POLYMERIC CONJUGATES OF 9-[2-(PHOSPHONOMETHOXY)ETHYL]PURINE WITH POTENTIAL ANTIVIRAL AND CYTOSTATIC ACTIVITY

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The other authors M. P., A. B., V. Š. and K. U. wish to dedicate this paper to Professor Antonín Holý on the occasion of his 70th birthday.

Syntheses and characterization of polymer conjugates of 9-[2-(phosphonomethoxy)ethyl] (PME) derivatives of adenine (PMEA), 2,6-diaminopurine (PMEDAP) and guanine (PMEG) are described. The phosphonate group of these acyclic nucleotide analogues was activated by reaction with triphenylphosphine and di(2-pyridyl) disulfide (TPP–PDS). The activated phosphonate reacted with a random copolymer containing *N*-(2-hydroxypropyl)methacrylamide (HPMA) and *N*-(3-methacrylamidopropanoyl)ethane-1,2-diamine (Ma-AP-ED) units. The phosphonamide bond between the nucleotide and polymer carrier proved to be relatively stable at physiological pH 7.4 while at pH 5.0 (corresponding to endosomal or lysosomal compartments of cells) underwent slow hydrolysis. The rate of hydrolysis (drug release) was shown to depend on the detailed structure of the heterocyclic base. The polymer–drug conjugates described in the paper represent a new family of antiviral and cytostatic drugs with potentially improved pharmacokinetics, sustained drug release and diminished non-specific toxicity.

Keywords: Drug delivery systems; Polymer-supported drugs; Cytostatics; Antivirals; Hydrolyses; Phosphonamide bond; HPMA copolymer; Acyclic nucleotide analogues; Acyclic nucleoside phosphonates.

It has become quite generally accepted that water-soluble polymer conjugates of some therapeutic agents (e.g., cytostatics or immunosuppressives) possess certain advantages over their low-molecular-weight counterparts. Prolonged drug circulation in blood, improved solubility of hydrophobic drugs, lower non-specific toxicity, especially in case of cytostatics, sup-

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pressed immunogenicity of polymer-modified proteins or peptides and possible targeting are the most significant benefits.

Hydrophilic polymers based either on poly(HPMA)[1–5] or poly(ethylene glycol) (PEG)[6–10] have been described as carriers of various low-molecular-weight drugs. The HPMA-based copolymers were successfully used as carriers of the anticancer drug doxorubicin attached to the polymer backbone via an enzymatically[11] or hydrolytically[12] cleavable bond. Some of these conjugates were also targeted by either polyclonal[13] or monoclonal[14] antibody to achieve a better tumour specificity and, in consequence, a higher therapeutic efficiency. High antitumour efficiency of the conjugates was verified[15,16] in mice bearing various murine tumour models, with 50–100% long-term survivors depending on the cell line and scheme and dosing of polymer drug administration.

In principle, any cytostatic drug with a suitable functional group can be covalently attached to a polymer carrier. A prerequisite for the biological activity of such a conjugate is usually degradability of the bond between the drug and the polymer in target tissue.

In this work, we describe synthesis and characterization of three phosphonate nucleotide analogues linked to a primary amine-containing polymer via a phosphonamide bond known to be hydrolyzed in acid medium[17], e.g. inside endosomes or lysosomes of target cells.

Many acyclic nucleotide analogues are known to show both antiviral and antitumour effects[18]. 9-[2-(Phosphonomethoxy)ethyl) (PME) derivatives of adenine (PMEA), 2,6-diaminopurine (PMEDAP) and guanine (PMEG) were selected to verify feasibility of their covalent attachment to copolymers based on HPMA bearing primary amino groups. We believe that the therapeutic activity of these drugs might substantially improve on their modification with a hydrophilic polymer as a consequence of prolonged circulation in blood, passive accumulation of the polymer conjugate in tumour tissue due to the enhanced permeability and retention (EPR) effect[19] and sustained release of the active compound in the target tissue. Additional targeting of these polymer drugs with a site-specific ligand, e.g. antibody, might further enhance their therapeutic potential.

EXPERIMENTAL

Methacryloyl chloride, 1-aminopropan-2-ol, 3-aminopropanoic acid, 2,2′-azobisisobutyronitrile (AIBN), *N*,*N*-dimethylformamide (DMF), *N*,*N*′-dicyclohexylcarbodiimide (DCC), 4,5-dihydrothiazole-2-thiol (TT), dimethyl sulfoxide (DMSO), ethane-1,2-diamine (ED) and pyridine were purchased from Fluka. 2,4,6-Trinitrobenzene-1-sulfonic acid (TNBSA) was purchased from Serva Feinbiochemica Heidelberg. All chemicals and solvents were of analytical

grade. Solvents were purified and dried by standard procedures. The reagents were used without further purification.

NMR spectra were recorded on a Bruker spectrometer (300 MHz). Determination of molecular weights was carried out by size-exclusion chromatography (SEC) on a Shimadzu HPLC system (Shimadzu, Japan) equipped with IR, UV and multi-angle light scattering DAWN DSP-F (Wyatt Technology Corp., U.S.A.) detectors using 0.3 M acetate buffer (pH 6.5) and Superose 6 column (Pharmacia) or TSK 3000 SW_{XL} column (Tosoh Bioscience). The calculation of molecular weights from light-scattering detector was based on the known injected mass presuming 100% mass recovery. The content of phosphonate drugs PMEA, PMEDAP and PMEG bound to the polymers was determined by UV spectrophotometry at 261 nm (ε = 14 000 l mol^{-1} cm^{-1}), 281 nm (ε = 8 800 l mol^{-1} cm^{-1}), and 252 nm (ε = 32 300 l mol^{-1} cm^{-1}), in H_2O, respectively. The amount of the phosphonate groups in the polymers was also verified by phosphorus analysis. The difference between the two methods was less than 1%; for the average value, see Table I. The primary amino group content in the copolymers was determined using TNBSA assay as described in literature[20]. The content of TT groups in the copolymers was measured spectrophotometrically at 305 nm (ε = 10 880 l mol^{-1} cm^{-1}), in DMSO. PMEA, PMEDAP and PMEG were prepared according to refs[21–23]. *N*-(2-Hydroxypropyl)methacrylamide (HPMA; **1**) was synthesized as described previously[3]. 3-Methacrylamidopropanoic acid was prepared by the reaction of methacryloyl chloride with 3-aminopropanoic acid in aqueous alkaline medium according to ref.[24]

3-(3-Methacrylamidopropanoyl)thiazolidine-2-thione (Ma-AP-TT) (**2**)

3-Methacrylamidopropanoic acid (5.0 g, 35 mmol) and 4,5-dihydrothiazole-2-thiol (4.37 g, 37 mmol) were dissolved in a mixture of ethyl acetate (72 m) and tetrahydrofuran (THF) (20 ml). DCC (8.64 g, 42 mmol) was dissolved in ethyl acetate (11 ml). After cooling to –15 °C, the solutions were mixed, DMAP (ca. 50 mg) was added and the resulting solution was kept at –15 °C for 1 h and then at 5 °C overnight. The reaction mixture was stirred at room temperature for 1 h, and then 100 µl of acetic acid was added. After 1 h stirring the precipitated *N*,*N*′-dicyclohexylurea (DCU) was filtered off, solvents were evaporated in vacuum and the oily residue was dissolved in ethyl acetate. A second crop of separated DCU was removed by filtration and the product Ma-AP-TT was purified by crystallization from ethyl acetate at –15 °C. The product was filtered off, washed with diethyl ether and dried in vacuum. Yield 6.18 g (69%) of yellow solid of m.p. 95–96 °C. For $C_{10}H_{14}N_2O_2S_2$ (258.4) calculated: 46.49% C, 5.46% H, 10.84% N, 24.82% S; found: 46.76% C, 5.26% H, 10.85% N,

TABLE I
Characteristics of polymer–drug conjugates

Polymer	Drug	M_w	M_w/M_n	Drug loading, wt.%
7a	PMEA	35400	2.28	4.7
7b	PMEDAP	49500	1.92	20.6
7c	PMEG	45000	1.81	3.4

24.46% S. ^{1}H NMR ($(CD_3)_2SO$): 1.83 s, 3 H (CH_3); 3.30–3.43 m, 6 H (CH_2S, CH_2-β, CH_2-α); 4.48 t, 2 H (CH_2N); 5.31 s, 1 H (CH_2=); 5.63 s, 1 H (CH_2=); 7.98 t, 1 H (NH).

Copolymer poly(HPMA-*co*-Ma-AP-TT) (**3**)

The title copolymer was prepared by solution radical copolymerization of HPMA (88 mole %) with Ma-AP-TT (12 mole %) in DMSO at 60 °C for 6 h. The concentration of comonomers in polymerization mixture was 13 wt.% and that of AIBN was 2 wt.%. Then the copolymer was precipitated into an acetone–diethyl ether (3:1) mixture, filtered off, washed with acetone and diethyl ether and dried in vacuum. The content of TT groups was 0.71 mmol/g.

Copolymer poly(HPMA-*co*-Ma-AP-ED) (**4**)

A solution of copolymer **3** (1 g, 0.71 mmol of TT) in methanol (10 ml) was added dropwise to a solution of ED (0.4 ml, 7.4 mmol) in methanol (10 ml) under stirring. The yellow solution of thiazolidine-2-thione derivative decolorized immediately. The solvent was evaporated under reduced pressure; the residue was dissolved in methanol (10 ml); the product was precipitated into an acetone–diethyl ether (3:1) mixture, filtered off, washed with acetone and diethyl ether and dried in vacuum. The polymer was dissolved in 50 ml of a mixture of methanol and 1 M aqueous NaCl (1:1), acidified with 0.1 ml of concentrated HCl and purified by SEC on Sephadex LH 20 with methanol as eluent. The polymer fraction was evaporated and lyophilized from water yielding 920 mg of the title copolymer. The content of NH_2 groups determined by TNBSA assay was 0.78 mmol/g.

N,*N*′-Dicyclohexylmorpholine-4-carboximidamide (**6**)

The title compound was prepared by a modified procedure according to literature[25]. A solution of *N*,*N*′-dicyclohexylcarbodiimide (25 g, 0.12 mol) and distilled morpholine (16 ml, 0.18 mol) in 1,4-dioxane (15 ml) was heated under reflux for 4 h. The solution was allowed to cool overnight. The resulting white crystals were removed by filtration, washed with cold dioxane and then with diethyl ether. The yield was 34.5 g (84%) of white crystals of m.p. 104–105 °C. For $C_{17}H_{31}N_3O$ (293.5) calculated: 69.58% C, 10.65% H, 14.32% N; found: 69.76% C, 10.77% H, 14.41% N.

Polymeric Conjugates of PME–Purine Derivatives (**7a**–**7c**)

A PME derivative (**5a**–**5c**) (345 mg, 1.2 mmol) and amidine **6** (352 mg, 1.2 mmol) were dissolved in a mixture of water (1 ml) and pyridine (6 ml). The solvent was removed under reduced pressure. The residue (amidinium phosphonate salt) was dissolved in pyridine (6 ml) and evaporated to dryness again. The solid dissolved in a mixture of pyridine (6 ml) and DMF (4 ml) was added to a solution of triphenylphosphine and di(2-pyridyl) disulfide (TPP–PDS) in pyridine (3 ml). The resulting mixture was added to a solution of copolymer poly(HPMA-*co*-Ma-AP-ED) (800 mg, 0.62 mmol of NH_2) in pyridine (5 ml) and DMF (15 ml). The reaction mixture was kept at 25 °C for 6 days over anhydrous Na_2SO_4 (1 g) with occasional stirring. The progress of the reaction was checked by SEC following an increase in absorbance of the polymer at 280 nm. The mixture was filtered, the crude polymer was precipitated into an acetone–diethyl ether (3:1) mixture, filtered off, dried in vacuum, dissolved in 50 ml of a mixture of methanol and 0.6 M phosphate buffer pH 7.4 (1:1) and purified by

SEC on Sephadex LH 20 with methanol as eluent. The polymer fraction was concentrated and the product was isolated by precipitation into diethyl ether yielding 900–920 mg of a white polymer **7a**–**7c**.

Hydrolytic Release of Phosphonate Drugs from Polymer Carrier

A polymer–drug conjugate **7a**–**7c** (5×10^{-4} M drug) was incubated in 0.07 M phosphate buffer pH 5.0 or pH 7.4 at 37 °C. The amount of released PME purine **5a**–**5c** were determined by SEC of the reaction mixture from the area of the corresponding peak (TSK 3000 SW_{XL} column in 0.3 M sodium acetate buffer, pH 6.5, flow rate 0.5 ml/min, UV detection at 260 nm).

RESULTS AND DISCUSSION

The aim of this work was to verify the feasibility of synthesis of a conjugate of a phosphonate nucleotide analogue with a hydrophilic synthetic polymer. Such a conjugate should enable prolonged drug circulation in blood and release of the active compound at a controlled rate in the biological environment. The phosphonamide bond between the drug and polymer carrier was chosen as a hydrolytically degradable pH-sensitive linkage.

Synthesis of Polymer Carrier

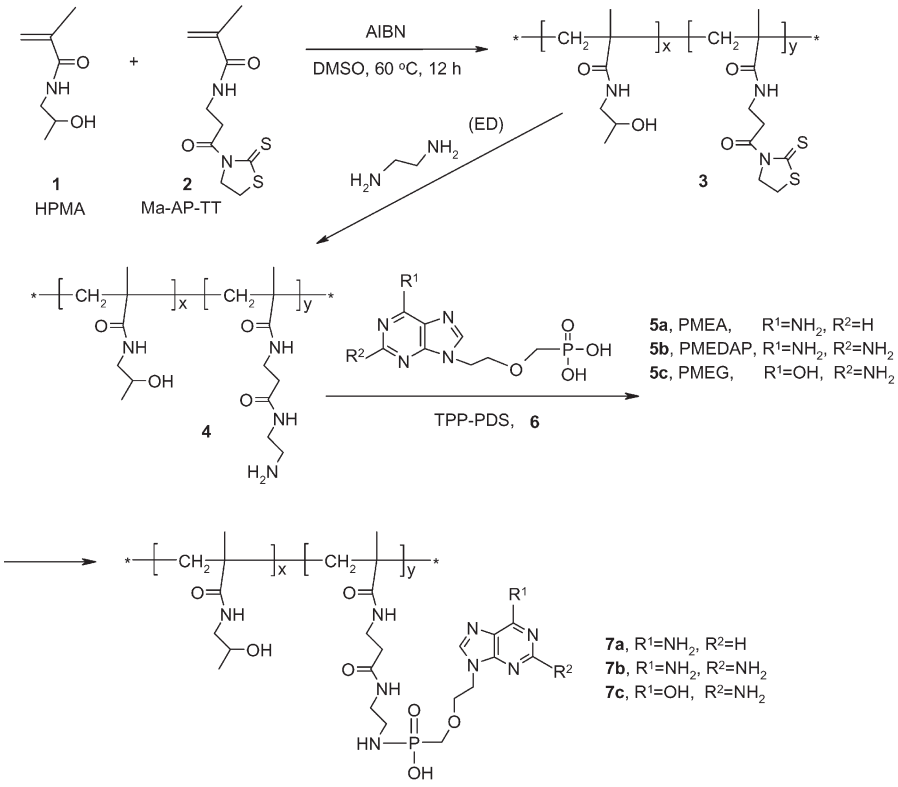
Polymer precursor **3**, bearing reactive thiazolidine-2-thione groups, was prepared by radical copolymerization of HPMA (**1**) and Ma-AP-TT (**2**) (Scheme 1) in DMSO solution. Molecular weight of the copolymers (drug carriers) was selected between 35–50 000 to achieve the effective EPR effect (accumulation in solid tumours) but still ensuring elimination of the polymer from body by glomerular filtration (threshold for elimination of HPMA copolymers is ca. 50 000). The copolymers containing TT reactive groups show low susceptibility to hydrolysis and high rate of aminolysis in aqueous solutions. The advantage of using TT copolymers[26] for the synthesis of polymeric drugs consists in a possible one-pot attachment of a drug and a targeting ligand (e.g., antibody) to the polymer carriers in aqueous medium. Recently, such synthesis of a human immunoglobulin-targeted polymer–doxorubicin conjugate was performed in our laboratory[27].

Polymeric amine **4** was obtained by aminolysis of **3** with large excess of ethane-1,2-diamine. No evidence of branching or crosslinking of polymer chains was observed by SEC by comparison of molecular weight distribution of the parent and aminolyzed polymers. It was found that purification of polymer **4** from excess of ED by preparative SEC was inevitable as we did

not succeed in removing free ED by simple precipitation or dialysis of the crude polymer.

Obviously, it would have been possible to prepare polyamine **4** also by direct copolymerization of HPMA with a suitable amine-containing monomer, (e.g. commercially available 3-aminopropylmethacrylamide). This approach should lead to similar results. However, we decided to use a versatile polymer precursor **3** that is used in our laboratory also for conjugation of other biologically active compounds.

The rationale for choice of β-alanyl spacer is based on its relative hydrophilicity and convenient copolymerization parameters of monomer **2** allowing preparation of copolymers within a wide range of comonomer mixture compositions.



SCHEME 1

Synthesis of the Polymer–Drug Conjugates

Polymer **4** was used for the attachment of phosphonate nucleotide analogues **5a**–**5c** (Scheme 1). The phosphonate function of the drugs was activated with TPP–PDS adduct according to the procedure described[28] for synthesis of phosphoric esters and phosphoramides of nucleotides. The advantage of this approach is its chemoselectivity towards monosubstituted phosphonate derivatives avoiding formation of disubstituted phosphonates.

We have used the same batch of polymer precursor **4** for preparation of all conjugates **7a**–**7c** (M_w = 28 000, M_w/M_n = 1.8). The different values in Table I may be possibly explained by partial branching caused by formation of certain minimal amount of disubstituted phosphonamides. Although formation of disubstituted phosphonates using TPP–PDS activation is not described in case of low-molecular weight derivatives, there is no information in literature regarding similar polymer analogous reactions. Presence of even a very low amount of disubstituted phosphonamides would lead to a substantial increase of M_w.

Our initial attempts to bind PME purines to polymer **4** in a heterogeneous reaction led either to no or very low loading of the drug. *N*,*N*′-dicyclohexylmorpholine-4-carboximidamide (**6**), forming amidinium salt with the phosphonate group[25], aided to significantly improve solubility of PME purines in a DMF–pyridine mixture and, consequently, to enhance the overall content of drugs in the conjugates. Despite the long reaction time and homogeneous reaction system, the final loading of the phosphonates in polymer conjugates was far from the theoretical amount 20 wt.% with the exception of polymer **7b**. In repeated experiments, PMEDAP proved to bind much more efficiently to polymeric amine **4** than PMEA and PMEG. We can speculate that the reason might consist in somewhat better solubility of PMEDAP leading to an equilibrium between the free and polymer-bound drug, shifted to the latter.

The unbound drugs had to be removed by preparative SEC in methanol as dialysis proved to be inefficient and led to partial release of phosphonates from the polymer.

Hydrolytic Release of Drugs from the Polymer Carrier

One of major advantages of polymer-bound drugs is, in most cases, their low biological activity or even inactivity during transport of the conjugate to the target site in organism, e.g. to a tumour or tumour cells. Especially in the case of cytostatic drugs (with their usually high non-specific toxicity),

this can minimize possible damage caused by the drug to healthy cells or tissue coming into contact with the drug. Consequently, a mechanism allowing drug release in the target site must be considered before such a polymer drug conjugate is designed and synthesized. It has been demonstrated previously that HPMA-based copolymers, with the antitumour drug doxorubicin bound via the acid-labile hydrazone bond, are capable of releasing doxorubicin inside endosomes of malignant cells while the conjugate is relatively stable during blood transport. It has been shown[29] using confocal microscopy that fluorescently labelled polymer conjugates remain inside the cells more than 48 h thus enabling acidolytic release of the drug.

In this work, we have used a similar strategy. We took advantage of susceptibility of the phosphonamide bond to acid hydrolysis. Figure 1 shows the release profiles of phosphonate drugs from polymers **7a**–**7c**. The amount of drug released within 48 h in phosphate buffer at pH 5.0 (corresponding to endosomes or lysosomes) from polymers **7a**–**7c** was 47% of PMEA, 35% of PMEDAP and 61% of PMEG. At physiological pH 7.4 (blood pH), PMEA (**7a**) and PMEG (**7c**) conjugates were practically stable (<2% of the drug released); only for the PMEDAP conjugate **7b** the degree of hydrolysis was 22%. This surprising difference in hydrolytic stability of the studied polymeric phosphonamides seems to indicate a significant influence of

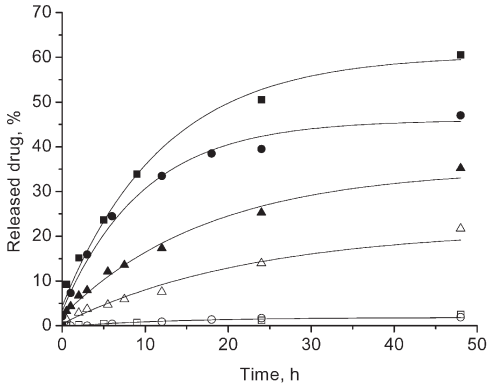


FIG. 1

Hydrolytic release of drugs PMEA, PMEDAP and PMEG from conjugates **7a**–**7c** incubated in 0.07 M phosphate buffers pH 5.0 or 7.4 at 37 °C. Concentrations of the drugs were 5×10^{-4} mol l^{-1}. The amount of released drug was determined by SEC of the reaction mixture, from the corresponding peak area (column TSK 3000 SW_{XL}, 0.3 M sodium acetate buffer, pH 6.5, flow rate 0.5 ml/min, UV detector 260 nm). ● PMEA (pH 5.0), ○ PMEA (pH 7.4), ▲ PMEDAP (pH 5.0), △ PMEDAP (pH 7.4), ■ PMEG (pH 5.0), □ PMEG (pH 7.4)

the detailed structure of the heterocyclic base on hydrolysis. Obviously, the larger is the difference in the hydrolytic rates at pH 5.0 and 7.4, the better should be the tumour-versus-blood selectivity of the polymeric drug. Unfortunately, a high drug loading was achieved only with PMEDAP using the procedure described above. Nevertheless, the achieved drug loading should be sufficient for evaluation of biological properties of the copolymers.

CONCLUSION

The feasibility of preparation of polymer–drug conjugates containing three different phosphonate nucleotide analogues with cytostatic and antiviral activity has been demonstrated. The phosphonamide bond between the polymeric carrier and drug proved to be susceptible to mild acid-catalyzed hydrolysis thus enabling controlled release of the free drug in media modelling target cell environment. The conjugates incubated in a buffer modelling the environment during blood transport were more resistant to hydrolysis (PMEDAP) or even stable (PMEA, PMEG). The rate of drug release significantly depended on the detailed structure of the heterocyclic base in the drug molecule. Cytostatic activities of the conjugates with PMEDAP and PMEG are under evaluation.

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