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Synthesis and Characterisation of a New Amphotericin B–Methoxypoly(ethylene Glycol) Conjugate

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Abstract—The reaction of methoxypoly(ethylene glycol)-4-nitrophenyl carbonate with amphotericin B has been used to prepare a new conjugate of amphotericin B (mPEG-AmB). A preliminary screening of in vitro antifungal activity has suggested that mPEG-AmB posseses a similar effect and a similar spectrum of activity as the conventional amphotericin B formulated with sodium desoxycholate. © 2001 Elsevier Science Ltd. All rights reserved.

Amphotericin B (AmB) is a polyene macrocyclic membrane-active antifungal antibiotic produced by *Streptomyces nodosus* M4575, which for as long as 40 years has been a salvaging medical drug in the treatment of systemic fungal diseases.[1] The drug is very efficient, having predominantly a fungicidal effect, and is particularly applied to immunosuppressed patients, including solid organ and bone marrow transplant (BMT)[2] recipients, acquired immunodeficiency syndrome (AIDS) persons[3] and patients with solid tumours and haematological malignancies.[4] However, the clinical application of AmB is hampered on one hand by its poor solubility in water and the lack of peroral drug formulation for systemic use and on the other hand by increased occurrence of adverse effects, especially nephrotoxicity.[5] Conjugates of AmB and its liposomal form have been prepared in order to increase the therapeutic index of AmB. These new drug formulations improve the pharmacological profile of AmB in terms of increased water solubility and decreased nephrotoxicity.[6] The conjugates used so far are non-covalent lipid complexes of the type of amphotericin B Lipid Complex (ABLC),[7] colloid systems composed of biodegradable phospholipid matrices [e.g., dimyristoylphosphatidyl choline (DMPC)[7]] of modified poly(ethylene glycols) [e.g., distearoyl-*N*-[(monomethoxy)poly(ethylene glycol)succinyl]phosphatidylethanolamine (DSPE-PEG)[8]] with AmB. Such forms of AmB usually possess lower in vitro antifungal activity as compared with the desoxycholate AmB, which is, however, outbalanced by better pharmacokinetic properties and possibility of higher dosages of these preparations.

In the literature, we have not found any case describing AmB bound by covalent bond to a carrier. At present, systems are being developed in which the drug is gradually released from a carrier in several phases in such a way that the individual bond types are split step by step, which ensures a relatively constant drug level in the organism. A drawback of such systems lies in the necessity of their multi-step synthesis.[9]

The aim of our work was to prepare, by a simple method, AmB bound to a suitable carrier in such a way that one part of AmB would be bound by covalent bonds and the other by non-covalent interactions.[7] During its application, the 'non-covalent' AmB would be released first.[10] The covalent bond should be of such nature that 'covalent' AmB would be released by action of hydrolytic enzymes.[11] As a carrier, we have chosen methoxypoly(ethylene glycol) (mPEG, $M_r = 5000$ g/mol) which fits two basic requirements: first, according to the

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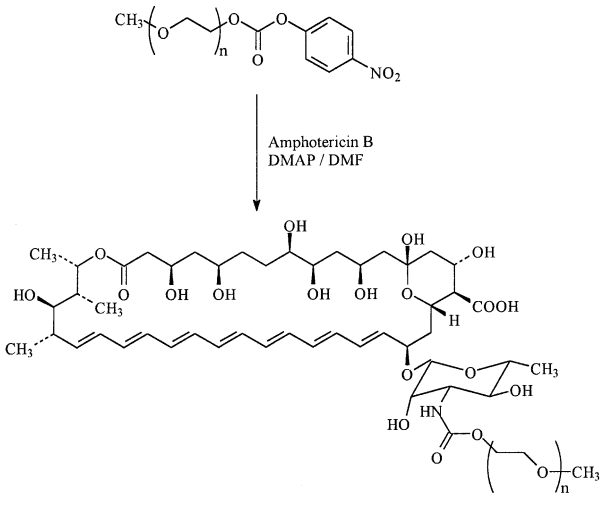
American Food and Drug Administration (FDA), mPEG is practically non-toxic, being easily eliminated from the organism through the kidneys,[12] and secondly, its hydrophilic character ensures solubilisation of hydrophobic molecules,[13] for example, it forms well-stabilised colloid systems.[14] The PEG derived polyene acts in vivo in its own right, either forming transmembrane pores,[15] or as a small molecule/ion shuttle across the membrane and the mode of action of the drug could thus be being altered.[16] The summarising paper[17] describes a number of possibilities of attachment of modified mPEG to an amino group. We have chosen the reaction of methoxypoly(ethylene glycol)-4-nitrophenyl carbonate[18] with the amino group of mycosamine, which forms a part of the AmB molecule (Scheme 1). The reaction is nucleophile-catalysed by dimethylamino-pyridine (DMAP). It proceeds by a known mechanism[19] with the 4-nitrophenoxide leaving group, and produces a relatively stable carbamate bond. At the reaction phase when one half of the 4-nitrophenol bound in methoxypoly(ethylene glycol)-4-nitrophenyl carbonate has been released, a more reactive substrate with an amino group [tris(hydroxymethyl)aminomethane] is added to become attached to mPEG. The hydroxyl groups of tris(hydroxymethyl)aminomethane attached to mPEG can thus bind another portion of AmB by means of intermolecular hydrogen bonds. The reaction was carried out in dimethylformamide in the following way.

Methoxypoly(ethylene glycol)-4-nitrophenyl carbonate (1.033 g, 0.2 mmol) and DMAP (25 mg, 0.2 mmol) were dissolved in dry dimethylformamide (3 mL), and the solution was treated with a suspension of AmB (210 mg, 0.22 mmol) in dimethylformamide (2 mL), which was added with stirring under inert atmosphere (argon) and with exclusion of light. The reaction mixture was thereafter stirred at room temperature for 20 h, during which period AmB gradually reacted and dissolved. The mixture was then treated with tris(hydroxymethyl)aminomethane (13 mg, 0.11 mmol) in dimethylformamide (0.5 mL). The reaction course was monitored by ^{1}H NMR spectroscopy (360.14 MHz, $CDCl_3$): gradual disappearance of the signal in the region of 7.39–8.26 ppm (the aromatic protons of AA′BB′ system of 4-nitrophenoxide group). The spectrum in the region of 0–7 ppm is unclear with broadened signals. After the reaction, the mixture was centrifuged (3000 rpm, 30 min), and the clear solution was poured in the cold ether (100 mL); the precipitated product was collected by filtration on a sintered glass filter under argon and washed with ether (3×40 mL). The raw product was dissolved in methanol (5 mL) and reprecipitated with ether (100 mL), collected by filtration and dried in a vacuum desiccator to give 1.1 g of a product readily soluble in water, methanol or chloroform (the solutions exhibit slight opalescence).



Scheme 1.

The UV–vis spectrum of an aqueous solution of the product shows the following maxima: 410, 386, 367 and 346 nm, and in the region mentioned it is practically identical with the spectrum of AmB itself. Quantitative analysis, that is comparison of the absorbance magnitudes of AmB and AmB-mPEG conjugate, showed that the molar ratio of AmB and methoxypoly(ethylene glycol) in the conjugate is 1:1 (i.e., 15.5% by wt AmB). Moreover, we determined the optical activity: $[\alpha]^{20}$ −49.6° (*c* 0.2, water). The IR spectrum[20] exhibits the typical band of valence vibration of C=O group in a carbamate bond, namely at 1710 cm^{-1}. Low molar mass polymers are usually well suited for mass spectrometry. Therefore, time of flight mass spectrometry (MALDI-TOF) was applied. However, the conjugate of mPEG-AmB did not give reasonable results using dithranol, HBA and NA matrices, not even after matrix modification with LiCl. The only detectable signal was due to the presence of unconnected methoxypoly(ethylene glycol) in the sample. The GPC analysis[21] showed 8.5% by weight free AmB, and the conjugate signal with the anticipated molecular mass of 5950 g/mol was overlapped by the signal of poly(ethylene glycol) itself. The UV detector could prove that the remaining AmB is bound to mPEG by a covalent bond (mPEG alone does not absorb in the UV region). Given the above facts, we can conclude that 45 mol% of overall AmB content is covalently bound in the conjugate and the rest of AmB is attached through non-covalent interactions. Thus, the AmB can be released in two steps: first the AmB held by non-covalent interactions and then that bound by covalent bonds, which means more or less fluent releasing of AmB analogous to a two-step cleavage of chemical bonds.[9]

The characterisation of mPEG-AmB involved a preliminary evaluation of its in vitro antifungal activity against 14 strains of human pathogenic fungi, using the broth microdilution test.[22] The values of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) after 48-h incubation varied within the limits from 2 to 32 mg/L (Table 1). A low increase in MIC after 48 h, as compared with MIC after 24 h, supports the idea that mPEG-AmB has a rather fungicidal nature, being particularly active against yeasts (Table 1). These results are comparable with the efficacy and spectrum of activity of conventional AmB. In addition, considering that the weight/volume concentrations were compared, in fact, in vitro antifungal

Table 1. In vitro antifungal activity of mPEG-AmB[a]

Fungal strain/Incubation time (h)		mPEG-AmB		AmB
		MIC (mg/L)	MFC (mg/L)	MIC (mg/L)
Candida albicans ATCC 44859	48	2	2	2
Candida albicans ATCC 90028	48	2	8	2
Candida krusei ATCC 6258	48	8	16	4
Candida krusei E28	48	8	32	4
Candida glabrata 20/I	48	4	16	2
Candida glabrata ATCC 90030	48	4	4	2
Candida parapsilosis ATCC 22019	48	2	4	2
Candida lusitaniae 2446/I	48	2	32	2
Candida tropicalis 156	48	4	4	4
Trichosporon beigelii 1188	48	2	32	0.25
Aspergillus fumigatus 231	48	2	8	0.5
Absidia corymbifera 272	48	16	32	4
Trichophyton mentagrophytes 445	120	8	8	1
Microsporum gypseum 27339	120	32	32	2

[a]Broth microdiluton test, M27-A.[19]

activity of the mPEG-AmB is about 10 times higher than activity of conventional AmB. The contribution of covalently bound AmB (45% mol of total AmB) to the in vitro antifungal activity and a possible effect of conjugated mPEG on the mechanism of action remain to be resolved.

Recently, some experiments showed that long-circulating liposome formulations of AmB prepared by the incorporation of polyethylene glycol-derivatised phospholipids have a favourable effect on the pharmacokinetics, in particular on liposome tissue distribution and the encapsulation rate of AmB.[23,24] The pharmacokinetics of covalently conjugated mPEG-AmB will have to be studied on the in vivo animal models to answer the question whether or not the pharmacokinetic properties are similar to those of the above-mentioned liposome formulations of AmB. Although the pharmacodynamics of mPEG-AmB are not yet known, the results of the in vitro susceptibility tests together with the physicochemical properties of the conjugate suggest that mPEG-AmB is a promising candidate for further research and development, including more detailed evaluation of its antifungal activity both in vitro and in vivo.

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References and Notes

1. Holz, R. W. In *Antibiotics;* Hahn, F. E., Ed.; Springer: 1979; Vol. 5, p 313.
2. Drakos, P. E.; Nagler, O.; Or, E.; Slavin, S. *Bone Marrow Transplant*. **1993**, *12*, 203.
3. Saag, M. S.; Powderly, W. G.; Cloud, G. A.; Brune, K. H.; Sabra, R. *New Engl. J. Med.* **1992**, *326*, 83.
4. Cole, S.; Zawin, M.; Lundberg, B.; Hoffman, J.; Bailey, L.; Ernestoff, M. *Am. J. Med.* **1987**, *82*, 662.
5. Heidemann, H. T.; Gerkens, J. F.; Spickard, W. A.; Jackson, E. K.; Workman, R. J. *Am. J. Med.* **1983**, *75*, 476.
6. Janoff, A. S.; Boni, L. T.; Poescu, M. C.; Minchey, S. R.; Cullis, P. R.; Madden, T. D.; Taraschi, T. F.; Gruner, S. M.; Shyamsunder, E. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 61227.
7. Janoff, A. S.; Perkins, W. R.; Saleton, S. L.; Swenson, C. E. *J. Liposome Res.* **1993**, *3*, 451.
8. Moribe, K.; Mararuyama, K.; Iwatsuru, M. *Int. J. Pharm.* **2000**, *201*, 37.
9. Greenwald, R.B.; Pendri, A.; Choe, Y. H.; PTC Int. Appl. WO 9930727, 1999; *Chem. Abstr.* **1999**, *131*, 59098.
10. Pahls, S.; Schaffner, A. *J. Infect. Dis.* **1994**, *169*, 1056.
11. Zalipsky, S. *Adv. Drug Delivery Rev.* **1995**, *16*, 157.
12. Herold, D. A.; Keil, K.; Bruns, D. E. *Biochem. Pharmacol.* **1989**, *38*, 73.
13. Harris, J. M. In *Poly(Ethylene Glycol) Chemistry*; Harris, J. M., Ed.; Plenum: New York and London, 1992; p 1.
14. Sedlák, M.; Antonietti, M.; Cölfen, H. *Macromol. Chem. Phys.* **1998**, *199*, 247.
15. Stadler, E.; Dedek, P.; Yamashita, K.; Regen, S. L. *J. Am. Chem. Soc.* **1994**, *116*, 6677.
16. Pregel, M. J.; Jullien, L.; Lehn, J. M. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 1637.
17. Zalipsky, S. *Bioconjugate Chem.* **1995**, *6*, 150.
18. Veronese, F. M.; Largajolli, R.; Boccu, E.; Benassi, C. A.; Schiavon, O. *Appl. Biochem. Biotechnol.* **1985**, *11*, 141.
19. Scriven, K. F. V. *Chem. Soc. Rev.* **1983**, *12*, 129.
20. IR spectra were measured using an FTS 6000 spectrometer (BIO-RAD Laboratories Europe, Krefeld, Germany) equipped with Golden Gate Single Reflection Diamond ART (SPCAC). The samples were measured in solid state.
21. Gel permeation chromatography measurement of the prepared conjugate was performed with HEMA-BIO columns (hydrophilic modified HEMA-Gel, particle size 10 μm porosity 40/100/300/1000) at room temperature using an RI detector (Shodex-R371, Thermo-Separatio products TSP, Germany) and UV–vis detector (260 nm, UV-1000, TSP, Germany). Acetic acid buffer (pH = 4.5) was used as the eluent at the flow rate of 1.0 mL/min. The columns were calibrated with a series of standard PEGs with varying molecular weights (PSS, Polymer Standard Service Gmbh, Mainz, Germany).
22. National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of yeasts: Approved Standard, NCCLS document M27-A, 771 E. Lancaster Avenue, Villanova, PA 19085, USA, 1997.
23. Moribe, K.; Tanaka, E.; Maruyama, K.; Iwatsuru, M. *Pharm. Res.* **1998**, *15*, 1737.
24. Otsubo, T.; Maruyama, K.; Maesaki, S., et al. *Antimicrob. Agents Chemother.* **1998**, *42*, 40.