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Polymeric anticancer drugs with pH-controlled activation

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Abstract

The paper is dealing with the synthesis and properties of new, nontargeted or antibody-targeted pH-sensitive polymer–doxorubicin (DOX) conjugates designed as anticancer drugs facilitating site-specific therapy. These conjugates are stable and inactive during transport in the body but activate inside target cells as a result of pH changes outside and inside the cells. Cytotoxicity of the conjugates depends on the detailed structure of the polymer and of the spacer between the drug and polymer carrier. In both protective and therapeutic regimes of drug administration, the in vivo antitumor activity of the pH-sensitive conjugates containing DOX was significantly enhanced (T-cell lymphoma EL 4, C57BL/16 mice) in comparison with the free DOX or classic PK1, the PHPMA–DOX conjugate clinically tested at present.

Keywords: HPMA copolymers; Poly(ethylene glycol); Doxorubicin; Drug targeting; Cytotoxicity; Antitumor activity

1. Introduction

Water-soluble synthetic drug carriers based on copolymers of *N*-(2-hydroxypropyl)methacrylamide (HPMA) or linear polymers consisting of poly(ethylene glycol) (PEG) blocks interconnected with oligopeptides are a group of potential drug delivery systems capable of delivering specific drugs to model tumors or tumor cells in mice (Ulbrich et al., 2000; Pechar et al., 2000). These polymer drugs, designed as lysosomotropic anticancer drug delivery systems, have to fulfil a number of requirements. In the ideal case, they have to be inactive during their transport in the body, they have to be small enough to extravasate from circulation, they should accumulate in a tumor and interact with receptors specific to tumor cells, they have to enter cells via fluid-phase, adsorptive or receptor-mediated pinocytosis and they have to be activated (drug released) in the cancer cell compartments. We have shown previously that polymer drugs can extravasate, accumulate in the solid tumors and enter the cell via pinocytosis (Duncan and Ulbrich, 1993; Hovorka et al., 2002). Anticancer drug doxorubicin (DOX) was attached to the polymer carrier through oligopeptide spacers susceptible to hydrolysis by lysosomal enzymes (classic conjugate). Since the intracellular release of DOX from the carrier is a prerequisite for anticancer activity of the conjugate in vivo, conjugates with ezymatically degradable spacers are very specific and efficient lysosomotropic drug delivery systems, especially if targeted with antibodies (Jelinkova et al., 1998; Rihova et al., 2000). Unfortunately, there are some drawbacks limiting practical application of the classic conjugates, such

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as complicated and expensive synthesis, branched structure of the molecule with a broad distribution of molecular weights, or the need of presence of lysosomal enzymes at the drug target.

Here, we describe the synthesis and properties of the antibody-targeted conjugates in which DOX is attached to a water-soluble polymer carrier via a simple hydrolytically labile spacer containing the hydrazone bond (hydrazone conjugates). The hydrazone linkage should enable the hydrolytically controlled release of doxorubicin from the carrier and its activation after transfer of the polymer drug from the blood circulation and extracellular environment (pH 7.4) into intracellular compartments (pH ∼ 5–6). In contrast to the classic conjugates, the presence of lysosomal enzymes in this case is not essential for biological activity of the hydrazone conjugates. Physicochemical and biological properties of the hydrazone conjugates are compared with those of the conjugates prepared by the classic DOX coupling with a polymer via amide bond and enzymatically degradable oligopeptide spacer.

2. Structure and synthesis of conjugates

Polymer–DOX conjugates under study contain four essential parts: (i) a water-soluble polymer forming the backbone of the whole system. It is responsible for a good water solubility of the system and for a long circulation in blood. In our experiments we used nondegradable HPMA copolymer (Fig. 1A and B) or biodegradable polymer formed by PEG blocks connected with N^{α},N^{ε}-diglutamyllysine tripeptide sequences (Fig. 1C). (ii) Cytotoxic drug doxorubicin (DOX) attached to the polymer through a biodegradable spacer. (iii) Biodegradable spacer facilitating release of the drug from the polymer inside the cell. Tetrapeptide GlyPheLeuGly, a substrate for lysosomal enzyme cathepsin B, diglycine and various amino acids terminated in hydrazone bonds were used as biodegradable spacers between the polymer chain and DOX. The spacers were designed to be stable in circulation at physiological pH (7.4) and degradable after uptake by cells as a result of acid hydrolysis in endosomes (hydrazones at pH ∼ 5–6) or enzymatic hydrolysis in secondary lysosomes (classic conjugates with GlyPheLeuGly spacer). (iv) Targeting moiety responsible for specific delivery of the conjugate to

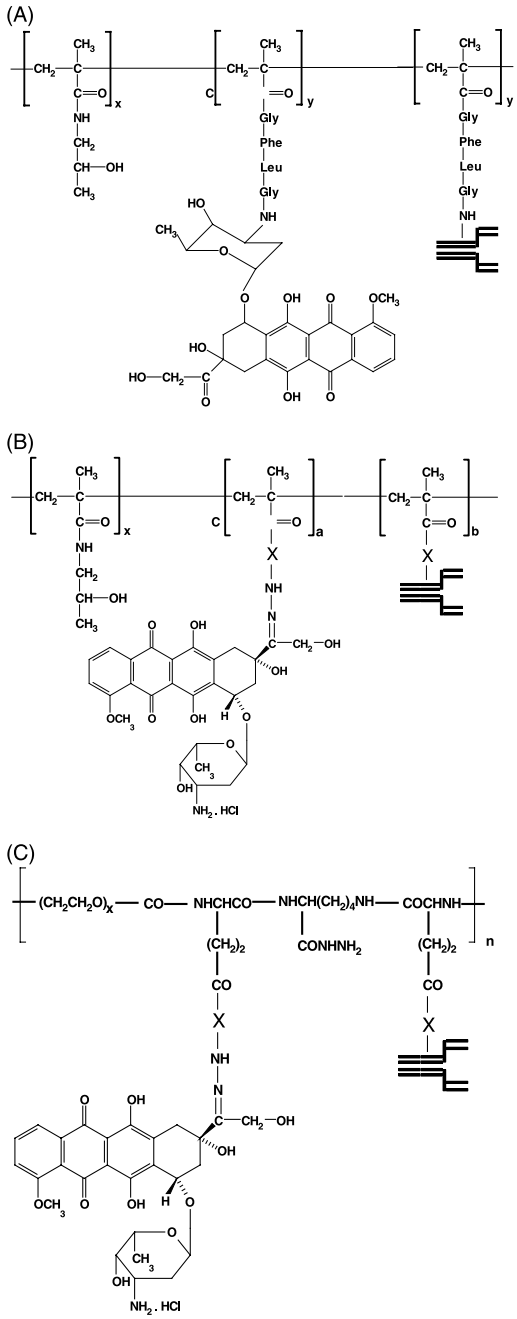

Fig. 1. Structure of the antibody-targeted polymer conjugates of doxorubicin. (A) Classic HPMA-based conjugate, (B) hydrazone HPMA-based conjugate, (C) hydrazone PEG-based conjugate. (X—spacer.)

the target cells was attached to the polymer backbone via a spacer. Specific polyclonal and monoclonal IgG-type antibodies were used for the synthesis of the conjugates. Structure of both types of conjugates is shown in Fig. 1.

HPMA-based conjugates were prepared by the reaction of DOX (nontargeted conjugates) or DOX and respective antibody (targeted conjugates) with the reactive polymer precursor—a copolymer of HPMA and methacryloylated oligopeptide or amino acid, bearing in its side chains 4-nitrophenoxy (classic conjugate) or hydrazide groups (hydrazone conjugates) (Etrych et al., 2001). Antibody was attached to the polymer through the three different linkages, a hydrazone linkage formed by direct coupling of the periodate-oxidized antibody with hydrazide groups of the polymer, or via disulfide or sulfide linkages resulting from the use of bifunctional coupling agents for conjugation of the antibody with the polymer precursor (Etrych et al., 2002).

Multiblock PEG-based conjugates were prepared as follows: PEG activated with phosgene and *N*-hydroxysuccinimide reacted with the triethyl ester of tripeptide N^{α},N^{ε}-diglutamyllysine (H-Glu-Lys-(H-Glu-)) to give a multiblock polymer degradable with lysosomal enzymes. The polymer was then converted to the corresponding polyhydrazide by hydrazinolysis of ethyl ester groups with hydrazine hydrate. Nontargeted conjugate was prepared by direct coupling of DOX with the hydrazide multiblock polymer. In the synthesis of targeted conjugates, a part of the polymer-bound hydrazide groups was modified with *N*-succinimidyl 3-(2-pyridyldisulfanyl)propanoate (SPDP) in the next step to introduce pyridyldisulfanyl groups for subsequent conjugation with a modified antibody. Doxorubicin was bound to the remaining hydrazide groups via acid-labile hydrazone bonds yielding a polymer precursor. Finally, human immunoglobulin IgG modified with 2-iminothiolane was conjugated to the polymer by substitution of the 2-pyridylsulfanyl groups of the polymer with SH groups of the antibody.

The polymer–drug conjugates and polymer–drug intermediates were freed from low-molecular-weight impurities (such as DOX, 4-nitrophenol, coupling agents) by gel filtration. All the polymers under study, including copolymers containing both the antibody and drug, were tested for the content of the free polymer, free drug or antibody. Polymer conjugates were characterized by the antibody content (estimated by amino acid analysis), the DOX content (UV spectrophotometry) and by molecular weight (M_w) and polydispersity (FPLC Pharmacia system equipped with RI, UV and multiangle light scattering DAWN DSP-F (Wyatt Co., USA) detectors).

3. Results and discussion

3.1. In vitro drug release

We have shown earlier that a prerequisite for anticancer activity of the polymer–DOX conjugates is release of the free DOX from the carrier. DOX can be released from the polymer carrier either after interaction of the conjugate with lysosomal enzymes (classic conjugate), or as a result of acid environment in endosomes (hydrazone conjugates). In the optimum case, the systems should be stable during its transport in blood circulation (pH 7.4). In the classic systems, the rate of DOX release from the conjugates incubated with lysosomal enzyme cathepsin B depends on the length and detailed structure of the spacer. Tetrapeptide GlyPheLeuGly was found to be one of the most suitable spacers. On the contrary, the glycylglycine spacer was nondegradable and corresponding conjugates were completely ineffective both in in vitro and in vivo experiments (Duncan et al., 1988, 1989).

Polymer conjugates under study are based on pH-labile polymer drug carrier systems containing the terminal hydrazone-bonded DOX moieties attached via amino acid or oligopeptide spacers, enzymatically degradable (GlyPheLeuGly) or nondegradable (Gly, GlyGly, β-Ala, 6-aminohexanoyl or 4-aminobenzoyl). The susceptibility of the conjugates to hydrolysis after incubation in buffers of different pH is shown in Figs. 2–4. Fig. 2 presents results of the DOX release from hydrazone HPMA-based conjugates incubated in phosphate buffer (pH 5.5) at 37 °C. Dependence of the rate of DOX release on the detailed conjugate structure, particularly on the spacer length and structure was studied. All the conjugates under study released DOX at significant rates. The highest rate of DOX release was observed for the conjugates with long aliphatic (6-aminohexanoyl) or aromatic (4-aminobenzoyl) spacer. In these cases more than

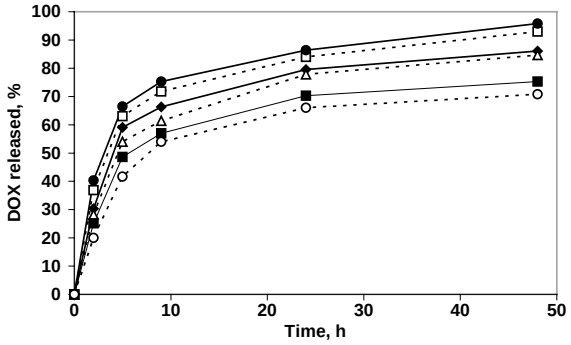

Fig. 2. In vitro doxorubicin release from hydrazone HPMA-based conjugates with different spacers incubated in phosphate buffer (pH 5.5) at 37 °C: (●) 6-aminohexanoyl spacer, (□) 4-aminobenzoyl spacer, (◆) Gly spacer, (△) GlyGly spacer, (■) GlyPheLeuGly spacer, (○) β-Ala spacer.

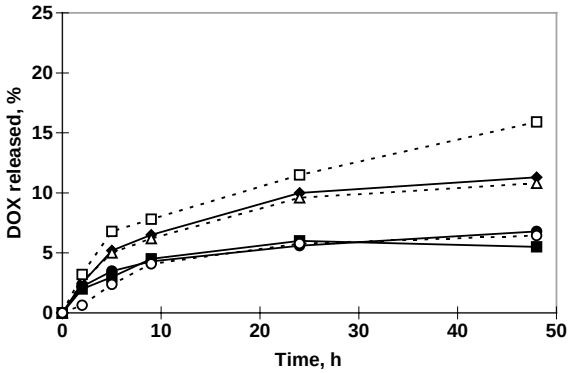

Fig. 3. In vitro doxorubicin release from hydrazone HPMA-based conjugates with different spacers incubated in phosphate buffer (pH 7.4) at 37 °C: (●) 6-aminohexanoyl spacer, (□) 4-aminobenzoyl spacer, (◆) Gly spacer, (△) GlyGly spacer, (■) GlyPheLeuGly spacer, (○) β-Ala spacer.

90% of DOX was released during 48 h. On the contrary, the lowest rate was observed with a conjugate containing β-alanine as a spacer, only 70% of DOX being released in the same time interval.

The results show that the rate of drug release can be controlled to a certain extent by the detailed structure of the spacer. Similar relations were observed after incubation of the conjugates in a buffer at pH 7.4 (Fig. 3).

However, in this case, the rates of DOX release were more than 10 times lower than in buffer at pH 5. This fact corroborates our hypothesis on stability of the linkage between the drug (DOX) and polymer carrier in bloodstream. Similar results, even more pronounced, were obtained after incubation of PEG-based conjugates under the same conditions (Fig. 4).

These results allow us to assume that the conjugates can release only very small amounts of free DOX in

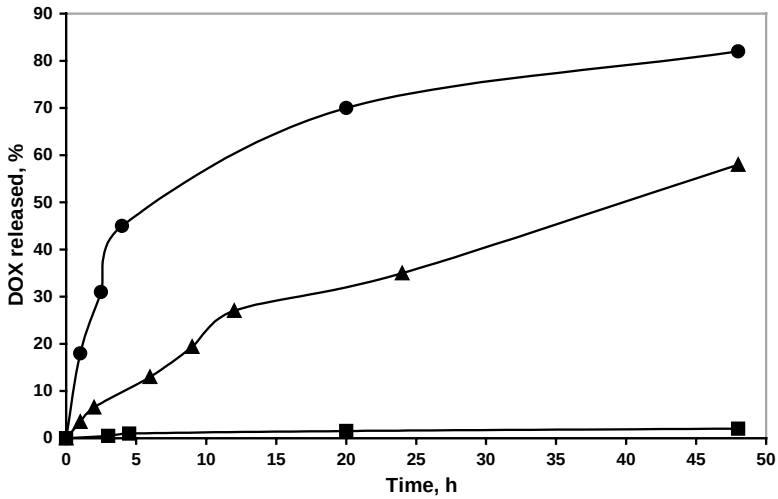

Fig. 4. In vitro doxorubicin release from PEG–DOX conjugates: PEG–DOX hydrazone conjugate incubated in phosphate buffer (a) at pH 5.5 (●) and (b) at pH 7.4 (■), PEG-DOX hydrazone conjugate containing GlyPheLeuGly spacer incubated with Cathepsin B (4×10^{-7} M) in phosphate buffer (pH 5.5) (▲).

the bloodstream (pH close to 7.4) during the transport to target cells and the conjugates fulfil the requirement for the stability of the conjugates during their transport in the body. A majority of the active drug could be released from the conjugate only after entering the target cells via pinocytosis due to a pH decrease in endosomes (pH ∼ 5–5.5). Moreover, DOX release from the conjugate with an enzymatically degradable spacer (GlyPheLeuGly) terminated in hydrazone bond is controlled by hydrolysis in endosomes or a combination of hydrolysis and enzymolysis in secondary lysosomes. In principle, DOX can be released in all cancer cells, i.e., also in the cells with only low concentrations of lysosomal enzymes (e.g., erythroblastoid cell lines) or even in the cells where lysosomal enzymes are missing.

3.2. In vitro cytotoxicity

Cytotoxicity of all the studied conjugates to various tumor cell models (BCL1 leukemia, 38C13 B-cell lymphoma, mouse T-cell lymphoma EL4, human colorectal carcinoma SW 620) was tested by (^{3}H)thymidine incorporation. Because conclusions arising from results of these tests were comparable, we present here only the results obtained with EL4 T-cell lymphoma. However, it is clear that sensitivity of various cancer cell lines to free and polymer-bound doxorubicin differs to some extent. Inhibition of cancer cell proliferation was clearly dose-dependent and the IC_{50}, concentration required for 50% inhibition of cell proliferation, was used to compare the sensitivity of T-cell lymphoma EL4 to tested polymer conjugates containing doxorubicin bound to the polymer carrier through hydrolytically (PEG- or HPMA-based hydrazone conjugates) or enzymatically (classic conjugates) cleavable linkages. The results of cytotoxicity tests are shown in Table 1.

Cytotoxicity of polymer–DOX conjugates to mouse T-cell lymphoma EL4. While there was no cytotoxic effect of the polymer carrier (IC_{50} > 100 μg DOX/ml), cytotoxicity of all polymer–DOX conjugates was significant and structure-dependent. The lowest cytotoxicity (highest IC_{50}) was found for the classic HPMA copolymer-bound DOX (IC_{50} = 19.10 μg DOX/ml), a conjugate containing the GlyPheLeuGly spacer and requiring enzymatic activation. Using the conjugate with the same tetrapeptide side chain to which DOX is bound through the hydrolytically cleavable hydrazone bond, an almost 150 times lower amount of doxorubicin is needed (IC_{50} = 0.13 μg DOX/ml) to achieve a comparable anti-proliferative effect. Accordingly, the conjugate containing doxorubicin bound to the GlyGly spacer through a hydrolytically cleavable hydrazone bond shows even a higher anti-proliferative capacity (IC_{50} = 0.08 μg DOX/ml). Quite interesting results were observed with conjugates containing spacers based on 6-aminohexanoic or 4-aminobenzoic acid, to which doxorubicin was bound also through hydrazone bonds. While the conjugate with the 6-aminohexanoyl spacer was four times less effective (IC_{50} = 0.34 μg DOX/ml) compared with conjugate with the GlyGly spacer, the conjugate with the 4-aminobenzoyl spacer exhibited a similar anti-proliferative activity to that of conjugate with the GlyGly spacer. Such an activity was 270 times higher than that observed with classic nontargeted conjugate. Cytotoxicity of the PEG-based conjugates with the GlyPheLeuGly spacer was very similar to that of HPMA copolymers, but cytotoxicity of the conjugate without spacer (DOX bound directly to the glutamic acid hydrazide) was even more pronounced and approached to the cytotoxicity of the free DOX. It has to be stressed that all the conjugates were always tested

Table 1
Cytotoxicity of polymer–DOX conjugates to mouse T-cell lymphoma EL4

Conjugate	IC_{50} (μg DOX/ml)
PHPMA–GFLG–DOX (classic)	19.1
PHPMA–GFLG–DOX–ATG (classic, targeted)	11.8
PHPMA–GFLG–DOX (hydrazone), M_w = 19,600	0.13
PHPMA–GFLG–DOX (hydrazone), M_w = 115,000	0.30
PHPMA–GG–DOX (hydrazone)	0.08
PEG–GFLG–DOX (classic)	8.0
PEG–DOX (hydrazone)	0.008
PHPMA–aminohexanoic acid–DOX (hydrazone)	0.34
PHPMA–aminobenzoic acid–DOX (hydrazone)	0.07
PHPMA–GFLG–DOX–ATG (hydrazone, targeted)	0.001
PHPMA–GFLG–$NHNH_2$ (precursor)	>100
DOX·HCl	0.01

together in one experiment to exclude or minimize experimental variations.

A reasonably high molecular weight of the antitumor drug conjugate is important for in vivo application in mice bearing solid tumors as it increases passive accumulation of the polymer-bound drug into the tumor bulk by the EPR effect. We have found that the anti-proliferative capacity of a higher molecular weight hydrazone conjugate ($M_w = 115{,}000$) (8) was lower compared to a lower molecular weight conjugate ($M_w = 19{,}600$). Surprisingly, the high-molecular-weight antibody-targeted conjugate ($M_w \sim 500{,}000$) exhibited a higher cytotoxicity effect than its corresponding nontargeted low-molecular-weight analogue. Cytotoxicity of the targeted systems depends on the method used for conjugation of the antibody with a polymer. Cytotoxicity of the conjugate targeted with antibody attached via the S–S bridge was the same or even higher than that of free DOX. Explanation of this high cytotoxicity effect of the conjugate is not simple: it cannot be explained only by the highest rate of DOX release during incubation. It is clear that hydrazone conjugates release DOX into the incubation media and this fact can influence results of cytotoxicity tests. In the cytotoxicity test, cells were incubated at pH 7.2; at this pH the conjugates release only very small amounts of the cytotoxic free DOX. Its instantaneous concentration is thus much lower than that of the free DOX used directly in the incubation media and this is why it cannot produce the same cytotoxic effect. Some changes in the mechanism of action of the free and conjugated drug in the cell can probably cause changes in the final effect. It seems that a combination of more factors is responsible for high cytotoxic activity of PHPMA–DOX hydrazone conjugates. Mechanism of DOX release from the classic and hydrazone conjugates is different (enzymatic release in lysosomes, pH-dependent hydrolysis in endosomes). This probably results in different mechanism of action of both types of conjugates. While classic conjugates probably cause necrosis, hydrazone conjugates, similar to free DOX, induce apoptosis. Detailed study is under way.

It is necessary to state in conclusion of this chapter that cytotoxicity test is a valuable tool for evaluation of the relationship between the structure of the conjugates and their biological effect helping to optimize the structure of the conjugates but serious evaluation of the potential of the conjugates in treatment of cancer can be made only using carefully selected in vivo tests in animal models.

3.3. In vivo biological activity

3.3.1. Blood clearance

Blood of Balb/c mice was collected in different time intervals after i.v. injection of free or polymer-bound

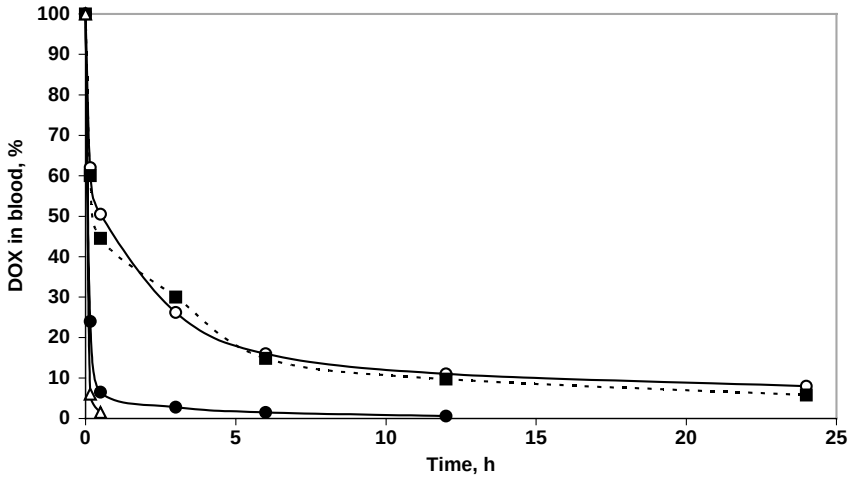

Fig. 5. Blood clearance of free DOX·HCl and polymer–DOX conjugates in mice: (△) DOX·HCl, (●) hydrazone conjugate with GlyPheLeuGly spacer, (■) classic conjugate, (○) antibody-targeted hydrazone conjugate.

DOX. Total DOX content was determined by HPLC after hydrolysis of samples and extraction of the formed aglycon with chloroform. The results are given in Fig. 5. All the polymer conjugates under study circulate in the bloodstream longer than free DOX. The half-time for clearance of the free DOX is less than 10 min while circulation of hydrazone conjugate is more extended. The longest persistence of DOX in circulation was observed for classic conjugate and antibody-targeted hydrazone conjugate. Faster blood clearance of the hydrazone conjugate than that of the classic structure can be ascribed to specific interactions of the hydrazide groups remaining in the conjugate and uptake of the conjugate in the body compartments. Also, the effect of DOX release and its fast elimination from bloodstream as a possible explanation cannot be omitted. A detailed study of the fate of hydrazone conjugates in mice is under way.

3.3.2. Antitumor activity

In vivo experiments were performed in mice using mouse T-cell lymphoma EL4 as tumor model and C57BL/6 mice. Mice were inoculated s.c. with 10^5 cells on day 0. The nontargeted or antibody-targeted (anti-Thy 1,2 antibody) conjugates of DOX or DOX·HCl were injected i.p. either in protective (on days 1, 3, 5, 7, 9) or therapeutic (on days 11, 13, 15, 18, 20, i.e., after the tumor became palpable) regime at a total dose equivalent to 25 mg DOX/kg. Classic conjugate was used as a reference sample. Free DOX was injected in a maximum tolerated dose of 12.5 mg DOX/kg. The groups of 5–10 mice were

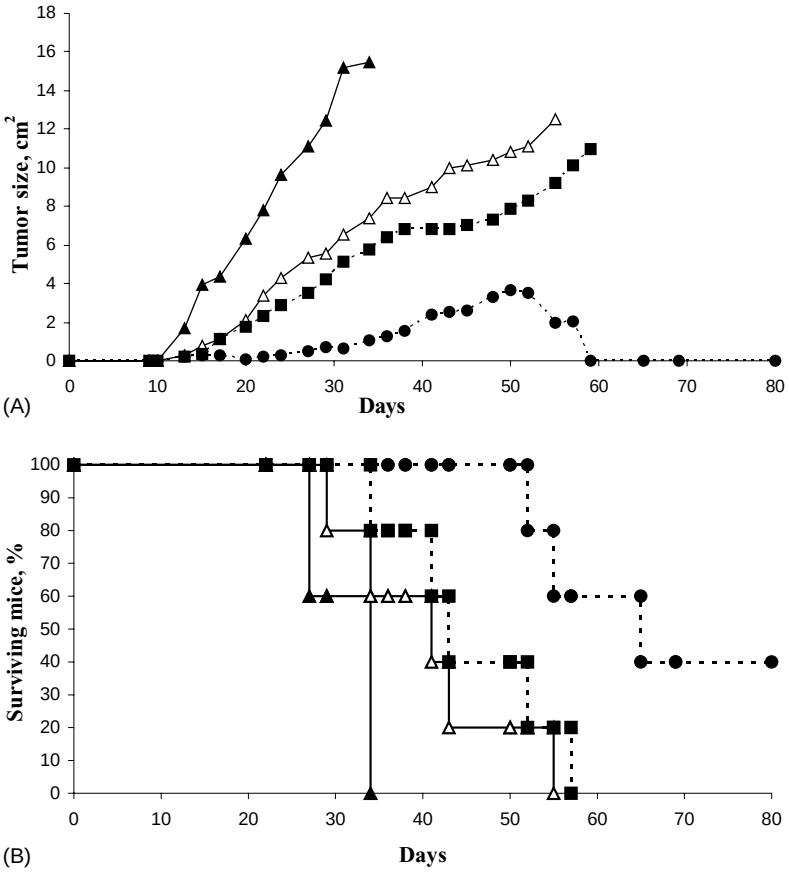

Fig. 6. In vivo effect of DOX·HCl and HPMA-based DOX conjugates (protective regime) on the growth of T-cell lymphoma EL4 (A) and on the survival of mice (B): (▲) control, (△) DOX·HCl, (■) classic conjugate, (●) hydrazone conjugate with GlyPheLeuGly spacer. $P < 0.01$ (Student's t-test).

used for each experiment. The animals were observed daily for signs of tumor progression. Tumor growth (tumor size in cm^2) and survival (the ratio of the mean survival of the test group T to that of the untreated control C, T/C) of experimental animals were checked.

As can be seen in Figs. 6 and 7, in contrast to low anticancer activities of free DOX and the classic conjugate, all the nontargeted HPMA-based hydrazone conjugates demonstrated a significantly better anticancer effect when tested on T-cell lymphoma EL4 in protective (Fig. 6) or therapeutic regime (Fig. 7).

In the protective regime, treatment of animals started the next day after cancer transplantation. In this case, the tumor in animals treated with polymer–DOX conjugates developed slowly or failed to grow at least in some animals treated with the hydrazone conjugate. There were 40% long-term survivors in the group of animals treated with nontargeted hydrazone conjugates containing the enzymatically degradable GlyPheLeuGly sequence as a spacer. The effect of hydrazone conjugate was significantly higher and the inhibition of tumor growth in treated mice was more pronounced than with free drug or the classic conjugate. In the therapeutic regime, the effect of polymer drugs was less pronounced; nevertheless, 20% animals treated with hydrazone conjugate containing the GlyPheLeuGly sequence survived more than 80 days.

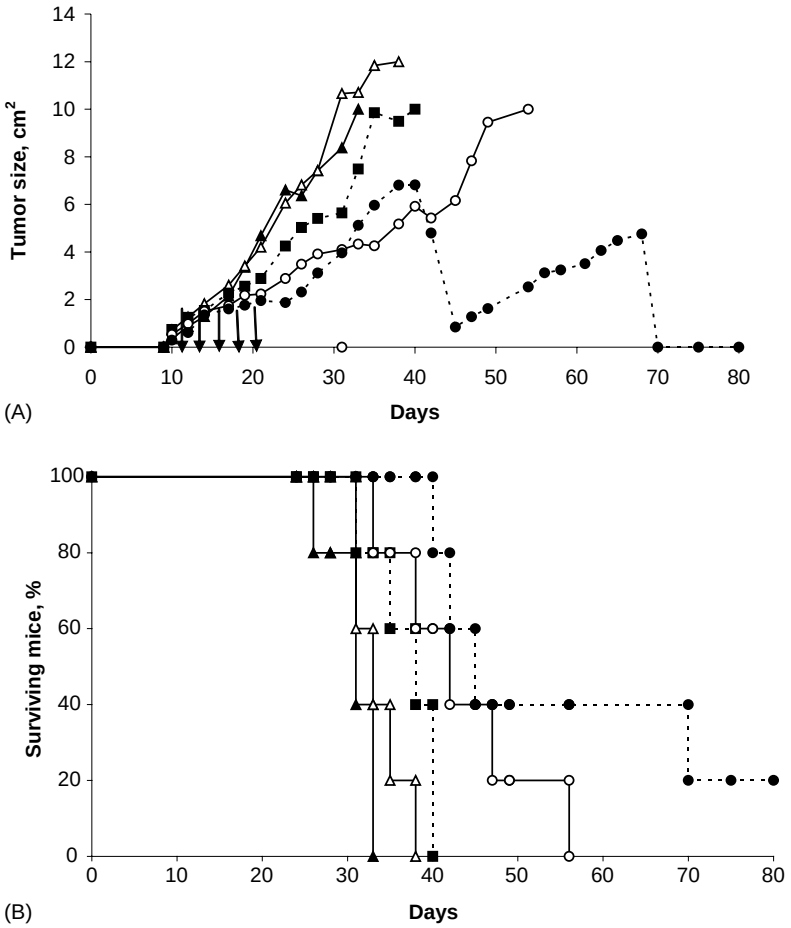

Fig. 7. In vivo effect of DOX·HCl and HPMA-based DOX conjugates (therapeutic regime) on the growth of T-cell lymphoma EL4 (A) and on the survival of mice (B): (▲) control, (△) DOX·HCl, (■) classic conjugate, (○) hydrazone conjugate with GlyGly spacer, (●) hydrazone conjugate with GlyPheLeuGly spacer. $P < 0.01$ (Student's *t*-test).

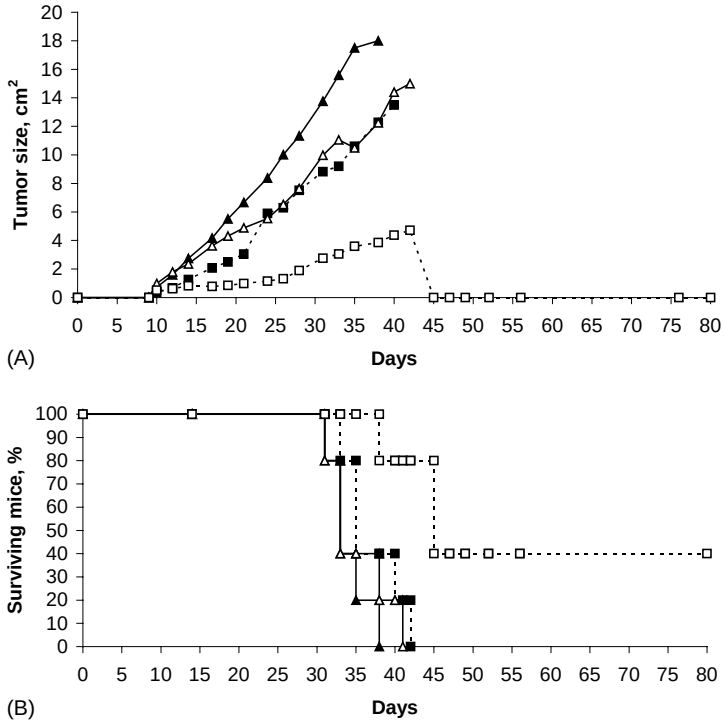

Fig. 8. In vivo effect of DOX·HCl and PEG–DOX conjugates (therapeutic regime) on the growth of T-cell lymphoma EL4 (A) and on the survival time of mice (B): (▲) control, (△) DOX·HCl, (■) PEG-DOX classic (GlyPheLeuGly spacer), (□) PEG-DOX hydrazone (GlyPheLeuGly spacer).

Hydrazone conjugate with the GlyGly spacer was less effective but superior to the effect of the classic conjugate. Unfortunately, the expected improvement of anticancer activity of the antibody-targeted systems observed in in vitro systems was not confirmed in vivo (results not shown).

Tumor growth in animals treated with PEG-based hydrazone conjugate containing the GlyPheLeuGly spacer was significantly inhibited in both protective and therapeutic regimes. The results obtained in the therapeutic regime of treatment of T-cell lymhoma EL4 in mice are shown in Fig. 8. While the effect of the PEG-DOX classic conjugate (bearing drug attached via an enzymatically degradable spacer and amide bond) on the tumor growth and survival of animals was negligible and comparable with free DOX, the hydrazone conjugate inhibited tumor growth efficiently and tumor in some cases failed to develop or disappeared. In the therapeutic regime, there were 40% of long-term survivors (longer than 80 days) in the experiment. This demonstrates a big potential of the system in treatment of cancer.

4. Conclusions

We have described the synthesis and properties of water-soluble drug delivery systems with pH-labile drug activation. The conjugates are relatively stable at blood pH (7.4) but release the active drug under mild acidic conditions (pH 5) modeling endosomal and lysosomal environment inside the cells. We have shown that such systems (HPMA-based and PEG-based) are more effective anticancer drugs than the free drug (doxorubicin, commonly used for cancer treatment in humans) or its earlier developed classic polymer conjugate. The hydrazone conjugates are efficient in the therapeutic regime of the treatment of advanced mouse cancer and show significant

inhibition of tumor growth and prolongation of mouse survival in experimental mice.

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