induction of the temporary leucopenia. Cyclophosphamide (“Biotex”, Saransk) was administrated at a dose 100 mg/kg/d, 3 day before (d.−3) and 1 day after (d.1) infection (d.0).

Animals were randomized into experimental and control groups, each containing 6 mice. Animals were infected intravenously with culture C. albicans (strain 14053 ATCC) (inoculum $3 \times 10^5$ CFU/mouse in volume of 0.1 ml). It is necessary to note, that doze C. albicans remained a constant in all experiments. The first intravenous introduction of tested preparations in corresponding doses in volume of 0.2 ml (with a speed of 0.2 ml/30 second) was carried out 30 minutes after infection.

Experiment has been planned in such a manner that in each experiment one dose (for AMB and for tested preparations) was used; each dose was entered daily within four days, since day of
infection (0, 1, 2 and 3 days). There was a group untreated at each experiment, a group of 
animals infected with *C. albicans* and a group of mice infected with *C. albicans* that received 
cyclophosphamide. Also there was a "placebo" group - intact not infected animals, which were 
intravenously (in the same volume as medical preparations) injected with 0.2 ml of diluent 
(phosphate buffer +5 % glucose (1:1)). “Placebo” have not shown any activity. *C. albicans* was 
ever found out in not infected animals.

After last injection of tested preparations, mice were weighed and sacrificed. Then, in 
sterile conditions, *C. albicans* burdens were determined by viable counting of homogenates from 
the kidneys. The kidneys, which were removed aseptically and weighed, pounded in porcelain 
mortars with sterile corundum, did dilutions of the received suspensions and sowed on Petri 
dishes with agar “Sabouraud”, incubated for 48 hours at temperature 35°C, then counted up 
developed colonies *C. albicans* and recalculated their quantity on 1 g of kidney’s tissue. The first 
dilution was $10^{-1}$. Zero result at this cultivation accepted for 5 CFU/g.

Statistical processing was carried out with the help of computer program Microsoft Office 
Excel 2003. Significant distinctions had $P \leq 0.05$ at comparison by Student t-criterion. Data are 
presented in Table 6.

RESULTS

Chemistry.

Our analysis of structure-activity relationship (SAR) for the series of genetically modified 
and semisynthetic polyene antibiotics of the AMB group is based on the comparison of 
properties of previously described derivatives of S44HP (13, 14) as well as novel derivatives of 
antibiotics S44HP (2), BSG005 (3), BSG022 (4) and BSG003 (6).

First, a series of semisynthetic derivatives of AMB, namely, N-(2-adamantyl)amide of 
AMB (1a), N-(2-hydroxyethyl)amide of AMB (1b), N-methylamide of AMB (1c), N-1-[di-(2-
hydroxyethyl)ethyl amide of AMB (1d), N-(hydroxymethyl, methoxycarbonyl)-methyl amide of AMB (amide of methyl ester of DL-serine) (1e), N-(methyl, methoxycarbonyl)-methyl amide of AMB (amide of methyl ester of L-alanine) (1f), N-(ethoxycarbonyl)methyl amide of AMB (amide of ethyl ester of glycine) (1g), N-(N’-2-hydroxyethyl)-piperazide of AMB (1h) and N-[( β-D-(galactosyl-1→4)-O-1-desoxy-D-fructos-1-yl] AMB (1i) were synthesized by the methods which have been described earlier for the synthesis of the S44HP derivatives: N-(2-adamantyl)amide of S44HP (2a), N-(2-hydroxyethyl)amide of S44HP (2b), N-methylamide of S44HP (2c), N-1-[di-(2-hydroxyethyl)ethyl amide of S44HP (2d), N-(hydroxymethyl, methoxycarbonyl)-methyl amide of S44HP (amide of methyl ester of DL-serine) (2e), N-(methyl, methoxycarbonyl)-methyl amide of S44HP (amide of methyl ester of L-alanine) (2f), N-(ethoxycarbonyl)methyl amide of S44HP (amide of ethyl ester of glycine) (2g), N-(N’-2-hydroxyethyl)-piperazide of S44HP (2h) and N-[( β-D-(galactosyl-1→4)-O-1-desoxy-D-fructos-1-yl] S44HP (2i), respectively (Table 2) (13). As it has been demonstrated previously using NMR method the interaction of antibiotic S44HP with an aldohexose carbohydrate led to an Amadori rearrangement product, i.e., a N-carbohydrate containing polyene antibiotic which exists in three equilibrium forms: linear keto form, cyclic (α+β) pyranose form and cyclic (α+β) furanose form (13). For simplification, only one, namely, cyclic (α+β) pyranose form of Amadori rearrangement products 1i or 2i is presented in Table 2.

Similarly, the 2-(N,N-dimethylamino)ethyl amides of BSG022 (DMAE-amide-BSG022) (4j) and BSG003 (DMAE-amide-BSG003) (6j), and a series of semisynthetic derivatives of BSG005, namely, N-fructosyl-BSG005 (3k), N-(4-N,N-dimethylanobenzyl)-BSG005 (3l), N,N-di-(3-aminopropyl)-BSG005 (3m) and N-(L-lysyl)-BSG005 (3n) were obtained by the methods described earlier (13). For the comparison of antifungal activities, corresponding derivatives of S44HP, namely, 2-(N,N-dimethylamino)ethyl amide of S44HP (2j) (Table 4), N-fructosyl-S44HP (2k), N-(4-N,N-dimethylanobenzyl)-S44HP (2l), N,N-di-(3-aminopropyl)-
S44HP (2m) and N-(L-lysyl)-S44HP (2n) (Figure 2) were obtained as it has been previously described (13). As it has been mentioned above, only one, namely, cyclic (α+β) pyranose form of Amadori rearrangement products 2k and 3k is presented on the Figure 2.

The properties of the novel compounds 1a-1i, 3k-3n, 4j and 6j are presented in Table 1.

Table 1.

Biological Evaluation.

Antifungal activities of biosynthetically engineered nystatin analogues, their novel semisynthetic derivatives, as well as semisynthetic derivatives of AmB were tested in comparison with AmB against two strains of yeast: Candida albicans, Cryptococcus humicolus and two strains of filamentous fungi (moulds): Aspergillus niger and Fusarium oxysporum. The results of MICs in vitro are represented in Tables 2 – 5. Compounds 1, 2, 2j, 3, 5, 6, and 6j were tested against additional number of yeasts (see Supplemental Material).

First, we analyzed the series of corresponding derivatives of antibiotics AMB (1a-1i) and S44HP (2a-2l), which allowed monitoring of the influence of the C7-C10 region structure on the antifungal activity (Table 2).

Table 2.

AMB (1) bears two OH groups in the positions C8 and C9 whereas S44HP (2) has two OH groups in the positions C7 and C10 (Fig. 1). As shown in Table 2, corresponding derivatives of AMB and S44HP have very similar in vitro antifungal activities. It suggests that the differences in the structures of the polyol regions of 1 and 2 have no drastic effect on the in vitro antifungal activity of these compounds. However, these modifications may influence pharmacological properties of these antibiotics. Earlier in vivo studies clearly demonstrated superior pharmacological properties of the biosynthetically engineered compound S44HP (2) over AMB (1) (11).
Next, we compared the influence of the structure of the polyol region on the antifungal activity of another group of antibiotics which have differences in the C9 – C10 region. Compound BSG005 (3), possessing OH group in position C10 had slightly higher activity than compound BSG019 (5), lacking OH group in that position C10 (Table 3). The shifting of the OH-group from position C10 (as in compound 3) to position C9 (BSG018) (7) decreased the antifungal activity significantly and resulted in almost complete loss of activity against C. *humicolus* and *F. oxysporum* (MICs ≥ 16 μg/ml). The order of decreasing activity could be presented as: BSG005 (3) > BSG019 (5) > BSG018 (7) (Table 3), that is consistent with the earlier data for *C. albicans*. (10).

**Table 3.**

Similar trend in antifungal activity change was found for the series of the DMAE-amides of antibiotics 2j, 4j and 6j (Table 4). Compound 2j was as active as AMB (1) against all yeast and molds tested. Compound 4j, lacking OH group in C10 position, being compared to AMB (1) and compound 2j, showed slightly lower antifungal activity against all 4 strains. Compound 6j, in which the OH-group was shifted from the position C10 (as in compound 3) to the position C9, (as in compound 7) demonstrated low activity against all test strains, especially *C. humicolus* (MIC> 16 μg/ml) and *F. oxysporum* (MIC 8 μg/ml). Thus, the change of positions of the hydroxyl groups in the C9-C10 region of S44HP led to dramatic changes in antifungal activity. Activity decreased in the following order: DMAE amide-S44HP (2j) > DMAE amide-BSG022 (4j) > DMAE amide-BSG003 (6j) (Table 4).

**Table 4.**

Essentially, the trend of the change of the activities of the derivatives 2j, 4j, 6j confirmed the results that have been obtained earlier against *C. albicans*, which can be presented in the following order of decreasing activity: S44HP (2) (C7-OH and C10-OH) > BSG022 (4) (7-OH) > BSG003 (6) (7-OH and 9-OH) (10). Thus, the same trend of change in the antifungal activity...
depending on the position of hydroxyls in the C9-C10 region was found in series of antibiotics bearing carboxylic (2, 4, 6) or methyl groups (3, 5, 7) in the position C16, as well as in the series of DMAE-amides 2j, 4j and 6j.

Similar results have been obtained previously for antibiotic of the AMB series: the elimination of one OH group in the C7-C10 region of AMB by biosynthetic engineering yielded 8-deoxyamphotericin B, which demonstrated slightly decreased activity against *Saccharomyces cerevisiae* (17).

The next part of our investigation was aimed at the determination of the influence of the substituents in the positions C16 and 3’-amino on the antifungal activity of polyenes. Antifungal activities of the new 3’-N-substituted derivatives of BSG005 (3k – 3n) in comparison with the corresponding derivatives of S44HP (2k – 2n) were evaluated (Fig.2, Table 5). These series of genetically engineered polyene macrolides have the same configuration in the C7-C10 region; compound S44HP (2) bears carboxylic group in the C16 position while BSG005 (3) has methyl group in this position (Fig. 2).

**Figure 2.**

Earlier, it has been shown that the replacement of the C16 carboxyl group in S44HP (2) with methyl group (BSG005, 3) does not decrease antifungal activity *in vitro* (12). Similarly, it has been demonstrated that the replacement of COOH in AMB for CH₃ group did not affect the antifungal activity of the resulting 16-decarboxy-16-methyl analogue of AMB (18). However, chemical modifications of the amino group of BSG005 (3k – 3n), and the comparison of 3k – 3n with the corresponding derivatives of S44HP (2k – 2n) showed different trends in changes of the antifungal activity (Table 5).

**Table 5.**

The N-fructosyl derivatives 2k and 3k had equal activity against two investigated filamentous fungi – *A. niger* and *F. oxysporum* (MIC = 2 and 8 μg/ml, respectively). The activity
of 2k against strains of yeasts *C. albicans* and *C. humicolus* was two folds higher than the activity of 3k (MIC = 1 and 2 μg/ml, respectively). Thus, the influence of the C16- COOH or -CH₃ in the case of semisynthetic derivatives bearing hydrophilic neutral sugar substituent on the amino group (2k or 3k) on the antifungal activity was not strongly pronounced.

In contrast to this, three other modifications of the mycosamine amino group of the polyene macrolides 2 and 3 yielded different results. The antifungal activities of the N-substituted derivatives 2l – 2n and 3l – 3n bearing amino group in the substituent showed marked difference. 4-N,N-Dimethylaminobenzyl derivative 3l was inactive against all four strains (MICs ≥ 16 μg/ml). The derivative 2l bearing the same moiety in the mycosamine amino group was inactive against *F. oxysporum* (MIC > 16 μg/ml), but had better activity (MIC = 2 – 4 μg/ml) against *C. albicans*, *C. humicolus* and *A. niger*. It suggests that the antifungal activity of polyene macrolides can be differentiated against certain fungi via specific combinations of modifications at C16 and the amino group of mycosamine.

The similar trend in the antifungal activity change was shown for the pair of the derivatives 2m and 3m. The compound N,N-di-(3-aminopropyl)-BSG005 (3m) was 4-8 fold less active than one of the most active derivatives N,N-di-(3-aminopropyl)-S44HP (2m) against all four strains tested. Contrary to this, the activity of N-(L-lysyl)-BSG005 (3n) was much higher (up to eight times) than the activity of N-(L-lysyl)-S44HP (2n) against all four strains. It should be noted that the similar semisynthetic analog of AMB - N,N-di-(3-aminopropyl)-AMB also showed higher antifungal activity compared to the parental antibiotic AMB (19).

It can thus be concluded that the activity of the discussed derivatives modified at the amino group depends on the structure of the starting compound, i.e. on the presence of C16-CH₃ or COOH group, and on the structure of the modifying substituent at the amino group of mycosamine.
Some of the studied compounds which showed high antifungal activities in *in vitro* experiments, namely, 3n, 5 and water-soluble form of compound 2j (L-glutamate salt of 2j) were chosen for *in vivo* investigations.

Maximum tolerated doses (MTD) were determined as it is described in were calculated with the method of “probit analysis” according to Litchfield – Wilcoxon by statistical analysis program “StatPlus-3.5.0. – 2005” (20). To investigate the *in vivo* efficacy of the tested compounds, a model of candidosis sepsis in leucopenic mice (cyclophosphamide-induced) was used (14, 21).

The highest *in vitro* activity of S44HP derivative 2j (L-glutamate salt) was confirmed by the study *in vivo* in the conditions of model candidosis on a leukopenic background. L-Glutamate of 2j was almost 10 times less toxic compared to AMB, and the effective dose (ED) was equal to 2.7 % of the MTD, whereas AMB had ED equal to 62 % of MTD (Table 6). L-Glutamate of 2j retained high activity in doses from 0.4 up to 4.0 mg/kg/day, despite of the leucopenic conditions induced by cyclophosphamide. Judging from the ED/MTD ratio, the compound 3n also had considerably better therapeutic index compared to AmB (Table 6). Compound (5) had the lowest toxicity (MTD 42.4 mg/kg/daily) and the lowest efficacy (ED 16.0 mg/kg/day) among the tested compounds, though ED/MTD ratio for compound 5 was better than for AMB (1) (Table 6).

Thus, activity of BSG019 (5) in the *in vitro* tests did not correlate well with the data obtained in the *in vivo* experiments. Most likely, the suboptimal pharmacokinetic properties of this compound can explain low activity in animal model.

**Table 6.**

**DISCUSSION**

Natural (Amphotericin B) (1) and bioengineered (2 – 7) polyene macrolide antibiotics and series of their semisynthetic derivatives represent a unique basis for the study of their structure-
antifungal activity relationship and understanding the roles of positions of hydroxyls in the C7-C10 region, amino group in mycosamine, and 16-C substituent (COOH or CH₃) in the antifungal properties of polyene antibiotics. The analysis of SARs for new polyenes may provide important information for the design of novel highly active and less toxic antifungal polyenes.

The mechanism of action of polyene macrolides is believed to be associated with their ability to interact with sterol-containing membranes and form pores (channels) through which ions are leaking out of cells, resulting in their death (6, 22). Detailed mechanisms of these interactions, however, remain to be investigated. Recently, a critical role of mycosamine and the 35-OH group of AMB molecule for its antifungal activity was shown, and it was stated that “Amphotericin primarily kills yeast by simply binding ergosterol” (23). However only antibiotics 1-3 and most of their semisynthetic derivatives have antifungal properties whereas antibiotics 4-7 that also have the C35-OH group and mycosamine moiety were low active. It demonstrates critical role of hydroxyl groups positions in the region C7-C10 for biological activity (functional groups on the macrolide cycle can influence conformation of the macrolide cycle, formation of hydrogen bonds network, intermolecular interactions, and ion channel formation).

The hypothesis that sterol binding is paramount to the antifungal activity of AMB has been supported by the testing a derivative of AMB without mycosamine moiety, that possessed the ability to bind ergosterol but lacked the capacity to form ion channels. It was concluded that a mycosamine-mediated direct binding interaction between AMB and ergosterol is required for both forming ion channels and killing yeast cells (24).

It is interesting to note that the derivatives of our antibiotics 1-3 (with beneficial positions of the hydroxyl groups in the region C7-C10) with various substituents at the amino group of mycosamine, including rather bulky hydrophobic or hydrophilic groups (see Tables 1 and 4) retained high antifungal activity. Though all of the studied derivatives have substituents which...
can be protonated (14, 19), the bulky substituents at the amino group might be barriers for the supposed interaction with ergosterol. It suggests that the amino group of mycosamine does not play a critical role in the interaction with ergosterol.

Studies of the antibiotics 2 and 3 showed that the replacement of the C16 carboxyl group for C16 methyl group does not decrease antifungal activity of polyene antibiotics in the *in vitro* tests if the 3’N-amino group of the antibiotic is unmodified. Our results are in agreement with previously found data for AMB and its analog - 16-decarboxy-16-methyl-AMB (18). However, the nature of the C16 substituent (COOH or CH₃, antibiotics 2 or 3) influences greatly the antifungal activity of the N-modified semisynthetic derivatives of the polyene macrolides.

Study of our library of semisynthetic polyene antibiotics led to the discovery of compounds, namely, N-(L-lysyl)-BSG005 (3n) and, especially, L-glutamate salt of 2-(N,N-dimethylamino)ethyl amide of S44HP (2j) with high antifungal activity that are comparable in the *in vitro* and *in vivo* tests to AMB, and have better toxicological properties.

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**Supplemental material**

# Supplemental material for this article may be found at [http://aac.asm.org/](http://aac.asm.org/).

**Author Contributions**

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Figures and tables.

Figure 1. The structures of polyene macrolide antibiotics amphotericin B (AMB) (1) and bioengineered nystatin analogues (2–7).

(1) Amphotericin B (AMB)

(2) R=COOH (S44HP)
(3) R=CH₃ (BSG005)
(4) R=COOH (BSG002)
(5) R=CH₃ (BSG019)
(6) R=COOH (BSG003)
(7) R=CH₃ (BSG018)

Figure 2. Structures of S44HP (2), BSG005 (3) and their semisynthetic derivatives.

X=NH₂     Y=COOH   (2) (S44HP)
X=HO     Y=COOH (2k) (BSG005)
X=HO     Y=CH₃ (3k)
X=HO     Y=COOH (2i) (BSG005)
X=HO     Y=CH₃ (3i)
X=HO     Y=COOH (2n) (BSG005)
X=HO     Y=CH₃ (3n)

Figure 2. Structures of S44HP (2), BSG005 (3) and their semisynthetic derivatives.
Table 1. The properties of the novel compounds 1a -1i, 3k-3n, 4j and 6j.

<table>
<thead>
<tr>
<th>Compound</th>
<th>TLC, Rf (system)</th>
<th>HPLC, Rt, min (system)</th>
<th>Brutto-formula</th>
<th>MW Calculated</th>
<th>MW Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>0.59 (I)</td>
<td>20.42 (A)</td>
<td>C_{57}H_{88}N_{2}O_{16}</td>
<td>1056.6134</td>
<td>1079.608e</td>
</tr>
<tr>
<td>1b</td>
<td>0.32 (II)</td>
<td>10.54 (A)</td>
<td>C_{49}H_{78}N_{2}O_{17}</td>
<td>966.5300</td>
<td>967.538</td>
</tr>
<tr>
<td>1c</td>
<td>0.31 (I)</td>
<td>11.14 (A)</td>
<td>C_{48}H_{76}N_{2}O_{16}</td>
<td>936.5195</td>
<td>959.600b</td>
</tr>
<tr>
<td>1d</td>
<td>0.57 (I)</td>
<td>19.88 (A)</td>
<td>C_{51}H_{82}N_{2}O_{18}</td>
<td>1010.5563</td>
<td>1011.568</td>
</tr>
<tr>
<td>1e</td>
<td>0.59 (I)</td>
<td>12.19 (A), C_{51}H_{80}N_{2}O_{19}</td>
<td>1024.5355</td>
<td>1047.560b</td>
<td></td>
</tr>
<tr>
<td>1f</td>
<td>0.55 (I)</td>
<td>12.99 (C)</td>
<td>C_{51}H_{80}N_{2}O_{18}</td>
<td>1008.5406</td>
<td>1031.532b</td>
</tr>
<tr>
<td>1g</td>
<td>0.47 (I)</td>
<td>13.66 (C)</td>
<td>C_{51}H_{80}N_{2}O_{18}</td>
<td>1008.5406</td>
<td>1031.550b</td>
</tr>
<tr>
<td>1h</td>
<td>0.55 (II)</td>
<td>6.06 (C)</td>
<td>C_{53}H_{83}N_{3}O_{17}</td>
<td>1035.5879</td>
<td>1058.656b</td>
</tr>
<tr>
<td>1i</td>
<td>0.15 (I)</td>
<td>11.65 (A)</td>
<td>C_{59}H_{93}NO_{27}</td>
<td>1247.5935</td>
<td>1248.604</td>
</tr>
<tr>
<td>3k</td>
<td>0.65 (III)</td>
<td>10.20 (A)</td>
<td>C_{53}H_{83}NO_{20}</td>
<td>1055.5665</td>
<td>1056.574</td>
</tr>
<tr>
<td>3l</td>
<td>0.85 (II)</td>
<td>25.39 (B)</td>
<td>C_{56}H_{86}N_{2}O_{15}</td>
<td>1026.6028</td>
<td>1049.601b</td>
</tr>
<tr>
<td>3m</td>
<td>0.02 (II)</td>
<td>10.07 (A)</td>
<td>C_{53}H_{89}N_{3}O_{15}</td>
<td>1007.6294</td>
<td>1008.542</td>
</tr>
<tr>
<td>3n</td>
<td>0.03 (I)</td>
<td>13.05 (A)</td>
<td>C_{53}H_{87}N_{3}O_{16}</td>
<td>1021.6086</td>
<td>1022.614</td>
</tr>
<tr>
<td>4j</td>
<td>0.32 (II)</td>
<td>13.57 (B)</td>
<td>C_{51}H_{83}N_{3}O_{15}</td>
<td>977.5824</td>
<td>978.600</td>
</tr>
<tr>
<td>6j</td>
<td>0.31 (II)</td>
<td>19.73 (B)</td>
<td>C_{51}H_{83}N_{3}O_{16}</td>
<td>993.5773</td>
<td>994.595</td>
</tr>
</tbody>
</table>


b* [M+Na]⁺.
Table 2. Antifungal activity (MIC, μg/ml) of the concurrent derivatives of AMB (1a – 1i) and S44HP (2a – 2i) compared with the parent antibiotics AMB (1) and S44HP (2) against *C. albicans* ATCC 14053 (A); *C. humicolus* ATCC 9949 (B); *A. niger* ATCC 16404 (C); *F. oxysporum* VKM F-140 (D).

<table>
<thead>
<tr>
<th>C7-C10 Polyol part</th>
<th>Am B derivatives</th>
<th>S44HP derivatives</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R¹     R²</td>
<td>Comp. A B C D</td>
</tr>
<tr>
<td>OH</td>
<td>H</td>
<td>1 1 1 4</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>1a</td>
</tr>
<tr>
<td>-NH-CH₃</td>
<td>H</td>
<td>1b</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>1c</td>
</tr>
<tr>
<td>-NH-CH₃</td>
<td>H</td>
<td>1d</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>1e</td>
</tr>
</tbody>
</table>
Table 3. Antifungal activity of AMB (1), BSG005 (3), BSG019 (5) and BSG018 (7).*

<table>
<thead>
<tr>
<th>MIC μg/ml</th>
<th>Compound/C7-C10 Polyol part</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMB (1)</td>
</tr>
<tr>
<td>C. albicans ATCC 14053</td>
<td>0.5</td>
</tr>
<tr>
<td>C. humicolus ATCC 9949</td>
<td>0.5</td>
</tr>
<tr>
<td>A. niger ATCC 16404</td>
<td>0.5</td>
</tr>
<tr>
<td>F. oxysporum VKM F-140</td>
<td>2</td>
</tr>
</tbody>
</table>

* Data represented in Tables 3 and 4 were obtained with a batch of medium RPMI 1640 different from the one in Tables 2 and 5.
Table 4. Antifungal activity of AMB (1) and DMAE-amides of S44HP (2j), BSG003 (4j) and BSG022 (6j).

<table>
<thead>
<tr>
<th>MIC μg/ml</th>
<th>Compound/C7-C10 Polyol part</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMB (1)</td>
</tr>
<tr>
<td>C. albicans ATCC 14053</td>
<td>0.5</td>
</tr>
<tr>
<td>C. humicola ATCC 9949</td>
<td>0.5</td>
</tr>
<tr>
<td>A. niger ATCC 16404</td>
<td>0.5</td>
</tr>
<tr>
<td>F. oxysporum VKM F-140</td>
<td>2</td>
</tr>
</tbody>
</table>
Table 5. Antifungal activities of BSG005 derivatives (3k – 3n) compared with the corresponding S44HP derivatives (2k – 2n), AMB (1), S44HP (2) and BSG005 (3) against C. albicans ATCC 14053 (A); C. humicolus ATCC 9949 (B); A. niger ATCC 16404 (C); F. oxysporum VKM F-140 (D).

<table>
<thead>
<tr>
<th>MIC μg/ml</th>
<th>S44HP derivatives</th>
<th>BSG-005 derivatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Comp. A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2k</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2l</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>2m</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2n</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 6. MTD and ED data for compounds 3n, 5, and L-glutamate of 2j in comparison with AMB (1).

<table>
<thead>
<tr>
<th>Compound</th>
<th>MTD (mg/kg/daily)</th>
<th>ED (mg/kg/daily)</th>
<th>ED/MTD, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.01 (1.88 + 2.23)</td>
<td>1.25</td>
<td>62</td>
</tr>
<tr>
<td>L glutamate of 2j</td>
<td>14.7 (12.8 + 16.8)</td>
<td>0.4</td>
<td>2.7</td>
</tr>
<tr>
<td>3n</td>
<td>4.48 (4.23 + 4.75)</td>
<td>1.25</td>
<td>28</td>
</tr>
<tr>
<td>5</td>
<td>42.8 (41.2 + 44.5)</td>
<td>16.0</td>
<td>37.3</td>
</tr>
</tbody>
</table>

MTD – maximal tolerated dose; ED – effective dose that means elimination of 99% of infectious agent from kidneys.