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RESEARCH ARTICLE

The Substantial Improvement of Amphotericin B Selective Toxicity Upon Modification of Mycosamine with Bulky Substituents

Edward Borowski[1], Natalia Salewska[1,2], Joanna Boros-Majewska[1,3], Marcin Serocki[3], Izabela Chabowska[1], Maria J. Milewska[2], Dominik Ziętkowski[1] and Sławomir Milewski[3,*]

[1]*BLIRT S.A., Gdańsk, Poland, Trzy Lipy 3, 80-172 Gdańsk, Poland;* [2]*Department of Organic Chemistry;* [3]*Department of Pharmaceutical Technology and Biochemistry, Faculty of Chemistry, Gdańsk University of Technology, 11/12 Narutowicza St., 80-233 Gdańsk, Poland*

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Abstract: ***Background*:** It is assumed that the unfavorable selective toxicity of an antifungal drug Amphotericin B (AmB) can be improved upon chemical modification of the antibiotic molecule.

***Objective*:** The aim of this study was verification of the hypothesis that introduction of bulky substituents at the amino sugar moiety of the antibiotic may result in diminishment of mammalian *in vitro* toxicity of thus prepared AmB derivatives.

***Methods*:** Twenty-eight derivatives of AmB were obtained upon chemical modification of the amino group of mycosamine residue. This set comprised 10 *N*-succinimidyl-, 4 *N*-benzyl-, 5 *N*-thioureidyl- and 9 *N*-aminoacyl derivatives. Parameters characterizing biological *in vitro* activity of novel compounds were determined.

***Results*:** All the novel compounds demonstrated lower than AmB antifungal *in vitro* activity but most of them exhibited negligible cytotoxicity against human erythrocytes and three mammalian cell lines. In consequence, the selective toxicity of majority of novel antifungals, reflected by the selective toxicity index (STI = EH_{50}/IC_{50}) was improved in comparison with that of AmB, especially in the case of 5 compounds. The novel AmB derivatives with the highest STI, induced substantial potassium efflux from *Candida albicans* cells at concentrations slightly lower than IC_{50}s but did not trigger potassium release from human erythrocytes at concentrations lower than 100 μg/mL.

***Conclusion*:** Some of the novel AmB derivatives can be considered promising antifungal drug candidates.

Keywords: Antifungal agent, amphotericin B, chemical modification, selective toxicity, hemotoxicity, potassium efflux.

1. INTRODUCTION

Polyene macrolide antibiotics constitute the group of the most potent broad-spectrum antifungals. A member of this group, heptaenic macrolide Amphotericin B (AmB), is the drug of choice for the treatment of disseminated fungal infections, especially in immunocompromised patients [1]. This antibiotic, consisting of a large polyene macrolide ring and an amino sugar mycosamine, combines most of the features expected for an "ideal" antifungal chemotherapeutic, including the high antifungal efficacy at low drug concentration, broad antifungal spectrum covering the multidrug-resistant fungal species, a fungicidal mode of action and a very limited potential of inducing fungal specific resistance [2]. The important disadvantage of AmB is its substantial mammalian toxicity, being a consequence of mechanism of antibiotic action. According to the two hypotheses on this mechanism, the "barrel-stave-pore" [3] and a "sterol sponge" [4], binding of AmB to sterol present in the fungal or mammalian cell membrane is the necessary condition for its biological effect. In the "barrel-stave-pore" mechanism, AmB:sterol complexes assemble into transmembrane barrel-like pores facilitating efflux of monovalent ions, especially K^+. This efflux, causing impairment of membrane barrier function, is considered a primary effect leading to cell death. A slightly higher affinity of AmB to fungal ergosterol than to mammalian cholesterol constitutes a molecular basis for the selective toxicity of this antibiotic [5]. In consequence, the therapeutic window of AmB is very narrow. This disadvantage has been only in part overcome upon construction of AmB complexes with lipids or liposomal formulations, such as Abelcet®, Amphotec® or AmBisome®, at the price of a substantial rise in treatment cost [6].

*Address correspondence to this author at the Department of Pharmaceutical Technology and Biochemistry, Faculty of Chemistry, Gdańsk University of Technology, 80-233 Gdańsk, Poland; Tel/Fax: +4858 3472107/+4858 3471144; E-mail: slamilew@pg.edu.pl

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Many examples of structural modifications of AmB molecule aimed at the improvement of its selective toxicity have been reported. Several AmB analogues modified in the polyol region of the aglycone were obtained upon biosynthetic engineering of the Nystatin-producing *Streptomyces noursei*, resulting in a series of AmB-mimicking 28,29-didehydronystatin derivatives, of which antibiotics S44HP and BSG005 demonstrated advantages over AmB in the *in vivo* tests [7, 8]. However, most modifications of AmB were made by chemical reactions and concerned the carboxyl group or the amino functionality of mycosamine. Some of the AmB derivatives thus obtained demonstrated improved selective toxicity, for example the AmB-benzoxaborole hybrids [9]. A substantial improvement was observed for *N*-D-ornithyl-AMB [10], *N*-methyl-*N*-D-fructopyranosyl-amphotericin B methyl ester [11] or *N*-piperidinepropionyl amphotericin B methyl ester [12]. *N, N*-di- (3-aminopropyl) AmB exhibited much higher than AmB antifungal activity and a slightly lower mammalian toxicity [13]. Substantial reduction of mammalian toxicity *in vitro* with retention or minimal decrease in antifungal potency has been achieved for 2'-deoxyAmB [14] but the conversion of AmB into its 2'-deoxy derivative is a multistep, costly procedure. The similar effect was shown for the derivatives modified at the carboxyl functionality, namely AmB urea derivatives [15] and conjugates of AmB with "molecular umbrellas" [16].

In our approach, we hypothesized that a substantial improvement of AmB selective toxicity could be achieved upon introduction of bulky substituents at the amino group of mycosamine, with retention of a positive charge at this site. Such an assumption was based on results of our previous studies, indicating that AmB derivatives with bulky substituents at the amino group, should form complexes with ergosterol or cholesterol of different geometries which could result in differential abilities to assemble into lethal transmembrane channels in ergosterol- or cholesterol-containing membranes [17]. On the other hand, the positive charge at mycosamine or in its close vicinity is the well -documented prerequisite for preservation of good antifungal *in vitro* activity [18]. In this work, we report on synthesis and biological properties of 28 AmB derivatives containing easily introducible bulky substituents at the amino group of mycosamine, fulfilling the above mentioned structural requirements.

2. MATERIALS AND METHODS

2.1. Synthesis of AmB Derivatives

Purification of final reaction products was carried out by preparative column liquid chromatography. Amphotericin B was from Sigma-Aldrich Co. (St. Louis, MO). Fmoc amino acids, amino acids and aromatic aldehydes were of commercial origin. Commercial grade reagents and solvents were used without further purification. The mobile phase was acetonitrile/water (20-70% acetonitrile gradient) containing 0.5% HCOOH. HRMS-ESI spectra were recorded on Aqilent Technologies 6540 UHD Accurate – Mass Q-TOF LC/MS apparatus. UV-VIS spectra were recorded on Lambda 45 Perkin Elmer spectrophotometer. HPLC analysis was performed on Agilent 1200 Series apparatus, using the Zorbax Eclipse XDB C_{18} column (4.6 × 150 mm). TLC solvent systems: A $CHCl_3$: MeOH: H2O (20:8:1, v/v); B $CHCl_3$: MeOH: H2O (25:8:1, v/v); C $CHCl_3$:MeOH:H_2O (30:8:1, v/v); D $CHCl_3$:MeOH:H_2O (10:6:1, v/v).

N-substituted maleimides were synthesized as described previously [19]. Isothiocyanates were prepared following the methodology described in previous works [20, 21]. *N, N*-dialkyl amino acids were prepared upon reductive alkylation of amino acid methyl esters with an appropriate aldehyde (1:4 molar ratio), followed by ester hydrolysis [22].

The general methods of synthesis of *N*-succinimidyl-, *N*-aminosuccinimidyl-, *N*-benzyl-, *N*-thioureidyl- and *N*-aminoacyl derivatives of AmB were described previously [23].

2.1.1. N-succinimidyl Derivatives of Amphotericin B

2.1.1.1. N-(N-benzylsuccinimidyl)-Amphotericin B ANS1

Yield 88%. TLC (C) R_f = 0.27; HPLC R_t = 15.480 min., purity 97.2%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; $E_{1cm}^{1\%}$ (MeOH, λ = 406 nm) = 1180; MS-ESI found m/z: 1109.3352 $[M-H]^-$; calculated for $C_{58}H_{82}N_2O_{19}$ 1110.1117.

2.1.1.2. N-(N-2,6-dimethylphenylsuccinimidyl)-Amphotericin B ANS2

Yield 92.2%. TLC (C) R_f = 0.30; HPLC R_t = 16.432 min., purity 95.3%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1147.4351 $[M+Na]^+$; calculated for $C_{59}H_{84}N_2O_{19}$ $[M-H]^-$ 1124.6663.

2.1.1.3. N-(N-2,4,6-trimethylphenylsuccinimidyl)-Amphotericin B ANS3

Yield 89.9%. TLC (C) R_f = 0.32; HPLC R_t = 16.576 min., purity 98.2%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1137.4155 $[M-H]^-$; calculated for $C_{60}H_{86}N_2O_{19}$ 1138.1753.

2.1.1.4. N-(N-2-tert-butylphenylsuccinimidyl)-Amphotericin B ANS4

Yield 91.9%. TLC (C) R_f = 0.22; HPLC R_t = 17.223 min., purity 99.1%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1175.4531 $[M-H]^-$; calculated for $C_{55}H_{84}N_2O_{19}$ 1076.6513.

2.1.1.5. N-(N-cyclohexylsuccinimidyl)-Amphotericin B ANS5

Yield 94.6%. TLC (B) R_f = 0.15; HPLC R_t = 17.567 min., purity 95.1%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1125.1167 $[M+Na]^+$; calculated for $C_{57}H_{86}N_2O_{19}$ 1102.6112.

2.1.2. N-aminosuccinimidyl Derivatives of Amphotericin B

2.1.2.1. N-{N-[3-(N,N-dimethylamino)-2,2-dimethylpropyl]-succinimidyl}-Amphotericin B ANS6

Yield 85.5%. TLC (A) R_f = 0.23; HPLC R_t = 10.682 min., purity 96.7%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1134.6259 $[M+H]^+$; calculated for $C_{58}H_{91}N_3O_{19}$ 1133.6241.

2.1.2.2. N-{N-[2-(piperidin-1-yl)ethyl]succinimidyl}-Amphotericin B ANS7

Yield 82.6%. TLC (A) R_f = 0.23; HPLC R_t = 10.852 min., purity 92.8%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm;

MS-ESI found m/z: 1132.5898 $[M+H]^+$; calculated for $C_{58}H_{89}N_3O_{19}$ 1131.6090.

2.1.2.3. N-[N-(2-morpholin-1-yl-ethyl)succinimidyl]-Amphotericin B ANS8

Yield 78.5%.TLC (A) R_f = 0.17; HPLC R_t = 10.627 min., purity 94.6%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1134.5891 $[M+H]^+$; calculated for $C_{57}H_{87}N_3O_{20}$ 1133.5833.

2.1.2.4. N-{N-[2-(4-methylpiperazin-1-yl)ethyl]succinimidyl}-Amphotericin B ANS9

Yield 80.4%. TLC (A) R_f = 0.22; HPLC R_t = 10.077 min., purity 96.3%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1147.6147 $[M+H]^+$; calculated for $C_{58}H_{90}N_4O_{19}$ 1146.6199.

2.1.2.5. N-{N-[(2-pyridin-4-yl)-methyl]succinimidyl}-Amphotericin B ANS10

Yield 89.7%. TLC (A) R_f = 0.25; HPLC R_t = 10.599 min., purity 92.2%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1113.5337 $[M+H]^+$; calculated for $C_{57}H_{87}N_3O_{19}$ 1112.5337.

2.1.3. N-benzyl Derivatives of Amphotericin B

2.1.3.1. N-benzyl-Amphotericin B AB1

Yield 86.9%. TLC (D) R_f = 0.81; HPLC R_t = 14.771 min., purity 96.5%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1012.3333 $[M-H]^+$; calculated for $C_{54}H_{79}NO_{17}$ 1013.6501.

2.1.3.2. N-[(pyridin-2-yl)methyl]Amphotericin B AB2

Yield 91.6%. TLC (D) R_f = 0.42; HPLC R_t = 13.750 min., purity 94.5%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1013.3258 $[M-H]^-$; calculated for $C_{53}H_{78}N_2O_{17}$ 1014.5226.

2.1.3.3. N-(4-tert-butylbenzyl)Amphotericin B AB3

Yield 90.7%. TLC (D) R_f = 0.87; HPLC R_t = 18.322 min., purity 99.7%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1068.5211 $[M-H]^-$; calculated for $C_{58}H_{87}NO_{17}$ 1069.6511.

2.1.3.4. N-(4-N,N-diethylaminobenzyl)Amphotericin B AB4

Yield 89.0%. TLC (D) R_f = 0.75; HPLC R_t = 12.175 min., purity 93.4%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1083.3544 $[M-H]^-$; calculated for $C_{58}H_{88}N_2O_{17}$ 1084.6177.

2.1.4. N-Thioureidyl Derivatives of Amphotericin B

2.1.4.1. N-(3-phenyl)-Thioureidyl-Amphotericin B ATU1

Yield 85.5%. TLC (A) R_f = 0.85; HPLC R_t = 15.385 min., purity 87.2%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1057.5432 $[M-H]^-$; calculated for $C_{54}H_{78}N_2O_{17}S$ 1058.5088.

2.1.4.2. N-[3-(pyridin-3-yl)-thioureidyl]-Amphotericin B ATU2

Yield 95.8%. TLC (A) R_f = 0.83; HPLC R_t = 12.186 min., purity 92.8%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1060.5225 $[M+H]^+$; calculated for $C_{53}H_{77}N_3O_{17}S$ 1059.5184.

2.1.4.3. N-{3-[3-(N,N-dimethylamino)propyl]-thioureidyl}-Amphotericin B ATU3

Yield 87.5%. TLC (A) R_f = 0.38; HPLC R_t = 11.952 min., purity 95.0%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1068.4765 $[M+H]^+$; calculated for $C_{53}H_{85}N_3O_{17}S$ 1067.6157.

2.1.4.4. N-{3-[2-(N,N-diethylamino)ethyl]-thioureidyl}-Amphotericin B ATU4

Yield 89.7%. TLC (A) R_f = 0.32; HPLC R_t = 12.021 min., purity 97.8%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1096.5578 $[M+H]^+$; calculated for $C_{55}H_{89}N_3O_{17}S$ 1095.6009.

2.1.4.5. N-{3-[(2-piperidin-1-yl)ethyl]-thioureidyl}-Amphotericin B ATU5

Yield 92.8%. TLC (A) R_f = 0.40; HPLC R_t = 12.082 min., purity 92.2%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1094.5634 $[M+H]^+$; calculated for $C_{55}H_{87}N_3O_{17}S$ 1093.6112.

2.1.5. N-aminoacyl and N-(N'-alkylamino)acyl Derivatives of Amphotericin B

2.1.5.1. N-D-phenylglycyl-Amphotericin B AAA1

Yield 87.0%TLC (A) R_f = 0.35; HPLC R_t = 13.323 min., purity 97.2%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1081.3461 $[M+Na]^+$; calculated for $C_{55}H_{80}N_2O_{18}$ 1056.5530.

2.1.5.2. N-[β-(pyridine-1-yl)-D-alanyl]Amphotericin B AAA2

Yield 85.3%. TLC (A) R_f = 0.70; HPLC R_t = 8.193 min., purity 92.6%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1072.9115 $[M-H]^-$; calculated for $C_{55}H_{83}N_3O_{18}$ 1073.6561.

2.1.5.3. N-(O^{γ}-tert-butyl-D-glutamyl)Amphotericin B AAA3

Yield 90.3%. TLC (A) R_f = 0.26; HPLC R_t = 14.861 min., purity 90.9%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1107.3614 $[M-H]^-$; calculated for $C_{56}H_{88}N_2O_{20}$ 1108.6122.

2.1.5.4. N-(O-tert-butyl-D-seryl)Amphotericin B AAA4

Yield 93.3%. TLC (A) R_f = 0.35; HPLC R_t = 13.720 min., purity 93.2%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1065.3437 $[M-H]^-$; calculated for $C_{54}H_{86}N_2O_{19}$ 1066.6444.

2.1.5.5. N-(O^{β}-tert-butyl-D-aspartyl)Amphotericin B AAA5

Yield 88.4%. TLC (A) R_f = 0.35; HPLC R_t = 14.714 min., purity 92.5%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1093.4937 $[M-H]^-$; calculated for $C_{55}H_{86}N_2O_{20}$ 1094.6504.

2.1.5.6. N-(2-fluoro-D-phenylalanyl)Amphotericin B AAA6

Yield 87.6%. TLC (A) R_f = 0.41; HPLC R_t = 14.143 min., purity 90.2%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm;

MS-ESI found m/z: 1087.2517 $[M-H]^-$; calculated for $C_{54}H_{81}FN_2O_{18}$ 1088.5561.

2.1.5.7. N-(N-methyl-α-methylalanyl)Amphotericin B AA-A7

Yield 91.1%. TLC (A) R_f = 0.20; HPLC R_t = 9.276 min., purity 90.7%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1021.3418 $[M]^+$; calculated for $C_{52}H_{81}N_2O_{18}$ 1021.6155.

2.1.5.8. N-(N-methyl-O$^{\gamma}$-tert-butyl-L-glutamyl)Amphotericin B AAA8

Yield 89.9%. TLC (A) R_f = 0.24; HPLC R_t = 9.895 min., purity 97.2%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1121.4531 $[M-H]^-$; calculated for $C_{57}H_{90}N_2O_{20}$ 1022.6831.

2.1.5.9. N-[(3-piperidin-1-yl)propionyl]amphotericin B (AAA9)

Yield 87.9%. TLC (A) Rf = 0.27; HPLC R_t = 11.681 min., purity 94.4%. UV-vis: λ_{max} (MeOH) 406; 382; 363 nm; MS-ESI found m/z: 1061.4053 $[M-H]^-$; calculated for $C_{55}H_{86}N_2O_{18}$ 1062.6734.

2.2. Microbial Strains and Culture Conditions

The reference strains used in this study were: Candida albicans ATCC 10231, Candida albicans SC 5314, Candida glabrata DSM 11226, Candida krusei DSM 6128, Candida parapsilosis DSM 5784, Candida stellatoidea CBS 1905, Candida dubliniensis CBS 7987, Candida lusitaniae DSM 70102, Candida guiliermondi DSM 11947, Candida tropicalis DSM 11953, Cryptococcus gatti 8395, Cryptococcus neoformans 8398, Trichoderma viride LOCK E159, Aspergillus fumigatus 10507, Aspergillus niger LOCK E201, Fusarium solani, 13932, Mucor circinelloides 9898 and Rhisopus oryzae 8542. C. albicans B3, B4, Gu4 and Gu5 clinical isolates were kindly provided by Joachim Morschhäuser, Würzburg, Germany. Gu4 and B3 are fluconazole-sensitive isolates obtained from early infection episodes, while Gu5 and B4 are the corresponding fluconazole-resistant isolates obtained from later episodes in the same patients treated with fluconazole [24]. Strains were grown at 30°C in YPD medium (2% glucose, 1% Yeast Extract and 1% Bacto Peptone) and stored on YPD agar plates containing 2% agar.

2.3. Determination of Antifungal *in vitro* Activity

The *in vitro* growth inhibitory activity of antifungals was quantified by determination of MIC values by the serial two-fold dilution method, using the 96-well microtiter plates. AmB and its derivatives were dissolved in DMSO and 5 µL aliquots of serial two-fold dilutions were added to individual wells so that the final DMSO concentration was 0.5% (v/v). Conditions of the assay were the same as outlined in the CLSI recommendations [25], except for the end-point read-out that was done by spectrophotometric determination of cell density at 531 nm. Turbidity in individual wells was measured with a microplate reader (Victor^3; Perkin Elmer). On the basis of obtained results, the diagrams of the relationship between A_{531} values and concentration of examined compound were drawn. From these graphs, the IC_{50} values were read, which were the interpolated concentrations of a tested compound, at which, the A_{531} value was exactly 50% of the A_{531} value for the control sample. MIC was defined as the lowest drug concentration that gave at least 80% decrease in turbidity, relative to that of the drug-free growth control.

2.4. Determination of Mammalian *in vitro* Cytotoxicity

Hemolysis assay and cytotoxicity determination were performed exactly as described previously [26].

2.5. Determination of Potassium Efflux

A. Yeast cells. *C. albicans* ATCC 10231 cells were grown overnight in YPD liquid medium at 30°C with vigorous shaking. The cells were harvested by centrifugation for 10 minutes at 1700 × g, washed three times with sterile TBS (Tris-HCl, pH 7.4 containing 0.9% NaCl) and dissolved in TBS to the final concentration of 2.0 mg of dry weight per mL. Kinetics of potassium leakage was followed with a potassium-selective combined electrode (PerfectION™, Mettler Toledo) linked with CPI-505 pH-ionmeter (Elmetron) in 25 mL cell suspensions. After 10 minutes of signal stabilization, 0.25 mL solutions of compounds tested in DMSO (100 × concentrated) were added and the measurement was continued for 30 minutes. The maximal potassium efflux (100%) was determined for cell suspension pre-boiled for 15 min (positive control).

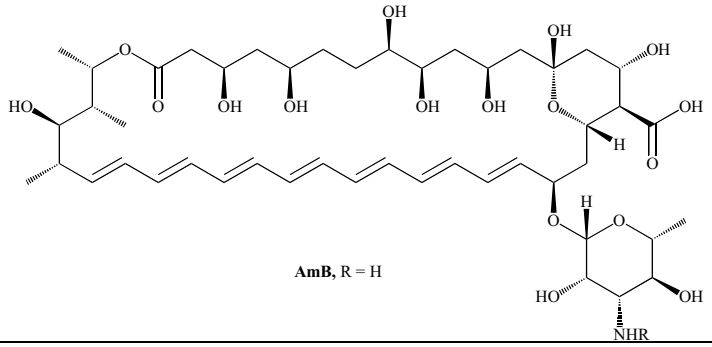
B. Erythrocytes. To the freshly prepared human erythrocytes (RBC) suspensions (50 mL, 2×10^7 cells/ mL, hemocytometer count) in saline, 100 × concentrated solutions of compounds tested in 0.5 mL of DMSO were added. Suspensions were incubated at 37°C for 30 min. Samples of 5 mL were collected at 5 min intervals and immediately filtered through the Whatman GF/A filters and filtrates were collected. Potassium concentration in filtrates was determined with the BWB-1 flame photometer. Erythrocyte suspension containing 0.1% Triton X-100 and 1% DMSO served as a positive control (100% efflux) and erythrocyte suspension containing 1% DMSO as a negative control (0% efflux).

The percent of K^+ ions released from *C. albicans* of cells or erythrocytes after 30 min in comparison with positive controls were plotted against log of compound concentration and EK_{50} values, *i.e.* compound concentration inducing 50% potassium release after 30 min, were determined from the curves obtained.

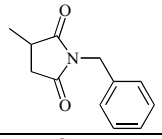
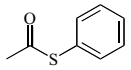
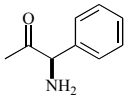
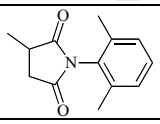
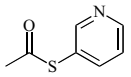
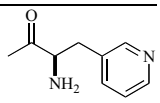
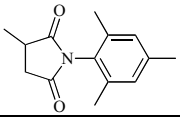
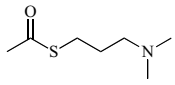
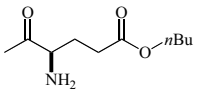
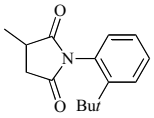
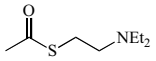
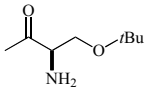
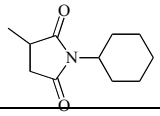
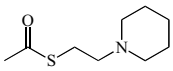
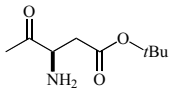
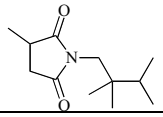
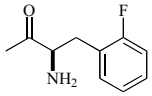
3. RESULTS

3.1. Chemistry

The synthetic strategies of AmB modification at its amino group applied in this work comprised: a/ the formation of *N*-succinimidyl analogs upon Michael-type addition of *N*-substituted maleimide derivatives; b/ the formation of *N*-benzyl analogs upon reductive amination with benzaldehyde derivatives; c/ the formation of *N*-thioureidyl analogs upon reaction with isothiocyanates; d/ the formation of *N*-aminoacyl analogs upon DCC-driven acylation with respective amino acid *N*-hydroxy-succinimides. All these reactions were performed under mild, preservative conditions, with no need for prior protection of any functionalities of the AmB



AmB, R = H

N-succinimidyl		*N*-thioureidyl		*N*-aminoacyl	
Symbol	**R =**	**Symbol**	**R =**	**Symbol**	**R =**
ANS1		**ATU1**		**AAA1**	
ANS2		**ATU2**		**AAA2**	
ANS3		**ATU3**		**AAA3**	
ANS4		**ATU4**		**AAA4**	
ANS5		**ATU5**		**AAA5**	
ANS6		*N*-benzyl		**AAA6**	
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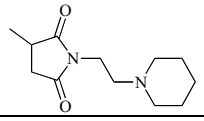
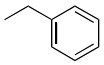
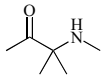
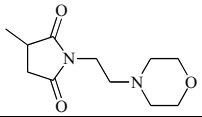
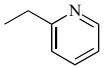
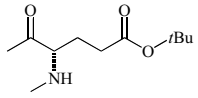
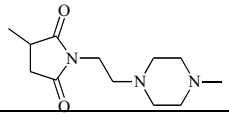
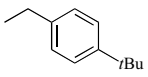
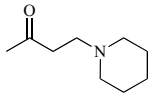
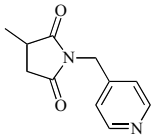
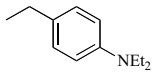
ANS7		**AB1**		**AAA7**	
ANS8		**AB2**		**AAA8**	
ANS9		**AB3**		**AAA9**	
ANS10		**AB4**			

Fig. (1). Structures of AmB and its derivatives.

molecule. The yields were generally good, in 78-98% range. The final products (28 compounds, Fig. (**1**) were purified by HPLC and their identity was unequivocally confirmed by HRMS and UV-vis spectroscopy. Almost all AmB derivatives specified in Fig. (**1**) are novel, except for the previously described *N*-benzyl-AMB (**AB1**) [27].

Table 1. Parameters of anticandidal *in vitro* activity and hemolytic activity and selective toxicity indexes of AmB and its novel derivatives. The IC_{50} and EH_{50} data are the means of 3 independent determinations ± SD.

-	Anticandidal *in vitro* Activity		Hemolysis EH_{50} [μg/mL]	STI (EH_{50} / IC_{50})
	MIC [μg/mL]	IC_{50} [μg/mL]		
AmB	0.25	0.19±0.02	2.06±0.06	10.8
ANS1	4.0	3.11±0.05	>100	>32.2
ANS2	2.0	1.55±0.07	>100	>64.5
ANS3	4.0	2.87±0.08	>100	>34.8
ANS4	4.0	3.22±0.07	>100	>32.0
ANS5	2.0	1.48±0.05	>100	>67.6
ANS6	4.0	3.11±0.02	>100	>32.2
ANS7	4.0	3.05±0.03	>100	>32.8
ANS8	4.0	2.95±0.07	38.36±0.87	13.0
ANS9	1.0	0.87±0.01	>100	>114.9
ANS10	8.0	5.26±0.09	>100	>19.0
AB1	1.0	0.76±0.01	3.50±0.12	4.6
AB2	0.5	0.32±0.02	3.93±0.08	12.3
AB3	4.0	3.28±0.89	>100	>30.5
AB4	1.0	0.66±0.03	>100	>151.5
ATU1	8.0	5.87±0.56	>100	>17.4
ATU2	2.0	1.48±0.07	>100	>67.6
ATU3	1.0	0.75±0.03	41.85±1.32	55.8
ATU4	2.0	1.57±0.04	>100	>63.7
ATU5	1.0	0.65±0.02	>100	>153.9
AAA1	2.0	1.76±0.12	>100	>56.8
AAA2	2.0	1.14±0.09	>100	>87.7
AAA3	4.0	3.28±0.08	>100	>30.5
AAA4	2.0	1.07±0.11	>100	>93.5
AAA5	4.0	2.86±0.14	>100	>35.0
AAA6	2.0	1.56±0.07	>100	>64.1
AAA7	0.5	0.34±0.02	12.55±0.78	36.9
AAA8	4.0	3.02±0.18	>100	>33.1
AAA9	0.5	0.39±0.02	14.03±0.66	36.0

3.2. Biological Evaluation

AmB and all its derivatives were tested for antifungal *in vitro* activity against the model *C. albicans* ATCC 10 231 strain and hemotoxicity against human RBC. The former was characterized by the MIC and IC_{50} values, determined in RPMI-1640 medium under conditions recommended by CLSI [25]. The quantitative parameter of hemotoxicity was EH_{50}, *i.e.* concentration of compound tested causing lysis of 50% of erythrocytes. The MIC, IC_{50} and EH_{50} values determined for all compounds are shown in Table **1**.

The fungistatic *in vitro* activity of all derivatives was lower than that of AmB and their MIC values were 2 – 32 times higher than that of the mother antibiotic but for 16 out of 28 derivatives, this increment was in 2 – 8-fold range

Table 2. Spectrum of antifungal *in vitro* activity of AmB and its selected 5 derivatives.

-	MIC [μg/mL]					
	AmB	ANS9	AB4	ATU5	AAA2	AAA4
***C. albicans* SC 5314**	0.25	1	0.5	2	1	1
***C. albicans* Gu4**	0.125	0.5	1	0.25	1	1
***C. albicans* Gu5**	0.5	1	1	2	4	1
***C. albicans* B3**	0.125	1	2	0.25	0.5	1
***C. albicans* B4**	0.25	1	1	4	4	1
C. glabrata	1	2	2	4	4	4
C. lusitaniae	0.125	0.5	1	1	2	2
C. krusei	0.5	2	2	2	4	4
C. parapsilosis	0.5	1	2	2	2	2
C. tropicalis	0.25	1	2	1	2	4
C. stellatoidea	0.25	2	1	1	2	2
C. dubliniensis	0.25	4	0.5	1	1	1
C. guiliermondi	0.25	4	2	1	1	4
C. gatti	0.25	1	1	2	2	4
C. neoformans	0.25	0.5	1	2	2	2
T. viride	0.5	2	4	2	4	4
A. niger	0.25	1	1	2	1	1
A. fumigatus	1	8	4	8	8	16
F. solani	0.25	4	2	16	>16	>16
M. circinelloides	0.25	2	2	2	2	4
R. oryzae	1	4	1	4	4	4

only. A much more significant difference between AmB and the derivatives was found for their hemotoxicity. Whereas AmB caused 50% lysis of erythrocytes at 2.06 μg/mL, only for 6 out of 28 derivatives their EH_{50} values were lower than 100 μg/mL. For the remaining 22 compounds, the EH_{50} values could not be actually determined, since the extent of hemolysis at 100 μg/mL was below 50% (9 – 22%) and poor solubility of these compounds at concentrations exceeding 100 μg/mL precluded determination of EH_{50} values. One may thus conclude that these derivatives were poorly hemolytic.

For all but one derivative, the EH_{50}/IC_{50} ratio (STI, selective toxicity index), which is a rational quantitative measure of selective toxicity under *in vitro* conditions, was higher than that of AmB. Outstanding STIs (close to 100 or higher) were found for 5 derivatives, namely *N*- {*N*-[2-(4-methylpiperazin-1-yl)ethyl]succinimidyl}-amphotericin B (**ANS9**), *N*-(4-*N,N*-diethylam-inobenzyl)amphotericin B (**A-B4**),*N*-{3-[(2-piperidin-1-yl)ethyl]-thioureidyl}-amphoteri-cin B (**ATU5**), *N*-[β-(pyridine-1-yl)-*D*-alanyl]amphoter-icin B (**AAA2**) and *N*-(*O*-*tert*-butyl-*D*-seryl)amp-hotericin B (**AAA4**). In all these cases, a relatively low MIC (1 or 2 μg/mL) was combined with poor hemolytic properties (EH_{50} > 100 μg/mL).

The antifungal spectrum of 5 derivatives exhibiting the best selective toxicity, *i.e.* the highest STI values, was compared to that of AmB against 15 yeasts (9 reference strains of the *Candida* genus, 4 clinical strains of *C. albicans*, 2 *Cryptococci*) and 6 filamentous fungi. Results of this comparison are shown in Table **2**.

Activity of 5 novel derivatives against human pathogenic yeasts was good (MICs in the 0.25 – 4 μg/mL range), although worse than that of AmB. The multidrug-resistant *C. albicans* strains Gu5 and B4, overproducing the drug-efflux pumps Cdr1p or Mdrp1, were equally or only slightly less sensitive than their respective counterparts Gu4 and B3, but the same regularity was found for AmB. The novel derivatives were less active than AmB against filamentous fungi and especially high MIC values (8 - >16 μg/mL) were found for **ATU5**, **AAA2** and **AAA4** against *Aspergillus fumigatus* and *Fusarium solani.*

Table 3. Cytotoxicity of AmB and some of its derivatives toward three mammalian cell lines in tissue culture. Data are the means of 3 independent determinations ± SD.

Compounds	IC_{50} [μg/mL]		
	HepG2	LLC-PK1	CCRF-CEM
AmB	5.4 ± 1.05	19.7 ± 8.05	4.30 ± 0.86
ANS1	>100	>100	>100
ANS2	>100	>100	>100
ANS3	>100	>100	>100
ANS4	>100	>100	>100
ANS5	73.3 ± 1.20	>100	50.1 ± 1.30
ANS6	>100	>100	>100
ANS7	>100	70.7 ± 2.30	>100
ANS9	>100	>100	>100
ANS10	>100	>100	>100
AB3	>100	>100	>100
AB4	>100	>100	>100
ATU1	30.0 ± 5.06	84.15 ± 3.66	0.78 ± 0.093
ATU2	>100	>100	>100
ATU4	>100	>100	80.9 ± 2.30
ATU5	>100	>100	>100
AAA1	>100	>100	>100
AAA2	>100	>100	>100
AAA3	>100	>100	>100
AAA4	>100	>100	>100
AAA5	>100	>100	>100
AAA6	>100	>100	>100
AAA8	>100	>100	>100

Cytotoxicity of 22 out of 28 novel derivatives demonstrating low hemolytic activity (EH_{50}>100 μg/mL, Table **1**) was assessed against Hep G2, LLC-PK1 and HEK-293T cells. Results shown in Table **3** clearly indicate that most of the derivatives tested were not cytotoxic (IC_{50} > 100 μg/mL). A substantial cytotoxic effect against all cell lines was noted only for AmB and **ATU1**. **ANS5** was cytotoxic against two cell lines, **ANS7** exhibited cytotoxicity against LLC-PK1 cells and **ATU4** against CCRF-CEM cells.

According to the "barrel-stave-pore" mechanism of action of AmB and its previously described derivatives, the selective toxicity of these compounds was to a large extent correlated with their differential potential of induction of potassium efflux from fungal and mammalian cells [5]. In this study, we measured the time- and concentration-dependent K^+ release from *C. albicans* and RBC induced by AmB and 12 selected novel derivatives. The set of compounds tested comprised 5 derivatives exhibiting the highest selective toxicity (**ANS9**, **AB4**, **ATU5**, **AAA2** and **AAA4**) and AmB + 7 derivatives demonstrating the lowest STI (**ANS8**, **ANS10**, **AB1**, **AB2**, **ATU1**, **AAA7** and **AAA9**). Cells suspended in TBS (*C. albicans*) or saline (RBC) were exposed to compounds tested and changes in the extracellular potassium level were monitored with potassium-selective electrode (RBC) or by flame photometry (*C. albicans*).

The exemplary kinetics of potassium efflux from yeast and erythrocytes induced by **AmB**, **AB1** and **AB4** are shown in Fig (**2AB**). **AmB** and **AB1** induced rapid efflux from both cell types, although the concentration-dependent difference between yeast cells and RBC was observed. On the contrary, for **AB4** the rapid efflux at low compound concentration was noted from yeast cells only.

The EK_{50} values, *i.e*. concentrations causing loss of 50% of intracellular potassium, estimated from dose response curves (not shown), are presented in Table **4**. All compounds

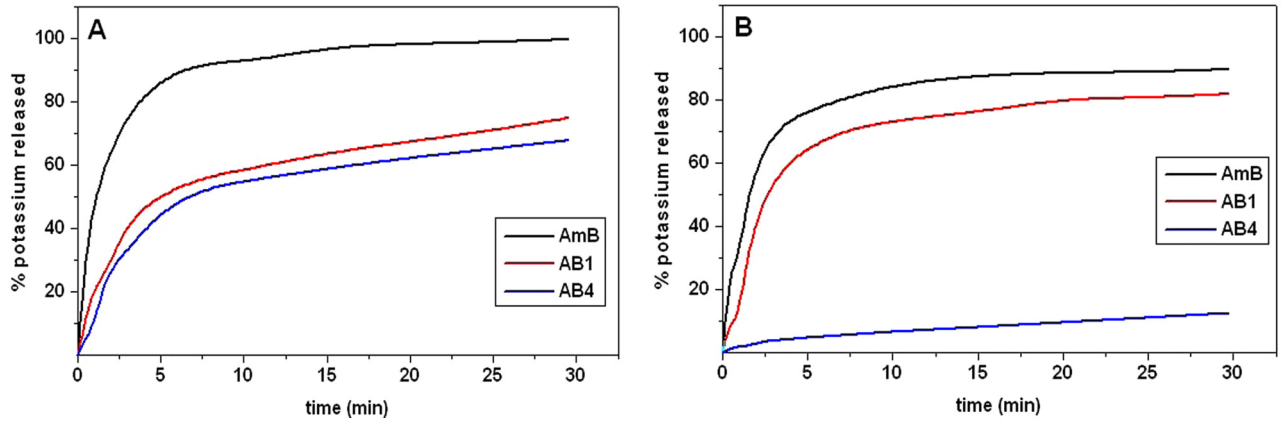


Fig. (2). Kinetics of potassium efflux from *C. albicans* (**A**) and RBC (**B**) induced by **AmB**, **AB1** and **AB4**. Compounds concentrations were 1 μg/mL in A and 10 μg/mL in B, respectively. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 4. Induction of potassium efflux from *C. albicans* cells and RBC by AmB and its selected derivatives. The data presented are the means of 3 independent determination of EK_{50} values ±SD.

Compounds	EK_{50} [μg/mL]	
	C. albicans ATCC 10 231	Erythrocytes
AmB	0.09±0.01	1.24±0.22
ANS8	2.64±0.15	15.34±1.87
ANS9	0.69±0.06	>100
ANS10	4.89±0.35	89.54±2.84
AB1	0.58±0.06	2.65±0.13
AB2	0.22±0.06	3.14±0.18
AB4	0.54±0.04	>100
ATU1	5.51±0.48	95.76±4.11
ATU5	0.56±0.07	>100
AAA2	0.99±0.08	>100
AAA4	1.01±0.09	>100
AAA7	0.28±0.02	8.56±0.76
AAA9	0.34±0.03	10.49±0.9

tested induced substantial potassium efflux from *C. albicans* cells at low concentrations (EK_{50} in the 0.09 – 5.51 μg/mL range). In all cases, the EK_{50} values were lower than the IC_{50} values presented in Table **1**. A substantial difference was found for potassium efflux from RBC. For the 5 AmB derivatives characterized by high STI values, EK_{50} could not be actually determined, because at the highest concentration applied, potassium efflux was lower than 50% (13 - 28% range). For 8 other compounds tested, the EK_{50} values were lower than the respective EH_{50} data presented in Table **1**.

4. DISCUSSION

Several approaches have been used previously to address the problem of high mammalian toxicity and poor solubility of Amphotericin B. A number of semisynthetic analogs have been generated over the past 20 years in an attempt to reduce this toxicity and increase solubility. Numerous AmB derivatives have been obtained by chemical modifications of C16 carboxyl (methyl ester and amides) or the amino group of mycosamine (*N*-acyl- and *N*-alkyl analogs) [5, 28]. It has been demonstrated that modification of the C16 carboxyl group of AmB or its analogue, 28,29-didehydronystatin, leading to charge suppression, reduces toxicity to mammalian cells, with little effect on antifungal activity [2, 29-31] but substantial improvement of selective toxicity has been achieved only recently for urea derivatives and conjugates with molecular umbrellas [15, 16]. None of modifications of the amino functionality of mycosamine, suppressing the positive charge at this site, resulted in improvement of selective toxicity of AmB [5, 28] but this effect has been achieved due to the bisalkylation with aminopropyl groups [13]. The early works of Wright *et al*. and results of previous studies in our group suggested that introduction of bulky substituents at

the amino group with concomitant charge retention, may lead to the improvement of selective toxicity of AmB and its methyl ester [10-12]. This assumption was based on the hypothesis that AmB derivatives with bulky substituents at the amino group should form AmB derivative:sterol complexes with geometry different from that of AmB:sterol complexes. This could result in the differential ability to aggregate into lethal transmembrane channels in ergosterol and cholesterol-containing membranes [17, 32]. On the other hand, the importance of the positive charge at the amino group for AmB:ergosterol interaction was previously shown [33].

Structures of most of the novel AmB derivatives described in this work were designed following the assumptions formulated above. All derivatives were synthesized using the unmodified AmB as a substrate and substituents at the amino group were introduced by simple reactions performed under mild conditions, namely the Michel-type addition of *N*-substituted maleimides (**ANS1-10**), the reductive amination of benzaldehydes (**AB1-4**), the addition to isothiocyanates (**ATU1-5**) and the DCC-driven acylation (**AAA1-9**). Two out of four reaction types have been already previously used for the preparation of AmB derivatives different from those reported in this work (except for **AB1**) [13, 18, 26, 34], *N*-aminoacyl derivatives of AmB methyl ester but not of AmB are known [35] and addition to isocyanates to form thioureidyl derivatives has been now used for the first time for derivatization of the AmB amino group. In each of the four sets of derivatives, there are 1 or 2 compounds in which the substituents introduced at the amino functionality are expected to provide the least steric hindrance (**ANS1**, **AB1**, **AB2**, **ATU1**, **ATU2 AA1** and **AA2**) and the remaining ones, in which the local steric hindrance is provided due to the bulky substituents, including *t*-butyl and cyclohexyl functionalities or the heterocyclic rings.

The expected improvement of the selective toxicity index in comparison with that of AmB has been achieved for all novel AmB derivatives containing bulky substituents and for 5 of them, their STI was at least 10-fold higher than that of AmB. Notably, the increase of STI was mainly due to the substantial reduction of hemotoxicity, whereas the antifungal *in vitro* activity of all novel derivatives was slightly lower than that of AmB. Accordingly, potassium efflux from *C. albicans* cells was triggered by the novel derivatives allow concentrations, while that from RBC at much higher ones. A possible molecular basis of STI increase may be a consequence of the steric hindrance generated in the vicinity of mycosamine amino group. Presence of any bulky substituent at this group could affect the interaction of the neighboring 2'-OH group with 3β-OH of cholesterol and ergosterol. This interaction was postulated to be important for the formation of AmB:sterol complexes [36, 37] but results of another later study showed that it is absolutely crucial for the formation of cholesterol:AmB complex, since 2'-deoksyAmB did not bind cholesterol at all, while its binding to ergosterol was only slightly disturbed [14]. Assuming that formation of the antibiotic:sterol complex is the prerequisite for both antifungal and hemolytic activity of AmB and its derivatives, it seems possible that presence of bulky substituents at the amino group of mycosamine decreases slightly AmB affinity to ergosterol and markedly that to cholesterol. In consequence, this difference would constitute a molecular basis for slightly diminished antifungal activity and substantially reduced hemotoxicity of novel AmB derivatives in comparison with the mother antibiotic. The similar explanation could be also valid for the previously observed improved selective toxicity of *N*-methyl-*N*-fructosyl-AmB methyl ester, developed in our group several years ago [11].

These features of novel AmB derivatives are in contrast with the biological properties of compounds obtained by Carreira and coworkers by bisalkylation of mycosamine [13, 18, 38]. Several AmB derivatives of this type demonstrated improved selective toxicity due to the higher than that of AmB antifungal *in vitro* activity (MIC values even 10-fold lower) and slightly lower hemotoxicity. It seems therefore, that the molecular basis of high STI of bisalkylated AmB derivatives might be different from that suggested above for the sterically hindered novel compounds.

The biological properties of *N*-succinimidyl and *N*-benzyl derivatives of AmB described in this work could be compared to those of the similar compounds containing less bulky substituents, described earlier. *N*-(*N*-ethylsuc-cinimidyl) AmB and *N*-(*N*-dimethylaminopropyl-succini-midyl)-amphotericin B demonstrated slightly lower antifungal activity and were slightly less hemolytic than AmB [34]. The antifungal activity of 11 *N*-benzyl derivatives of AmB described by Belakhov and Shenin was the same or lower than that of AmB but their hemolytic properties are not known [27].

Low *in vitro* cytotoxicity of most of the novel AmB derivatives against hepatocytes and kidney cell lines seems especially advantageous for them as the drug candidates in light of the well-known nephrotoxicity of AmB and its lipid preparations [39]. Moreover, these compounds should be much cheaper than known liposomal AmB preparations demonstrating lower nephrotoxicity than AmB. Another advantage is their better solubility in aqueous solutions than that of the mother antibiotic. However, further study, especially under *in vivo* conditions, is needed to warrant the use of these compounds for clinical applications. Such studies are now in progress.

CONCLUSION

Introduction of bulky substituents at the amino group of mycosamine residue in Amphotericin B generates derivatives exhibiting substantially improved selective toxicity, resulting mainly from the strongly reduced hemolytic potential. This effect is most likely due to the induced steric hindrance which may affect the creation of the hydrogen bond between 2'-OH of mycosamine and 3β-OH of cholesterol present in the mammalian cell membranes, thus preventing the formation of the AmB derivativ-e:cholesterol complex and assembly of the potassium-perme-able transmembrane channels. Facility and simplicity of synthesis and the advantageous biological properties of some of the novel AmB derivatives make them promising drug candidates for antifungal chemotherapy.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the corresponding author, [SM], upon reasonable request.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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