New targeting system for antimycotic drugs: β-Glucosidase sensitive Amphotericin B–star poly(ethylene glycol) conjugate

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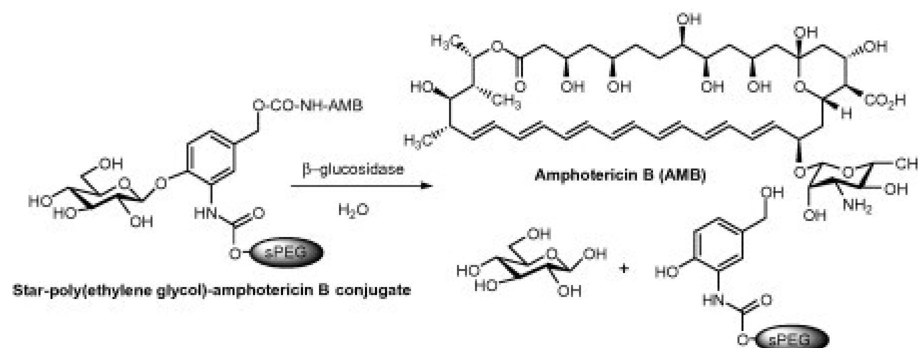
Abstract

A new targeting potentially intravenous conjugate Amphotericin B (AMB)–star poly(ethylene glycol) (sPEG) (M = 25,160) has been synthesized and characterized. It contains a β-D-glucopyranoside molecular switch which is sensitive to β-glucosidases (E.C.3.2.1.21). The β-glucosidase-catalyzed release of AMB from the polymeric carrier was proved in vitro by means of spectrophotometry and HPLC.

Graphical abstract

The prepared conjugate of Amphotericin B with star poly(ethylene glycol) represent a new targeting system which is sensitive to β-glucosidases (E.C.3.2.1.21).



Keywords: Drug targeting; Amphotericin B; Controlled release in vitro

Amphotericin B (AMB) (Fig. 1) is a polyene macrocyclic membrane-active antifungal antibiotic drug used in treatment of system fungal infections.[1] It is a potent salvaging medical drug that is applied in the cases of immunosuppressive patients after transplantations of organs, those with acquired immune deficiency syndrome (AIDS) or with tumors and hematological malignities. However, the clinical application of AMB is restricted by both its poor solubility in water and some side effects, particularly nephrotoxicity.[1] The pharmaceutical dosage forms used in practice are non-covalent complexes of the type Amphotericin B lipid complex (ABLC), which are colloidal systems composed of biodegradable phospholipid matrixes.[2] These types of formulations represent a solution to

the solubility problems of AMB, but the release of AMB takes place already in the patient's blood circulation system, which results in a general impact on his/her organism inclusive of the undesirable side effects.

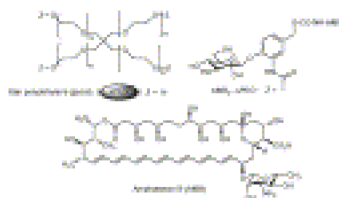
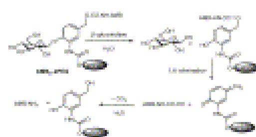


Figure 1. Structure of β-glucosidase sensitive Amphotericin B–star poly(ethylene glycol) conjugate (AMB_4–sPEG).

The efforts to increase the therapeutic index of AMB led to development of conjugates with polymeric carriers: polysaccharides,[3a] poly(ethylene glycol)[3b] and [3c] and poly(ethylene glycol)-*b*-poly(L-lysine).[3d] The last mentioned types of conjugates[3c] and [3d] were suggested with the aim to achieve the release of AMB from polymeric carrier in a targeted way, that is, in the areas having locally lowered pH value due to pathological processes.[4] The rate of release of AMB (acid-catalyzed hydrolysis of imine linkage) is controlled by the pH value of the milieu and by substitution of benzene ring attached to the polymeric carrier.[3c] and [3d]

The aim of this work was to prepare and characterize a conjugate that would release AMB selectively—only in the area of the attacked organ. The proposed structure of conjugate (AMB_4–sPEG) (Fig. 1) was inspired by the finding that many parasitic fungal pathogens, such as species of *Aspergillus*, *Candida* or *Trichosporon* families possess specific hydrolases β-glucosidases (E.C.3.2.1.21) in their enzymatic outfit.[5]

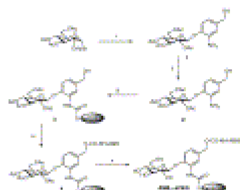
In the case of human organism, β-glucosidases only occur in some bacteria present in intestinal micro-flora.[6] This finding was previously used for achieving controlled release of some drugs from per oral intestinal pro-drugs.[6] However, there exists no natural occurrence of β-glucosidases in healthy human tissues.[7] The basic linker in AMB_4–sPEG is the β-glucosidic bond of a substituted phenyl-β-D-glucopyranoside molecule. With regard to its biocompatibility and binding capacity, we chose star poly(ethylene glycol) [pentaerythritol poly(ethylene glycol)ether, M = 20,000] (sPEG) as the polymeric carrier.[8] The AMB_4–sPEG conjugate involves two types of carbamate bond: the connecting units is linked to polymer by stable carbamate bond, while AMB is linked to the phenyl-β-D-glucopyranoside fragment by unstable carbamate bond. The enzymatic hydrolysis of β-glucosidic bond should produce glucose, and the subsequent 1,6-elimination should give the carbamic acid of Amphotericin B, which is quickly decomposed to release free Amphotericin B. The remaining molecule of polymeric carrier with covalently bound fragment of substituted 4-hydroxybenzylalcohol is then excreted from the organism (Scheme 1).



Scheme 1. Principle of release of Amphotericin B form AMB_4–sPEG.

Similar principle of activation for low-molecular pro-drugs was described earlier in the case of hydrolysis of β-D-glucuronides, where β-D-glucuronic acid is split off by action of β-D-glucuronidases (E.C.3.2.1.31). This principle is being used in the so-called Antibody Directed Enzyme Prodrug Therapy (ADEPT).[9]

The key part of the AMB$_4$–sPEG molecule is the enzyme-sensitive linker derived from substituted β-D-glucopyranoside, which was prepared by the following reaction sequence.[10], [11] and [12] First, the Koenigs–Knorr reaction[10] of 2,3,4,6-tetra-*O*-acetyl-α-d-glucopyranosyl bromide with 4-hydroxymethyl-2-nitrophenol[9c] was used to prepare 1-(4-hydroxymethyl-2-nitrophenyl)-(2,3,4,6-tetra-*O*-acetyl)-β-D-glucopyranoside (**1**).[11] The hydrogenation of glucopyranoside **1** on Adams catalyst gave 1-(2-amino-4-hydroxymethylphenyl)-(2,3,4,6-tetra-*O*-acetyl)-β-D-glucopyranoside (**2**)[12] (Scheme 2).



Scheme 2. Synthesis of AMB$_4$–sPEG. Reagents and conditions: (i) 2-nitro-4-hydroxymethylphenol/Ag_2O/acetonitrile/ultrasound 5 h/25 °C/77%; (ii) PtO_2/H_2/ethyl acetate/3 h/25 °C/97%; (iii) sPEG–O–CO–O–C_6H_4–NO_2/DMAP/DMF/48 h/65 °C/84%; (iv) a—4-nitrophenyl chloroformate/toluene/72 h/25 °C; b—AMB/DMAP/DMF/144 h/25 °C/66%; (v) a—KCN/methanol/5 h/25 °C, b—Amberlite 120-H/methanol/10 min/25 °C/83%.

The β-configuration of glucopyranosides **1** and **2** was confirmed by ^{1}H–^{13}C HMQC NMR spectra.[13] The anomeric carbon atom in derivative **1** exhibits a shift at 98.4 ppm, which corresponds with the doublet of anomeric hydrogen atom at 5.68 ppm with the coupling constant J = 7.9 Hz. Derivative **2** exhibits the anomeric carbon atom at 99.1 ppm, which corresponds with the doublet of anomeric hydrogen atom at 5.33 ppm (J = 7.9 Hz). The values of coupling constant of anomeric hydrogen atom coupled with the neighboring vicinal hydrogen atom indicate that the two atoms are in axial positions, that is, the glucose fragment of both derivatives **1** and **2** assumes the β-configuration [β-4C1(D)].

The next reaction step involves reaction of pentaerythritol tetra(4-nitrophenyl carbonyl) poly(ethylene glycol) (sPEG–O–CO–O–C_6H_4–NO_2) with glucopyranoside **2** to form a carbamate bond.[14] The hydroxymethyl group of polymer **3** was then activated with 4-nitrophenyl chloroformate and resulting intermediate reacted with Amphotericin B. The polymer **4** obtained in this way contains four AMB molecules linked by labile carbamate bond.[15] Due to the instability of AMB it was impossible to deprotect the hydroxyl groups of glucose by means of carboxyesterase (pH 5; 40–50 °C).[16] Therefore, the deprotection of acetylated hydroxyl groups of polymer **4** was carried out by catalytic amount of potassium cyanide. Potassium cyanide was removed using of an ion exchanger after the reaction[17] (Scheme 2). The course of deprotection was monitored by ^{1}H NMR spectroscopy. The multiplet of CH_3– in acetyl groups at 2.02 ppm gradually disappeared. The final conjugate AMB$_4$–sPEG was characterized[18] by microanalysis and GPC[19] was adopted for determination of M_w. The HPLC[19] was used for estimation of conjugate purity. The content of free AMB was below 1 mol %. Furthermore, by means of UV–vis spectroscopy (typical maxima corresponding to polyene system of AMB[18]) it was proved that the content of AMB in the conjugate AMB$_4$–sPEG corresponds to the molar ratio of 1:4 (sPEG/AMB). The prepared conjugate is very well soluble in water and forms finely opalescent solutions.

The next part of our work was focused on preliminary in vitro testing of the conjugate synthesized in various media. First, the stability of conjugate was studied in phosphate buffer.[19] The monitoring of UV–vis spectra showed no observable spectral changes in the phosphate buffer of pH 7.4 during a period of 18–20 h. For a model medium imitating the tissue attacked by fungal pathogen we chose β-glucosidase (E.C.3.2.1.21) (*Aspergillus niger*) in phosphate buffer with pH 7.4. Spectral recording of the conjugate showed an increase in the

absorbance at the wavelengths of 367, 386 and 409 nm (Fig. 2). Formation of substituted 1,4-quinonemethide (Scheme 1) should be visible in the wavelength range of 280–320 nm[20]; however, this region is overlapped by the absorption band of β-glucosidase (245–330 nm). The spectral records clearly show an increase in the absorption band at 409 nm, which is specific[21] for monomeric AMB. Such increase with time corresponds with the rate of release of AMB from the carrier. The rate of release of AMB under the experimental conditions adopted[19] obeys the kinetic equation of the pseudo-first order, and the half-life of release of AMB is $\tau_{1/2}$ = (103 ± 4) s. The release of AMB from the conjugate was also confirmed by means of HPLC.[21] The results described indicate that the behavior of the AMB_4–sPEG conjugate in the presence of buffered solution of β-glucosidase corresponds to Scheme 1.

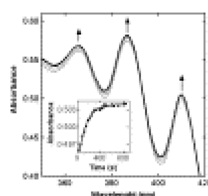


Figure 2. Record of time changes of spectrum for enzymatic hydrolysis of AMB_4–sPEG conjugate (3×10^{-6} M) catalysed by β-glucosidase (2 mg/1 mL; 66.6 U/g) in phosphate buffer (pH 7.4, 2×10^{-2} M) at 37 °C. The inset represents time dependence of absorbance at 409 nm [$\tau_{1/2}$ = (103 ± 4) s].

In contrast to the pharmaceutical dosage forms of AMB used for intravenous application nowadays, we have designed a fundamentally new targeting AMB conjugate that selectively releases AMB by enzymatic action of β-glucosidase. The results of preliminary study in vitro allow the presumption that targeted release of AMB will take place in the area of expected activity of fungal pathogen. This new system which we have suggested can be used more generally, that is, also for other antifungal medicaments. However, its practical applicability will need further tests on animal models and their critical evaluation.

Acknowledgments

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11 *1-(4-Hydroxymethyl-2-nitrophenyl)-(2,3,4,6-tetra-O-acetyl)*-β-D-*glucopyranoside (**1**):* A mixture of 2,3,4,6-tetra-*O*-acetyl-α-d-glucopyranosyl bromide (3.08 g; 7.5 mmol), 4-hydroxymethyl-2-nitrophenol[9c] (1.27 g; 7.5 mmol), Ag_2O (7.3 g; 31.5 mmol) and dry acetonitrile (65 mL) was stirred by means of ultrasound at the temperature of 25 °C for a period of 5 h. The crude product was purified chromatographically (silica gel/ethyl acetate). Yield: 2.7 g (77%), mp 198–200 °C, TLC: (Silicagel plates Merck), ethyl acetate, R_F = 0.55; $[\alpha]_D^{20}$ +19.4° (0.5 g/100 mL, CH_3OH). ^1H NMR (500.13 MHz, DMSO-d_6): 2.03 (s, 3H), 2.06 (s, 3H), 2.07 (s, 3H), 2.09 (s, 3H), 4.17–4.20 (m, 1H), 4.26–4.30 (m, 1H), 4.32–4.35 (m, 1H), 4.57 (d, 2H, *J* = 5.72 Hz), 5.09 (t, 1H, *J* = 9.7 Hz), 5.12–5.16 (m, 1H), 5.44–5.49 (m, 2H), 5.68 (d, 1H, *J* = 7.9 Hz), 7.44 (d, 1H, *J* = 8.7 Hz), 7.68 (dd, 1H, *J* = 1.9 Hz, 8.7 Hz), 7.85 (d, 1H, *J* = 1.9 Hz). ^{13}C NMR, 125.76 MHz, DMSO-d_6: 20.31, 20.37, 20.48, 20.58, 61.46, 61.68, 67.98, 70.25, 71.24, 71.81, 98.44, 117.94, 122.37, 132.01, 138.54, 140.35, 147.24, 168.94, 169.43, 169.72, 170.13. Calcd for $C_{21}H_{25}NO_{13}$ (499) (%): C, 50.50; H, 5.05; N, 2.80. Found: C, 50.50; H, 4.97; N, 2.98.

12 *1-(2-Amino-4-hydroxymethylphenyl)-(2,3,4,6-tetra-O-acetyl)*-β-D-*glucopyranoside (**2**):* A solution of glucopyranoside **1** (1 g; 2 mmol) in ethyl acetate (150 mL) with an addition of PtO_2 (0.1 g; 0.4 mmol) was hydrogenated under a mild overpressure of hydrogen (ca 5 kPa) at the temperature of 25 °C for 3 h. Yield: 910 mg (97%), mp 135–138 °C, TLC: (Silicagel plates Merck), ethyl acetate, R_F = 0.32; $[\alpha]_D^{20}$ −30.4°

(0.5 g/100 mL, CH_3OH). ^{1}H NMR (500.13 MHz, DMSO-d_6): 2.03 (s, 3H), 2.06 (s, 3H), 2.08 (s, 3H), 2.09 (s, 3H), 4.10–4.14 (m, 1H), 4.23–4.29 (m, 2H), 4.36 (d, 2H, J = 5.5 Hz), 4.61 (br s, 2H), 5.02–5.06 (m, 2H), 5.08–5.12 (m, 1H), 5.34 (d, 1H, J = 7.9 Hz), 5.5 (t, 1H, J = 9.52 Hz), 6.51 (dd, 1H, J = 1.60 Hz, 8.2 Hz), 6.71 (d, 1H, J = 1.6 Hz), 6.87 (d, 1H, J = 8.2 Hz). ^{13}C NMR (125.76 MHz, DMSO-d_6): 20.43, 20.51, 20.61, 20.66, 61.79, 62.91, 68.28, 70.79, 71.17, 71.79, 99.11, 113.33, 114.51, 115.87, 138.05, 138.19, 142.49, 169.46, 169.70, 170.11. Calcd for $C_{21}H_{27}NO_{11}$ (469) (%): C, 53.73; H, 5.80; N, 2.98. Found: C, 53.70; H, 5.72; N, 2.88.

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14 *Polymer **3**:* A mixture of pentaerythritol tetra(4-nitrophenyl carbonyl) poly(ethylene glycol) M = 20,348 (NOF Corp.) (1 g; 0.05 mmol), glucopyranoside **2** (1 g; 2 mmol) and 4-*N*,*N*-dimethylaminopyridine (30 mg; 0.25 mmol) in anhydrous DMF (10 mL) was heated at the temperature of 65 °C for 100 h. The crude polymer was twice recrystallized from propan-2-ol. Yield 920 mg (84%). ^{1}H NMR (500.13 MHz, $CDCl_3$) 2.02 (m, 12H; $(CH_3)_4$), 3.42–3.72 (m, 392H; $(CH_2–CH_2–O)_{98}$), 3.69 (m, 2H; CH_2), 3.75 (m, 2H; CH_2), 4.21 (m, 2H; CH_2), 4.28 (m, 2H; CH_2), 5.01 (m, 2H; CH_2), 5.13 (m, 1H; CH), 5.27 (m, 1H; CH), 6.73 (m, 1H; CH) 6.88 (m, 2H; CH_2), 7.04 (m, 1H; CH), 7.45 (s, 1H, CONH). Calcd for $C_{997}H_{1920}N_4O_{504}$ (22,030) (%): C, 54.35; H, 8.79; N, 0.25. Found: C, 54.72; H, 8.92; N, 0.36; M_w/M_n = 1.18.

15 *Polymer **4**:* An anhydrous solution of polymer **3** (1.1 g; 0.05 mmol) and 4-nitrophenyl chloroformate (500 mg; 2.5 mmol) in toluene (40 mL) was kept at the constant temperature of 50 °C for a period of 48 h, whereupon the toluene was distilled off, and the residue was mixed with diethyl ether (250 mL), filtered and washed with diethyl ether (10 × 50 mL). After drying in desiccator, this intermediate (ca 1 g) was dissolved in dimethylformamide (8 mL) with addition of Amphotericin B (185 mg, 0.20 mmol), 4-*N*,*N*-dimethylaminopyridine (12 mg; 0.1 mmol) and trioctylamine (350 mg; 1 mmol). The mixture was stirred in the dark in argon atmosphere at room temperature for several days. The end of reaction was estimated by means of GPC monitoring. The crude polymer was purified by reprecipitation from diethyl ether. Yield 850 mg (66%); UV–vis: λ_{max} (nm) 346, 367, 386, 409. Calcd for $C_{1198}H_{2204}N_8O_{576}$ (25,830) (%): C, 55.29; H, 8.60; N, 0.43. Found: C, 55.56; H, 8.89; N, 0.59; M_w/M_n = 1.22.

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18 *AMB_4–sPEG conjugate:* A mixture of polymer **4** (260 mg; 0.01 mmol) and KCN (6.5 mg; 0.1 mmol) in methanol (10 mL) was stirred at the temperature of 25 °C for a period of 5 h, whereupon Amberlite IR–120H ion resin (1 g) was added. After 10 min, the ion exchanger was removed by decantation, and methanol was distilled off under reduced pressure at the temperature of 35 °C. Yield 230 mg (83%); UV–vis: λ_{max} (nm) 346, 367, 386, 409. Calcd for $C_{1198}H_{2204}N_8O_{576}$ (25,160) (%): C, 55.23; H, 8.70; N, 0.45. Found: C, 55.61; H, 9.08; N, 0.52; M_w/M_n = 1.23.

19 *General methods/equipment:* (a) The ^{1}H NMR spectra were calibrated with respect to the middle signal in the multiplet of solvent (δ = 2.55; DMSO-d_6) or (δ = 7.24; $CDCl_3$). The ^{1}H NMR spectra of polymers were measured with relaxation delay of 6 s and an acquisition time of 4 s: Tocco, G.; Begala, M.; Delogu, G.; Meli, G.; Picciau, C.; Podda, G. *Synlett* **2005**, 1296. The ^{13}C NMR spectra were measured in standard way using broad-band proton decoupling and/or pulse sequence APT. The ^{13}C NMR spectra were calibrated with respect to the middle signal in the multiplet of solvent (δ = 39.6; DMSO-d_6) or (δ = 77.0; $CDCl_3$).The pulse sequence *gs* ^{1}H–^{13}C HMQC was measured with the CH interaction of 145 Hz.

(b) Gel permeation chromatography (GPC) was used for the estimation of M_w of the polymers. The gel permeation chromatography measurements of the prepared polymers **3**, **4** and conjugate AMB_4–sPEG were performed with HEMA–BIO columns (hydrophilic modification HEMA–Gel, particle size 10 μL, porosity 40/100/300/1000) at room temperature using an RI detector and UV–vis detector. Redistilled water (pH 7.1) was used as the eluent. The columns were calibrated with a series of standard PEGs with varying molecular weights (PSS, Polymer Standard Service GmbH, Mainz, Germany).

(c) The purity and stability of conjugate AMB_4–sPEG was estimated by HPLC using LiChroCART® 125 × 4 mm column packed with LiChrospher® 100 RP–18 e 5 μm (Merck) and eluted with mobile phase of acetonitrile with 20 mM chelaton II.

(d) The stability of AMB_4–sPEG conjugate was monitored spectrophotometrically using an HP UV–vis 8453 Diode Array apparatus and 1-cm closable quartz cell in the cell holder kept at the constant temperature of 37 °C. The cell was charged with 2 mL phosphate buffer added from a pipete (pH 7.4; 2×10^{-2} M), and after reaching the said temperature, 15 μL methanolic solution of AMB_4–sPEG conjugate was injected, so that the final concentration of the substrate was about 3×10^{-6} M. The enzymatic hydrolysis of AMB_4–sPEG was monitored by the same methodology; however, the used solution was β-glucosidase (2 mg/mL) (E.C.3.2.1.21; Fluka; 66.6 U/g; *A. niger*) in phosphate buffer (pH 7.4, 2×10^{-2} M). The measured absorbance-time (A_τ) dependence was used to calculate the half-life of enzymatic hydrolysis ($\tau_{1/2} = \ln 2/k_{obs}$) and the observed rate constant (k_{obs}(s^{-1})): $k_{obs}\tau = \ln \Delta A + \text{const.}$, where $\Delta A = (A_0 - A_\tau)$.

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