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Synthesis of pH-sensitive amphotericin B–poly(ethylene glycol) conjugates and study of their controlled release in vitro

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Dedicated to Professor Jaromír Kaválek on the occasion of his 70th birthday.

Abstract—New intravenous conjugates of amphotericin B (AMB) with poly(ethylene glycols) (PEG) (M = 5000, 10,000, 20,000) have been synthesized and characterised. The intermediate PEGs possess a 1,4-disubstituted benzene ring with aldehyde group at the end of the chain. The benzene ring is connected with PEG at its 4-position (with respect to the aldehyde group) by various functional groups (ether, amide, ester). Reaction of terminal aldehyde group of the substituted PEGs with AMB gave conjugates containing a pH-sensitive imine linkage, which can be presumed to exhibit antimycotic effect at sites with lowered pH value. All types of the conjugates are relatively stable in phosphate buffer at physiological conditions of pH 7.4 (37 °C), less than 5 mol% AMB being split off from them within 24 h. For a model medium of afflicted tissue was used a phosphate buffer (pH 5.5, 37 °C), in which controlled release of AMB from the conjugates takes place. The imine linkage is split to give free AMB with half-lives of 2–45 min. The rate of acid catalysed hydrolysis depends upon substitution of the benzene ring; however, it does not depend on molecular weights of the PEGs used. The conjugates with ester linkage undergo enzymatic splitting in human blood plasma and/or blood serum at pH 7.4 (37 °C) with half-lives of 2–5 h depending on molecular weights of the PEGs used (M = 5000, 10,000, 20,000). At first, the splitting of ester linkage produces the relatively stable pro-drug, that is, 4-carboxybenzylideniminoamphotericin B, which is decomposed to AMB and 4-formylbenzoic acid in a goal-directed manner only at pH 7 ($t_{1/2}$ = 2 min, pH 5.5, 37 °C). A goal-directed release of AMB is only achieved by acid catalysed hydrolysis of imine linkage, either from the polymeric conjugate or from the pro-drug released thereof. The LD_{50} values determined in vivo (mouse) are 20.7 mg/kg and 40.5 mg/kg for the conjugates with ester linkage (M = 10,000 and 5000, respectively), which means that they are ca. 6–11 times less toxic than free AMB.

1. Introduction

The AIDS epidemic, cancer chemotherapy, pervasive use of antibiotics and immunosuppressive drugs used for organ transplantation have all contributed to the emergence of fungal pathogens[1] like *Candida albicans*, *Aspergillus fumigatus*, *Cryptococcus neoformans*, etc. Polyene macrocyclic antibiotics, particularly amphotericin B (AMB), have been found to be effective and extensively used drugs in treatment of both pre-systemic and systemic fungal infections.[2] Amphotericin B was isolated by Trejo and Bennett[3] half a century ago from the *Streptomyces nodosus* found along the Orinoco River in Tebladora (Venezuela). However, clinical applications of AMB are limited by its poor solubility in water and by possible serious organ toxicity, particularly nephrotoxicity, which appears to be dose dependent.[4] With the aim of increasing the therapeutic index of AMB, a number of formulations and conjugates have been adopted, which enable solubilization of AMB in aqueous media and also ensure continuous dosage usually accompanied by lowered toxicity.[5] The clinically applied formulations are non-covalent complexes of the type of amphotericin B lipid complex (ABLC[6]), which are colloidal systems composed of biodegradable phospholipid matrices (e.g.,

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dimyristoylphosphtidylcholine (DMPC)[6] or modified poly (ethylene glycols) [e.g., distearoyl-*N*-[(monomethoxy)poly(ethylene glycol)succinyl)]-phosphatidylethanolamin (DSPE-PEG)][6]with amphotericin B. Such lipid formulations of amphotericin B often significantly increase antifungal activity in vitro as compared with common amphotericin B.[7] Our previous paper[8] suggested a synthesis of a conjugate for intravenous application in which 50 mol% AMB is bound by covalent carbamate bond to methoxy poly(ethylene glycol) (mPEG) and the remaining 50 mol% is bound non-covalently in this conjugate. This non-covalent bond is most probably realised by π–π interaction and hydrogen bonds between the covalently bound AMB and the monomeric AMB in similar way[9] as it is in the aggregated forms of AMB. Our conjugate exhibits very good water solubility and an antimycotic spectrum similar to that of the liposomal and deoxycholate formulations.[8] At present, we are finishing pharmacokinetic studies in vivo of this conjugate on animal models. Our study[8] was continued by the team of R.B. Greenwald,[10] who prepared conjugates of AMB with poly(ethylene glycol) (PEG) in which AMB is bound by means of labile carbamate bond that is decomposed in blood plasma by elimination mechanism to give free AMB ($t_{1/2}$ = 1.5–3 h, blood plasma, rat). This labile bond between PEG and AMB is constructed in such a way that the first slowest step of release consists in enzymatic reaction in which blood hydrolases split the ester linkage. In the second step, the pro-drug thus formed undergoes a fast base-catalysed 1,6-benzyl elimination[11] giving quinonemethide and carbamic acid of AMB. The quinonemethide is then immediately hydrated, and the carbamic acid of AMB is rapidly decomposed to carbon dioxide and free AMB.[10,11] This system of release of active substance appears to be suitable for goal-directed distribution of cytostatics containing primary amino group, namely in the delivery systems that in their polymeric carrier contain a bound monoclonal antibody possessing affinity to specific glycoprotein surface of tumour-transformed cells.[12] However, this principle cannot be applied generally to all groups of drugs. For example, in the case of PEG-AMB conjugates this system of 1,6-benzyl elimination results in relatively fast splitting off of free AMB directly into the blood circulation, which rapidly increases its concentration in kidneys, and this is undesirable due to its nephrotoxicity. The AMB released in blood undergoes subsequent distribution[13] between the present lipoproteins (high-density-lipoprotein (HDL) and low-density-lipoprotein (LDL)). Previously, it has been found out[14] that the increased level of complexes of AMB with LDL causes nephrotoxic effects of AMB.

The aim of the present paper is synthesis and characterisation of a new type of AMB-PEG conjugates in which AMB is bound in such a way as to prevent splitting off of free AMB in blood circulation during intravenous applications: AMB should be released in a goal-directed manner only at the site of activity of fungal pathogens. One of the methods of achieving such goal-directed antimycotic action in such cases is based on the fact that a number of pathogens, inclusive of fungal agents, induce such biochemical processes in the afflicted tissue[15] that lead to local lowering of pH value as much as to pH ≈5. This piece of knowledge has been utilised recently in constructing very efficient pH-sensitive lipid formulations (liposomes) of nystatin.[16] An analogous principle was also used for pH-sensitive spacers between the drug and carrier that enable release of an active drug from the carrier in a tumour tissue, either slightly acidic extracellular fluids or, after endocytosis, in endosomes or lysosomes of cancer cell.[17–19] For the pH-sensitive bond between amino group of mycosamine AMB and PEG, we have selected imino group, which should be sufficiently stable at pH values that are usual in blood and tissues pH 7.4–7.6), for example, in blood plasma or blood serum. On the other hand, the bond should be very easy to hydrolyse at lowered pH value. The chosen carriers are poly(ethylene glycols) (mPEG M = 5000 and PEG M = 10,000, 20,000), which can be chemically substituted very easily[20,21] and are non-toxic, non-immunogenic, non-antigenic, that is, resistant to recognition by the immunity system of organism.[22] Figure 1 presents the structures of our selected conjugates: α-methoxy-ω-(4-AMB-iminomethylphenyloxy)poly(ethylene glycol) (**1**), in which the poly(ethylene glycol) chain is bound to the benzene ring by means of ether linkage. This linkage should not be split by usual components of blood plasma. The 4-position of the benzene ring of the conjugate carries the pH-sensitive imino group connecting the conjugate with AMB. The second suggested conjugate, that is, α-methoxy-ω-(4-AMB-iminomethylphenylcarboxamino)-poly(ethylene glycol) (**2**), contains linkages that are not split in blood plasma, namely amide group directed to the poly(ethylene glycol) chain and pH-sensitive imino group directed to AMB. The third type of our suggested conjugates includes α-methoxy-ω-(4-AMB-iminomethylphenylcar-

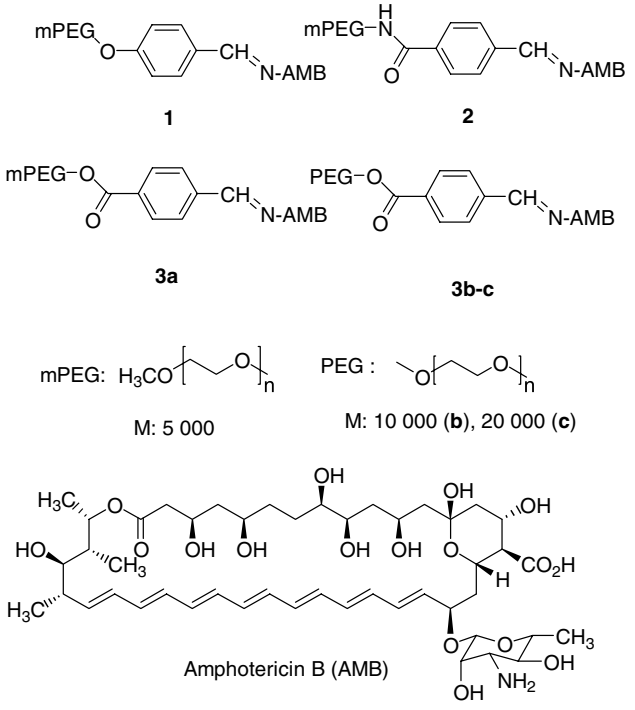


Figure 1. Structures of PEG amphotericin B conjugates **1**, **2**, **3a**–**c**.

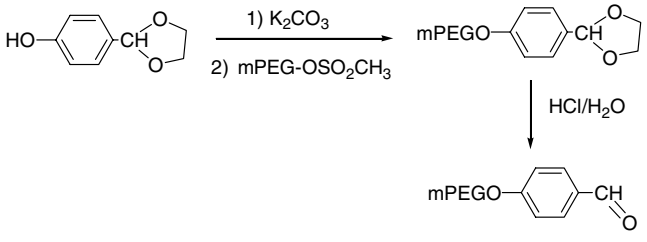
bonyloxy)poly(ethylene glycol) (**3a**) and α,ω-bis(4-AMB-iminomethylphenylcarbonyloxy)poly(ethylene glycols) (**3b–c**), which in contrast to the previous derivatives have ester linkage between PEG chain and 4-iminomethylbenzoic acid that can be split by blood hydrolases. Apart from synthesis and characterisation of the suggested conjugates, it was also the aim of this paper to study the behaviour of the conjugates in blood plasma and/or blood serum and in phosphate buffers (pH 7.4; 5.5). The paper also includes determination of toxicity of the synthesized conjugates on mouse model in vivo in order to enable evaluation of anticipated advantages of the above-mentioned conjugates.

2. Results and discussion

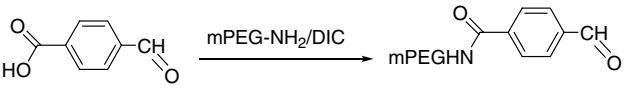
2.1. Synthesis of substituted poly(ethylene glycols) and conjugates

First, we synthesized substituted poly(ethylene glycols) having a terminal benzene ring with aldehyde group. The benzene ring is bound to the poly(ethylene glycol) chain at 4-position with respect to the aldehyde group by means of various bridges (ether, amide, ester) (Fig. 1). α-Methoxy-ω-(4-formylphenyloxy)poly(ethylene glycol) (M = 5000) was prepared by nucleophilic substitution of methanesulfonic group in α-methoxy-ω-methanesuphonylpoly(ethylene glycol)[23] with anion of 4-(1,3-dioxolan-2-yl)phenol.[24] The reaction was carried out in melt (65 °C) using a 15-fold excess of 4-(1,3-dioxolan-2-yl)phenol in the presence of potassium carbonate as basic catalyst (Scheme 1). Deblockade of aldehyde functional group was carried out by hydrolysis in 1 M hydrochloric acid. After extraction with dichloromethane, the crude product containing ca 5% free 4-hydroxybenzaldehyde was twice recrystallized from propan-2-ol; yield 68 % pure product.

α-Methoxy-ω-(4-formylphenylcarboxamino)poly(ethylene glycol) (M = 5000) was prepared by reaction of α-amino-ω-methoxypoly(ethylene glycol) with 4-formylbenzoic acid, which was pre-activated with diisopropylcarbodimide (DIC); yield of reaction 86% (Scheme 2). α-Methoxy-ω-(4-formylphenyloxy)poly(ethylene glycol) (M = 5000) and α,ω-bis(4-formylphenylcarbonyloxy)-poly(ethylene glycol)[25] (M = 10,000) or α,ω-bis(4-formylphenylcarbonyloxy)poly(ethylene glycol)[25] (M = 20,000) were prepared similarly in accordance with the literature.[25] The synthesized starting substituted PEGs were characterised by means of ^{1}H NMR. Comparison



Scheme 1. Synthesis of α-methoxy-ω-(4-formylphenyloxy)poly(ethylene glycol).



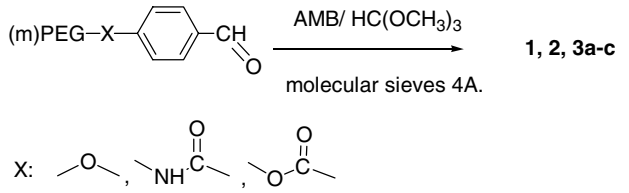
Scheme 2. Synthesis of α-methoxy-ω-(4-formylphenylcarboxamino)poly(ethylene glycol).

of integral intensities of hydrogen atoms of poly(ethylene glycol) chain and hydrogen atoms of aromatic system and/or of CH=O or CONH groups showed that the terminal functional group(s) (OSO_2CH_3, NH_2 or OH) of poly(ethylene glycol) chain were transformed practically quantitatively. Infrared spectroscopy revealed typical bands of the functional groups at 3225, 1693 cm^{-1}(CONH), 1701 cm^{-1} (CHO) and 1735 cm^{-1} for the ester functional group. The syntheses of conjugates **1**, **2**, **3a–c** made use of the reaction[25] of the aldehyde functional group present in the benzene ring of substituted PEG with the primary amino group of amphotericin B in the presence of activated molecular sieves 4A and trimethyl orthoformate giving imino linkage (Schiff's base) (Scheme 3).

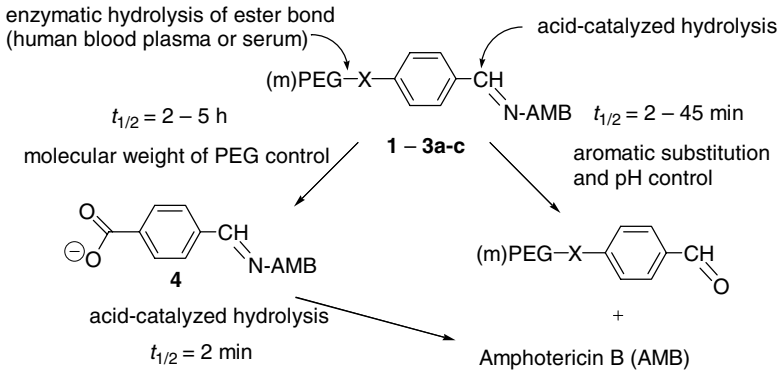
The yields of conjugates varied in the interval of 74–92%. The conjugates were characterised by means of infrared spectroscopy; the spectra exhibited the following bands: 1735 cm^{-1} (ester), 1719 cm^{-1} (lactone), 1638 cm^{-1} (imine). Using HPLC and GPC, we estimated the purity of conjugates; the content of free AMB was <1 mol%. Moreover, UV/vis spectroscopy showed that the AMB content in conjugates **1**, **2**, **3a** corresponds to the molar ratio of 1:1 (AMB/PEG) and in conjugates **3b–c** to 2:1 (AMB/PEG). The UV/vis spectra of all the conjugates measured in water exhibit identical typical maxima corresponding to the polyene system of AMB (346, 367, 386, 409 nm). In an independent way, we also synthesized 4-carboxybenzylideniminoamphotericin B (**4**), that is, the pro-drug formed by splitting of ester linkage of conjugates **3** by hydrolases of blood plasma and/or blood serum (Scheme 4). 4-Carboxybenzylideniminoamphotericin B (**4**) was prepared by reaction of AMB with 4-formylbenzoic acid in dimethylsulfoxide at similar conditions to those used for conjugates **1–3**; composition of the product was verified by means of ESI-MS, showing the molecular peak $[M+H]^+$ m/z 1057.2. IR spectrum showed typical bands of lactone at 1719 cm^{-1} and imine at 1638 cm^{-1}.

2.2. Study of hydrolytic behaviour of conjugates in phosphate buffers, human blood plasma and human blood serum

A key part of the research consisted in investigation of behaviour of synthesized conjugates in various media



Scheme 3. Synthesis of PEG amphotericin B conjugates **1**, **2**, **3a–c**.



Scheme 4. Survey of controlled release of AMB from conjugates.

imitating conditions that control the stability of solutions of the conjugates and their behaviour in organism. First, we studied stability of all the conjugates **1**–**3a**–**c** in phosphate buffers and then in the medium of human blood plasma and/or human blood serum. On the basis of monitoring of time changes in UV/vis spectra, we found out that in solutions of phosphate buffer at the value of pH 7.4, (7×10^{-2} M, 37 °C) all types of conjugates are relatively stable, very slow hydrolysis being only observed in the case of imino linkage: less than 5 mol% AMB is split off from the conjugates during 24 h. All the conjugates were constructed in such a way as to release AMB in goal-directed manner only at spots with pathologically lowered pH value. The model medium imitating the afflicted tissue (characterised by the lowered pH value) was a phosphate buffer solution[26] (pH 5.5; 7×10^{-2} M). During the hydrolysis of conjugate **3a**, we observed absorbance decrease at 346 nm (corresponding to concentration drop of conjugate) and absorbance increase at 367, 386 and 409 nm. The absorption band at 409 nm is specific[9] for monomeric AMB, and its time increase corresponds to the rate of its formation (Fig. 2).

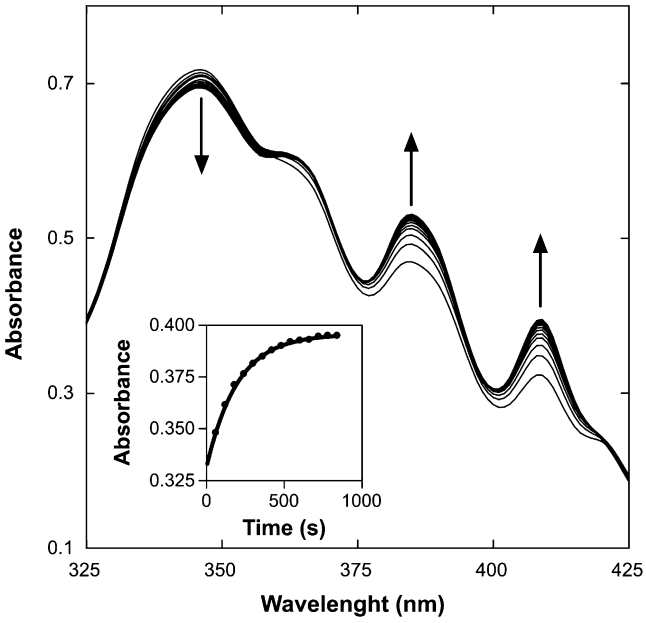


Figure 2. Time changes of spectrum of conjugate **3a** in phosphate buffer (pH 5.5; 7×10^{-2} M, 37 °C). The inset represents the absorbance–time dependence during hydrolysis of imino linkage in **3a** at 409 nm ($t_{1/2}$ = (124 ± 8) s).

Similar spectral record was also obtained during monitoring of hydrolysis of the other conjugates **1**–**3b**–**c**. Conjugates **2**, **3a**–**c** in phosphate buffer medium (pH 5.5; 7×10^{-2} M) undergo a relatively fast splitting of imino linkage giving free AMB, the half-lives of individual conjugates being as follows: **2**: $t_{1/2}$ = (1.8 ± 0.2) min; **3a**: $t_{1/2}$ = (2.1 ± 0.1) min. We also found out that the acid-catalysed hydrolyses of conjugates **3b**: $t_{1/2}$ = (2.4 ± 0.2) min and **3c**: $t_{1/2}$ = (2.2 ± 0.4) min proceed at comparable rates, which means that the molecular weight of poly(ethylene glycol) carrier has virtually no effect on these rates. Conjugate **1** with different substitution in benzene ring exhibits a rate decrease of acid-catalysed hydrolysis of imino linkage ($t_{1/2}$ = (45 ± 1) min). The rate difference of hydrolyses of conjugate **1** as compared with conjugates **2**, **3a**–**c** corresponds to the effect of substituent at 4-position of benzene ring upon the acid-catalysed hydrolysis of imines.[27] Conjugates **1** and **2** are hydrolysed at measurable rates only in the imine moiety, the difference between rates of their acid-catalysed hydrolyses being approximately one order of magnitude. Conjugates **3a**–**c**, in contrast to conjugates **1** and **2**, contain two types of hydrolysable linkages, namely ester and imine linkage (Fig. 1). If the same conditions (pH 5.5, 7×10^{-2} M) were adopted when monitoring the stability of ester of methoxy-ω-(4-formylphenyloxy)poly(ethylene glycol), it was found that the hydrolysis of ester linkage proceeds immeasurably slowly in this case, which confirmed the fact that in the phosphate buffer medium the splitting of conjugates exclusively concerns their imino linkage and gives AMB.

Furthermore, using HPLC, we monitored the stability and/or decomposition of all the conjugates in media of human blood plasma or human blood serum. Results of the kinetic measurements indicate that conjugates **1** and **2** are relatively stable in both blood serum and blood plasma (pH 7.4), like in phosphate buffer (pH 7.4). Conjugates **3a**–**c** in blood serum and/or blood plasma undergo enzymatic hydrolysis of their ester linkage by action of blood hydrolases, which is indicated by the decrease in concentration of the starting conjugate.

The kinetic dependence shown in Figure 3 indicates that the results of kinetic measurements of hydrolysis of conjugate **3a** in blood plasma and in blood serum were mutually comparable within experimental error. However, the determination of concentration of the conjugates in blood plasma was loaded with larger error and worse reproducibility (Fig. 3). From the standpoint of analysis, it appeared more appropriate to monitor the concentration decrease of starting conjugates than to follow the AMB increases, since AMB interacts with the present lipoproteins,[28] which results in concentration changes of free AMB that are not connected with the kinetic course of decrease in the starting conjugates.

The kinetic dependence shown in Figure 3 also allows calculation of half-life (2.1 ± 0.1 h) of enzymatic hydrolysis of conjugate **3a** in human blood serum. The half-life found by us is comparable with literature data,[10,12] reporting splitting of ester linkages in poly(ethylene glycol) (M = 5000) in rat blood serum with the half-life values of 1–3 h. However, in our case the splitting in blood plasma or serum produces the relatively stable pro-drug **4**, that is, AMB with bound 4-formylbenzoic acid, which can subsequently release AMB only at the conditions of lowered pH value, that is, at the spot of damaged tissue. Our kinetic measurements confirmed (phosphate buffer, pH 5.5, 7×10^{-2} M, 37 °C) that the half-life of acid-catalysed hydrolysis of pro-drug **4** ($t_{1/2}$ = (2.15 ± 0.01) min) is comparable with the half-lives of acid-catalysed hydrolyses of conjugates **3a**–**c** (Scheme 4). Moreover, we found out that the half-lives of enzymatic hydrolyses of ester linkage by blood hydrolases in plasma and/or serum depend on molecular weights of individual conjugates: **3a**: $t_{1/2}$ = (2.1 ± 0.2) h; **3b**: $t_{1/2}$ = (3.0 ± 0.3) h; **3b**: $t_{1/2}$ = (5 ± 0.2) h; (PEG: M = 5000, 10,000, 20,000) (Figs. 3 and 4).

From these results it is obvious that molecular weight of carrier controls the rate of enzymatic release of pro-drug

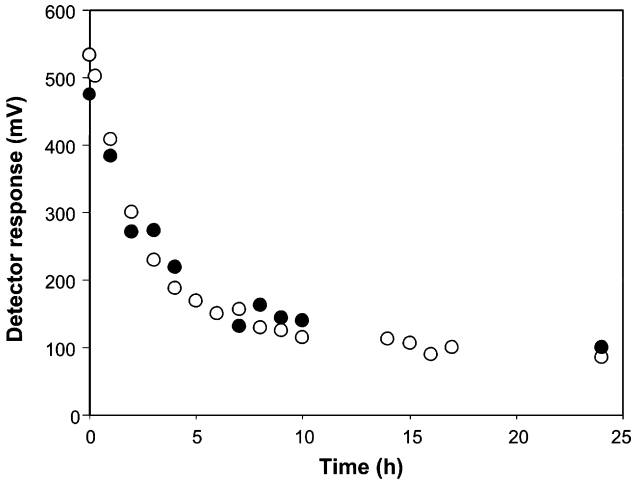


Figure 3. Concentration–time dependence of conjugate **3a** in human blood plasma (●) (pH 7.4) and in human blood serum (○) (pH 7.4) at 37 °C. The conjugate decrease represents the enzymatic hydrolysis of ester linkage by blood hydrolases ($t_{1/2}$ = 2 h).

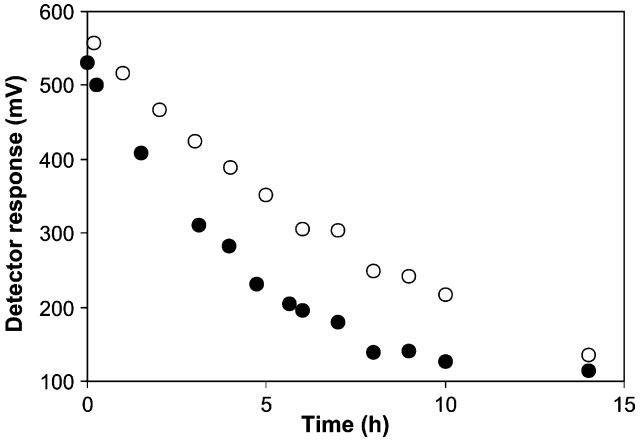


Figure 4. Concentration–time dependence of conjugate **3b** (●) ($t_{1/2}$ = (3.0 ± 0.3) h) and **3c** (○) ($t_{1/2}$ = (5.0 ± 0.3) h) in human blood serum (pH 7.4) at 37 °C.

4 (Scheme 4). Application of PEGs with higher molecular weight results in retardation of release of pro-drug **4** and can be favourable from the point of view of prolongation of the drug effect, not only for the reason of generally known[12] slower excretion of PEGs with higher molecular weight by kidneys.

2.3. Study of toxicity of conjugates 3a and 3b

Acute toxicity was tested for AMB as a control and for two conjugates (**3a** and **3b**).

Mice (NMRI, weight 25–30 g) were purchased from Biotest Ltd. Konarovice, Czech Republic. For each compound tested, the mice were divided into 6 groups of 10 males, and the compound was administered intravenously via the tail vein at doses carefully determined after a series of preliminary trials. Most deaths occurred 5 min to 24 h after iv administration of the tested compound. When compared to control, PEGylation increased LD_{50} 11 times in the case of **3a** conjugate and about five times in the case of **3b** conjugate (see Table 1).

Both the toxicities of conjugates determined in vivo and the results of hydrolytic studies in vitro agree with the presumption that our suggested system of two-stage independent release of AMB minimizes the level of free AMB and its complexes with LDH in blood circulation and thereby reduces the risk of toxic damage to the patient.[14] In conclusion, it can be stated that the conjugates prepared by us represent promising candidates for new intravenous forms of AMB suitable for further tests on animal model.

Table 1. Effect of amphotericin PEGylation on acute iv toxicity in mice

Compound	Control (AMB)	**3a**	**3b**
LD_{50} mouse iv (mg/kg)	3.7	40.5	20.7
95% confidence intervals (mg/kg)	3.2–4.2	38.1–43.1	15.4–32.3

3. Experimental

3.1. Materials

Unless otherwise stated, all the poly(ethylene glycols), amphotericin B, other chemicals and solvents were obtained from Fluka or Aldrich and used without further purification.

3.2. Analytical methods

3.2.1. The ^{1}H NMR spectra were measured in deuteriochloroform ($CDCl_3$), on a Bruker AMX 360 apparatus and a Bruker 500 Avance apparatus.

3.2.2. Positive-ion electron-spray ionisation (ESI) mass spectra were measured on an Esquire 3000 ion trap analyzer (Bruker Daltonics, Bremen, Germany) in the *m*/*z* range of 50–1500.

3.2.3. The IR spectra were measured using Nicolet Impact 400 spectrometer. The samples (0.2 mg) were measured in KBr pellets (200 mg).

3.2.4. Gel permeation chromatography measurements of the prepared conjugates **1**–**3** were performed with HEMA–BIO columns (hydrophilic modified HEMA–Gel, particle size 10 μm, porosity 40/100/300/1000) at room temperature using 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).

3.2.5. Study of acid-catalysed hydrolysis of imine linkage in compounds **1**–**3a**–**c**, **4** in phosphate buffers. The kinetic measurements were performed on an HP UV/vis 8453 Diode Array apparatus in 1 cm closable quartz cell kept at a temperature of 37 °C. The cell was charged with 2 ml phosphate buffer (pH 7.4, 7×10^{-2} M or pH 5.5, 7×10^{-2} M), and after reaching the above-mentioned temperature, 10-20 μl methanolic solutions of conjugates **1**–**3a**–**c** or pro-drug **4** were injected to make the final concentration of substrate ca 5×10^{-5} M. The measured time dependences of absorbance (A) were used to calculate the pseudo-first-order observed rate constants (k_{obs} (s^{-1})) using the equation: $k_{obs}t = \ln \Delta A + \text{const.}$, where $\Delta A = (A_\infty - A_t)$ or $(A_t - A_\infty)$.

3.2.6. Study of enzymatic hydrolysis of ester linkage of conjugates **3a**–**b** in human blood plasma and human blood serum by means of HPLC. A micro-test-tube was charged with 500 μl plasma or serum sample (obtained from healthy donors of plasma at transfusion station) and 500 μl aqueous solution of conjugates **3a**–**c** to make the final conjugate concentration of 1×10^{-1}M. The samples were incubated at 37 °C. After the incubation, they were gradually (at definite time intervals: 0 (immediately), 1, 2 to 10 h and after 24 h) stabilized with 100 μl of 4% $ZnSO_4 \cdot 7H_2O$. The mixture was shaken for 1 min and then centrifuged at 14000 rpm for 3 min. The supernatant (20 μl) was then introduced on a LiChroCART® 125 × 4 mm column packed with LiChrospher® 100 RP-18e 5 μm (Merck) and eluted with mobile phase of acetonitrile with 20 mM chelaton II. The measured time dependences of the analyser responses (mV) were used to calculate the pseudo-first-order observed rate constants (k_{obs} (h^{-1})) similarly as in above cases.

3.3. Synthesis of α-methoxy-ω-(4-formylphenyloxy)poly(ethylene glycol)

A melt of 5 g (1 mmol) α-methoxy-ω-methanesuphonylpoly(ethylene glycol),[23] 2.5 g (15 mmol) 4-(1,3-dioxolan-2-yl)phenol[25] and 2 g (15 mmol) potassium carbonate was stirred and heated under argon atmosphere at 65 °C for 48 h. After cooling, the reaction mixture was dissolved in 200 ml dichloromethane, filtered with Celite and evaporated on a water bath. The dry evaporation residue was dissolved in 150 ml of 1 M hydrochloric acid and refluxed for 4 h. After cooling, the solution was saturated with sodium chloride and extracted with 5 × 60 ml dichloromethane. The combined extracts were dried and evaporated, and the residue was twice recrystallized from propan-2-ol. Yield 3.4 g product (68%). ^{1}H NMR ($CDCl_3$, ppm) δ: 3.36 (s; 3H, CH_3-O); 3.52-4.27 (m; 460H, $(CH_2–CH_2–O)_{115}$); 7.17 (d; J 7.7 Hz, 2H-arom); 7.97 (d; J 7.7 Hz, 2H-arom); 9.94 (s; 1H, CH═O). IR (KBr) ν: 3439, 2945, 2887, 2741, 1701, 1467, 1360, 1343, 1280, 1201, 1149, 1110, 1061, 963, 843, 531 cm^{-1}.

3.4. Synthesis of α-methoxy-ω-(4-formylphenylcarboxamino)poly(ethylene glycol)

α-Amino-ω-methoxypoly(ethylene glycol) (5 g; 1 mmol),[21] 0.6 g (4 mmol) 4-formylbenzoic acid and 0.05 g (0.4 mmol) 4-dimethylaminopyridine were dissolved in 50 ml dry dichloromethane, and the obtained solution was cooled to 0 °C. The reaction mixture was treated with cold solution of 0.5 g (4 mmol) diisopropylcarbodiimide in 10 ml dichloromethane added drop by drop during 10 min. Then the reaction mixture was stirred at 0 °C for another 4 h, whereupon it was slowly warmed to room temperature. After 24 h, diisopropylurea was filtered off and the filtrate was evaporated; the residue was recrystallized from propan-2-ol. Yield 4.3 g product (86%). ^{1}H NMR ($CDCl_3$, ppm) δ: 3.36 (s; 3H, CH_3-O); 3.52–4.27 (m; 460H, $(CH_2$-CH_2-$O)_{115}$); 4.51 (m; 2H, CH_2–NH–CO); 6.48 (m; H, NH–CO); 7.96 (d; J 7.8 Hz, 2H-arom); 8.22 (d; J 7.8 Hz, 2H-arom); 10.11 (s; 1H, CH═O). IR (KBr) ν: 3439, 3225, 2945, 2887, 2741, 1701, 1693, 1467, 1360, 1343, 1280, 1201, 1149, 1110, 1061, 963, 843, 531 cm^{-1}.

3.5. General procedure of synthesis of conjugates 1, 2, 3a–c

α-Methoxy-ω-(4-formylphenyloxy)poly(ethylene glycol) (0.1 mmol; M = 5000), α-methoxy-ω-(4-formylphenylcarboxamino)poly(ethylene glycol) (0.1 mmol; M = 5000), α-methoxy-4-(formylphenylcarbonyloxy)poly(ethylene glycol)[25] (0.1 mmol; M = 5000), α,ω-bis(4-formylphenylcarbonyloxy)poly(ethylene glycol)[25] (0.1 mmol; M = 10,000), or α,ω-bis(4-formylphenylcarbonyloxy)poly(ethylene glycol)[25] (0.1 mmol; M = 20,000) was dissolved in 5–10 ml trimethyl orthoformate, and the solution

formed was treated with an equivalent amount (0.1 or 0.2 mmol) of saturated solution of amphotericin B in dimethylformamide with addition of 0.2 g activated molecular sieves 4A. The reaction mixture was stirred under argon atmosphere at room temperature for several days with exclusion of light. The end of reaction was established for the individual cases by means of GPC. Finally, the reaction mixture was filtered, and the filtrate was evaporated in vacuum to remove trimethyl orthoformate. The distillation residue was poured in 250 ml dry diethyl ether, and the precipitated product was collected by filtration. The conjugates prepared were dissolved in dichloromethane and reprecipitated with diethyl ether.

3.5.1. α-Methoxy-ω-(4-AMB-iminomethylphenyloxy)poly-(ethylene glycol) (1). Yield 450 mg (74%). IR (KBr) *v*: 3428, 2945, 2885, 2741, 2695, 1719, 1637, 1467, 1360, 1343, 1280, 1201, 1149, 1110, 1061, 963, 843 cm^{-1}.

3.5.2. α-Methoxy-ω-(4-AMB- iminomethylphenylcarboxamino)poly(ethylene glycol) (2). Yield 500 mg (54%). IR (KBr) *v*: 3439, 3225, 2945, 2887, 2741, 1719, 1693, 1638, 1467, 1360, 1343, 1280, 1201, 1149, 1110, 1061, 963, 843, 531 cm^{-1}.

3.5.3. α-Methoxy-ω-(4-AMB- iminomethylphenylcarbonyloxy)poly(ethylene glycol) (3a). Yield 510 mg (86%). IR (KBr) *v*: 3439, 2945, 2887, 2741, 1735, 1719, 1638, 1467, 1360, 1343, 1280, 1201, 1149, 1110, 1061, 963, 843, 531 cm^{-1}.

3.5.4. α,ω-Bis(4-AMB- iminomethylphenylcarbonyloxy)-poly(ethylene glycol) (3b). Yield 980 mg (83%). IR (KBr) *v*: 3439, 2945, 2887, 2741, 1735, 1719, 1693, 1638, 1467, 1360, 1343, 1280, 1201, 1149, 1110, 1061, 963, 843, 531 cm^{-1}.

3.5.5. α,ω-Bis(4-AMB- iminomethylphenylcarbonyloxy)-poly(ethylene glycol) (3c). Yield 2 g (92%). IR (KBr) *v*: 3439, 2945, 2887, 2741, 1735, 1719, 1693, 1638, 1467, 1360, 1343, 1280, 1201, 1149, 1110, 1061, 963, 843, 531 cm^{-1}.

3.6. Synthesis of 4-carboxybenzylideniminoamphotericin B (4)

Amphotericin B (93 mg; 0.1 mmol) and 15 mg (0.1 mmol) 4-formylbenzoic acid were dissolved in 2 ml dry dimethylsulfoxide and 3 ml trimethyl orthoformate with addition of 0.1 g activated molecular sieves 4A. After 48 h (25 °C), the reaction mixture was filtered, and the filtrate was distilled in vacuum at the temperature of 30 °C to remove the trimethyl orthoformate. After addition of 400 ml dry diethyl ether, the obtained solid was collected by filtration, washed and dried in vacuum desiccator. Yield 90 mg (84%). IR (KBr) *v*: 3439, 3097, 2838, 2679, 2560, 2345, 1719, 1638, 1610, 1575, 1560, 1505, 1430, 1393, 1293, 1125, 934, 885, 790, 762, 686, 532, 461 cm^{-1}; ESI-MS (M = 1056.2): $[M+H]^+$ *m/z* 1057.2.

3.7. Acute toxicity

Acute toxicity was tested for amphotericin B (Bristol-Myers Squibb) as a control and for two PEGylated derivatives (**3a**, **3b**). For each compound tested, the mice were divided into 6 groups of 10 males, and the compound was administered intravenously via the tail vein at doses carefully determined after a series of preliminary trials. Toxic signs and mortality were monitored for 14 days after administration. LD_{50} values and the corresponding confidence limits were determined by non-linear regression using GraphPad Prism (version 4; GraphPad software, Inc., San Diego CA, USA).

4. Conclusion

In contrast to the previous idea of covalent-noncovalent conjugates and conjugates based on 1,6-benzyl elimination, which release free AMB in blood circulation, we have suggested such conjugates that selectively release AMB only at the site of expected operation of fungal pathogen, that is, tissue with lowered pH value. The goal-directed release can only take place by acid-catalysed hydrolysis of imino linkage, either in the polymeric conjugate or in the liberated pro-drug **4**, which is split off enzymatically from the carrier by action of blood hydrolases. The molecular weight of carrier controls the rate of enzymatic liberation of pro-drug **4**. From the LD_{50} values determined *in vivo* on mouse model for conjugates **3b** and **3a** it follows that they are ca 6–11 times less toxic than free AMB. The above-described system of release of AMB from conjugates **3** minimizes the level of free AMB and hence also that of its complexes with LDH in blood circulation, which results in lowered toxicity of the conjugates as compared with free AMB. Our suggested conjugates **3** of amphotericin B with poly(ethylene glycols) represent a new system of two-stage independent release of AMB from polymeric carrier. However, its appropriateness for practical applications will need further studies on animal models and their critical evaluation.

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