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HPMA Copolymer Conjugates of Paclitaxel and Docetaxel with pH-Controlled Drug Release

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Abstract: In this paper we describe the synthesis, physicochemical characteristics and data on the biological activity of polymer prodrugs based on the anticancer drugs paclitaxel (PTX) and docetaxel (DTX) conjugated with a water-soluble *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer drug carrier. The drugs were derivatized and then attached to the polymer backbone via a spacer that is stable under physiological conditions (pH 7.4) and hydrolytically degradable in mild acidic environments (e.g., endosomes, pH $\sim$ 5). Polymer−drug conjugates were designed to achieve prolonged blood circulation and release of the active compound in target cells. Six types of conjugates differing in the structure of the keto acid (levulic, 3-(acetyl)acrylic acid) and 4-(2-oxopropyl)benzoic acid-containing spacer or in the amount of drug bound to the HPMA copolymer were synthesized. In all the conjugates, the linkage susceptible to hydrolytic cleavage was formed by the reaction of the carbonyl group of a drug derivative with the hydrazide group-terminated side chains of the polymer. *In vitro* incubation of the conjugates in buffers resulted in much faster release of drugs or their derivatives from the polymer at pH 5 than at pH 7.4 with the rate depending on the detailed structure of the spacer. Conjugates containing drugs acylated with levulic acid were tested for their anticancer activity *in vivo* using two murine models. The PTX-containing conjugate showed better antitumor efficacy in the 4T1 model of mammary carcinoma than the parent drug and its derivative. The DTX-containing conjugate demonstrated high activity in treating EL4 T cell lymphoma. The treatment with the polymer conjugates was devoid of side toxicity. In both models, we achieved complete regression of established tumors accompanied by a durable tumor resistance in most of the cured animals.

Keywords: *N*-(2-Hydroxypropyl)methacrylamide (HPMA) copolymer; docetaxel; paclitaxel; murine lymphoma; mammary carcinoma; tumor resistance

Introduction

During the last three decades, water-soluble polymers have become useful drug delivery systems[1–4] suitable as carriers, mainly for anticancer or anti-inflammatory agents. Conjugation or mixing of drugs with polymers usually alters their distribution in the body, prolongs their circulation time and reduces their degradation in the body fluid, thereby decreasing drug-related side effects and increasing drug efficacy.[5]

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Coupling the hydrophobic drug to a water-soluble polymer allows for the preparation of a hydrophilic conjugate while retaining the activity of the drug. To obtain a tumor-specific or a tumor cell-specific drug delivery, the important parameters are proper selection of the polymer carrier, the type of linkage between the drug and carrier and, if applicable, proper selection of a targeting moiety and its binding procedure.[6−9] The drug can be released from the polymer by a specific enzymatic cleavage mechanism[10,11] (e.g., cleavage of the GFLG sequence by lysosomal enzymes) or by pH-controlled chemical hydrolysis[12−14] (hydrazone bond, *cis*-aconityl spacer).

Paclitaxel (PTX; Taxol, Bristol Myers Squibb) and docetaxel (DTX; Taxotere, Sanofi Aventis) are anticancer agents frequently used in treatment regimes for patients with breast, ovarian, prostate, lung, and other cancers. PTX was derived from natural sources (Pacific yew tree, genus *Taxus*), while DTX is a more active synthetic derivative of PTX. At higher concentrations they interfere with disassembly of microtubules, thereby inhibiting cell division and inducing cell death.[15] DTX is more efficient in inducing tubule polymerization and thus is more effective than PTX *in vitro* and *in vivo*.[16] As with many other chemotherapeutic agents, metronomic dosing schedules with taxanes have been shown to exert antiangiogenic activity in solid tumors.[17,18]

One of the major disadvantages of these drugs is their prominent hydrophobic character. Taxanes must be administered in oily formulations using mixtures of ethanol and Cremophor EL (PTX) or polysorbate 80 (DTX). The solvents were shown to induce serious adverse reactions in a considerable proportion of patients. Moreover, a significant decrease of the drug effectiveness due to the presence of Cremophor EL was reported in several tumor models.[19]

PTX was tested in various formulations to enhance its clinical applicability and to avoid the unwanted adverse reactions related to the solubilizing agent, but only some of them demonstrated improved *in vivo* efficacy. For instance, a therapeutic formulation of PTX-containing nanoparticles with an albumin shell called Abraxane (ABI-007; Abraxis Bioscience) showed better pharmacokinetic and toxicity profiles as well as superior therapeutic efficacy in MTD regimens when compared with the parent drug Taxol.[20−22] It also showed a better antiangiogenic effect at metronomic dosages.[19] Approved by the FDA in 2005, Abraxane is the first chemotherapy in its class, and it is used for treatment

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of cancers in patients who do not respond to classical first-line chemotherapy. Various types of nanoparticles containing DTX have shown high encapsulation efficiency and anticancer effects in experimental model systems and are currently under development.[23,24]

PTX has also been prepared as a polymer-bound prodrug using several synthetic polymers. The drug was bound to poly(ethylene glycol) (PEG) by a noncleavable ester, a 7-carbamate bond or a pH-labile amino acid linker (Ala, Gly).[25,26] The conjugate of PTX and poly(glutamic acid) (PGA) showed better anticancer activity than the parent drug, and it is now in phase III clinical trials as Opaxio (CT-2103, paclitaxel polyglumex, formarly known as Xyotax, Cell Therapeutics).[27,28] In Opaxio the drug is linked to the polymer carrier via an ester bond using the hydroxyl group at the 2-position. The drug is released due to enzymatic degradation of the polymer backbone first by cathepsin B to form PTX diglutamate. This intermediate is further degraded by an unknown exopeptidase to form PTX monoglutamate, Glu-PTX, which is finally hydrolyzed to generate the active drug.[27,29] A different PTX−HPMA copolymer conjugate has the drug coupled via an ester bond to the enzymatically degradable tetrapeptide linker GFLG. Upon incubation of the conjugate with lysosomal enzymes, the drug was released from the carrier, and it was documented that the release was essential to achieve anticancer efficacy. PTX−HPMA copolymer conjugate PNU166945 was tested in phase I clinical trials.[30] The results showed drug-related toxicities, including significant bladder toxicity that could be explained by instability of the ester bond of the conjugate during its blood circulation and urine-excretion phase. Clinical trials were permanently discontinued mainly because of significant neurotoxicity seen in preclinical testing in rats and also in a proportion of patients.

In sum, conjugation of PTX to a polymer carrier could be a way to achieve a water-soluble prodrug with more favorable pharmacokinetics and better bioavailability than the parent drug. However, clinical application of the polymer prodrug requires proper design of the conjugate including proper selection of the polymer and its structure, its M_w with a narrow distribution of molecular weights, the structure and selective degradability of the spacer, and the optimal content of the linked drug. Indeed, these characteristics must be verified using appropriate animal tumor models.

In this paper we describe the synthesis, characterization and preliminary results of the evaluation of the biological properties of the PTX- and DTX-bearing water-soluble polymer prodrugs based on HPMA copolymers. In the conjugates, the PTX and DTX derivatives were attached to the polymer via pH-sensitive hydrazone bonds selected with the aim to enable controlled release of PTX, DTX, or their derivatives from the carrier after passage of the polymer prodrug from the blood circulation and extracellular environment (pH 7.4) into intracellular compartments (pH ∼ 5). In contrast to the other polymer-based prodrugs of taxanes, in this case the presence of lysosomal enzymes is not essential for the drug release and consequent biological activity of the conjugate.

Materials and Methods

Chemicals. 1-Aminopropan-2-ol, 3-acetylacrylic acid (AAK), 4-(dimethylamino)pyridine, methacryloyl chloride, 2,2′-azobis(isobutyronitrile) (AIBN), methyl 6-aminohexanoate hydrochloride (ah-MeO), *N*,*N*′-dimethylformamide (DMF), *N*,*N*′-dicyclohexylcarbodiimide (DCC), *N*-ethyldiisopropylamine, dimethyl sulfoxide (DMSO), *tert*-butyl carbazate, hydrazine hydrate, 4-(2-oxopropyl)benzoic acid (OPB), 4-oxopentanoic acid (levulic acid, LEV), 3-(acetyl)acrylic acid (AAC) and trifluoroacetic acid (TFA) were purchased from Fluka (Switzerland). 2,4,6-Trinitrobenzene-1-sulfonic acid (TNBSA) was purchased from Serva (Germany). Paclitaxel (PTX) and docetaxel (DTX) were purchased from Aurisco (China).

Synthesis of Monomers and Derivatives of PTX and DTX. *N*-(2-Hydroxypropyl)methacrylamide (HPMA) was synthesized as described in ref 2 with the exception that Na_2CO_3 was used instead of $NaHCO_3$. Mp 70 °C; elemental anal.: calcd, C 58.72, H 9.15, N 9.78; found, C 58.98, H 9.18, N 9.82.

6-Methacrylamidohexanohydrazide (Ma-ah-$NHNH_2$) was prepared by the reaction of methyl 6-aminohexanoate hydrochloride with methacryloyl chloride followed by hy-

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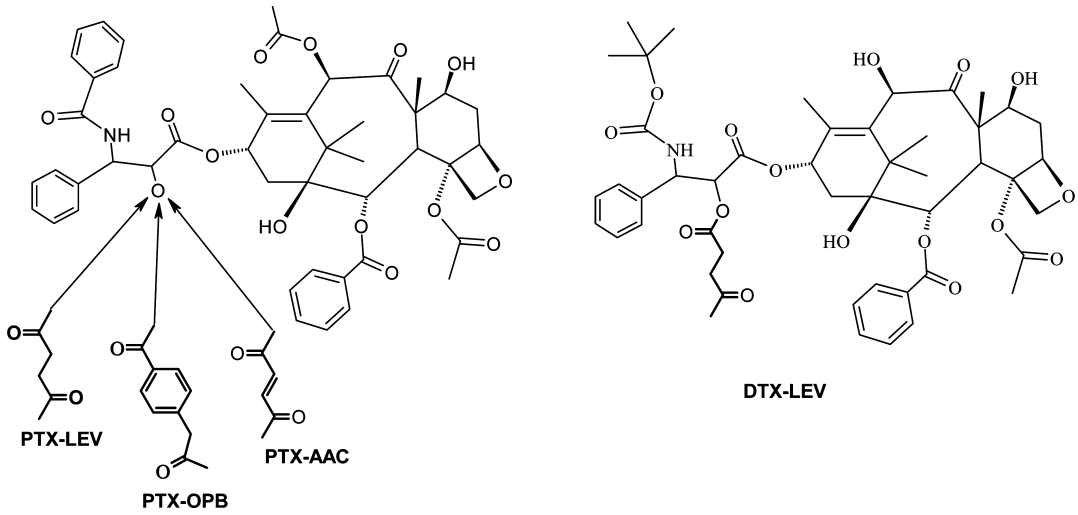

Figure 1. Structure of PTX and DTX derivatives.

drazinolysis of the methyl ester as described in ref 31. Mp 79−81 °C; elemental anal.: calcd, C 56.32, H 8.98, N 19.70; found, C 56.49, H 8.63, N 19.83.

The derivative of PTX with levulic acid (PTX-LEV, see Figure 1) was synthesized by esterification of the hydroxyl group on C2′ of PTX with levulic acid as follows: DCC (37.5 mg, 0.182 mmol) was dissolved in 200 μL of DMF, and levulic acid (19.37 mg, 0.166 mmol) was dissolved in 100 μL of DMF. Both solutions were mixed together and cooled for 20 min to −18 °C. Then a solution of PTX (100 mg, 0.117 mmol) and 14 mg of DMAP (0.117 mmol) in 300 μL of DMF was added. The reaction proceeded at 4 °C for 20 h, and its course was monitored by TLC (TLC plate with silica gel F254); R_f (PTX) = 0.60, R_f (PTX-LEV) = 0.48, R_f (levulic acid) = 0.80 using a mixture of ethyl acetate:hexane 2:1 as mobile phase. The reaction mixture was purified from free PTX on a column filled with silica gel 60 (column 2 × 30 cm, eluent ethyl acetate:hexane = 2:1) with UV detection (240 nm). Eluent containing pure compound (PTX-LEV) was collected, and the solvent was evaporated. The resulting waxy solid was triturated with diethyl ether, and the product was isolated by filtration. The yield was 86 mg (72%). The chemical structure of the PTX-LEV derivative was determined and proved by NMR spectroscopy. The derivative was distinguished by the presence of characteristic peaks in the ^{1}H NMR spectra: two multiplets corresponding to the O═C−CH_2−CH_2−C═O group were observed at 2.6 and 2.75 ppm (see detail spectra in Figure SI1 in the Supporting Information). The purity of PTX-LEV (95.4%) was determined by HPLC (peak at 11.86 min (λ = 240 nm)). MS (APCI): m/z 950.1 $[M - H]^-$.

The derivative of PTX with 4-(2-oxopropyl)benzoic acid (PTX-OPB, see Figure 1) was synthesized according to a similar procedure as was used for PTX-LEV: OPB (55 mg, 0.31 mmol) and DCC (100 mg, 0.46 mmol) were dissolved in a mixture of 1 mL of DMF and left at −18 °C for 20 min. Then PTX (200 mg, 0.23 mmol) and DMAP (28 mg, 0.23 mmol) dissolved in 0.6 mL of DMF were added, and the reaction mixture was left at 4 °C for 24 h. The reaction course was monitored by TLC (alufolien with Silica gel F254); R_f (PTX) = 0.60, R_f (PTX-OPB) = 0.70, R_f (OPB) = 0.30 using a mixture of ethyl acetate:hexane 2:1 as mobile phase. The reaction mixture was purified on a column filled with silica gel 60 (column 2 × 30 cm, eluent ethyl acetate: hexane = 2:1) with a UV detector (λ = 240 nm). PTX-OPB was obtained after evaporation of the solvent, washing with diethyl ether, filtration and drying under vacuum. The yield was 174 mg (71%). The chemical structure of the PTX-OPB derivative was determined and proved by NMR spectroscopy. The derivative was distinguished by the presence of characteristic peaks in the ^{1}H NMR spectra: additional signals from the aromatic ring were observed at 7.4−7.45 ppm (see detail spectra in Figure SI1 in the Supporting Information). HPLC showed 94% purity (peak maximum at 12.3 min). MS (APCI): m/z 1212.25 $[M - H]^-$.

The derivative of PTX with 3-(acetyl)acrylic acid (PTX-AAC, see Figure 1) was synthesized according to a similar procedure as was used for PTX-LEV: AAC (18.7 mg, 0.17 mmol) and DCC (37.5 mg, 0.26 mmol) were dissolved in a mixture of 0.4 mL DMF and left 20 min at −18 °C. Then PTX (100 mg, 0.12 mmol) and DMAP (14 mg, 0.12 mmol) dissolved in 0.3 mL of DMF were added, and the reaction mixture was left at 4 °C for 24 h. The course of the reaction was monitored by TLC (alufolien with silica gel F254); R_f (PTX) = 0.60, R_f (PTX-AAC) = 0.85, R_f (AAC) = 0.10, using a mixture of ethyl acetate:hexane 2:1 as mobile phase. The reaction mixture was purified on a column filled with silica gel 60 (column 2 × 30 cm, eluent ethyl acetate:hexane = 2:1) with a UV detector (240 nm). PTX-AAC was obtained after evaporation of the solvent, washing with diethyl ether, filtration and drying under vacuum. The yield was 71 mg (63%). The chemical structure of the PTX-OPB derivative was determined and proved by NMR spectroscopy.

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Table 1. Characteristics of Polymer Conjugates

polymer conjugate	drug derivative	M_w	M_w/M_n	drug content (wt %/mol %)	free drug (mol % of drug)	R_H[b] (nm)
1[a]		27000	1.67			4.3
2	PTX-LEV	33500	1.65	7.6/1.5	0.15	6.3
3	PTX-LEV	35400	1.71	14.8/2.9	0.13	6.4
4	PTX-OPB	33800	1.75	8.6/1.7	0.14	7.8
5	PTX-AAC	33000	1.70	8.8/1.7	0.21	6.1
6	DTX-LEV	34200	1.62	8.2/1.6	0.15	6.3
7	DTX-LEV	36100	1.54	16.3/3.2	0.22	6.4

[a] Polymer precursor with 5.7 mol % of hydrazide groups used for the synthesis of polymer conjugates. [b] Hydrodynamic radius in aqueous solution.

The derivative was distinguished by the presence of characteristic peaks in the ^{1}H NMR spectra: two doublets corresponding to the −CH═CH− protons were observed at 6.82 and 7.08 ppm (see detail spectra in Figure SI1 in the Supporting Information). HPLC showed 95.3% purity (peak maximum at 12.58 min). MS (APCI): *m*/*z* 948.33 [M − H]$^-$.

The derivative of DTX with levulic acid (DTX-LEV, see Figure 1) was synthesized accordingly to a similar procedure as was used for PTX-LEV: LEV (38.1 mg, 0.33 mmol) and DCC (100 mg, 0.51 mmol) were dissolved in 0.5 mL of DMF and left at −18 °C for 20 min. Then DTX (200 mg, 0.25 mmol) and DMAP (30 mg, 0.25 mmol) dissolved in 0.5 mL of DMF were added, and the reaction mixture was left at 4 °C for 24 h. The course of the reaction was monitored by TLC (alufolien with silica gel F254); R_f (DTX) = 0.65, R_f (DTX-LEV) = 0.90, R_f (LEV) = 0.60 using ethyl acetate as mobile phase. The reaction mixture was purified on a column filled with silica gel 60 (column 2 × 30 cm, eluent ethyl acetate) with a UV detector (240 nm). DTX-LEV was obtained after evaporation of the solvent, washing with diethyl ether, filtration and drying under vacuum. The yield was 74 mg (68%). The chemical structure of the PTX-OPB derivative was determined and proved by NMR spectroscopy. The derivative was distinguished by the presence of characteristic peaks in the ^{1}H NMR spectra: a strong signal at 1.46 ppm from the tBu group was observed (see detail spectra in Figure SI2 in the Supporting Information). HPLC showed 95.3% purity (peak maximum at 12.58 min). MS (APCI): *m*/*z* 904.25 [M − H]$^-$.

The purity of the monomers and derivatives was examined by a Shimadzu HPLC system equipped with a reverse-phase column (Chromolith Performance RP-18e; 100 × 4.6 mm; water−acetonitrile; gradient, 0−100% acetonitrile) with UV−vis detection (Shimadzu SPD-M10A vp) (240 nm).

Synthesis of Polymer Precursors. Random copolymers of HPMA with Ma-ah-NHNH$_2$ containing free hydrazide groups were prepared by radical copolymerization of the monomers in methanol (AIBN, 0.6−1.0 wt %; monomer concentration 18 wt %; molar ratio HPMA:Ma-ah-NHNH$_2$ 93:7; 60 °C; 17 h). Example of polymerization: HPMA (2.0 g, 14 mmol), Ma-ah-NHNH$_2$ (227 mg, 1.06 mmol) and AIBN (96 mg, 0.58 mmol) were dissolved in methanol (12.7 mL). The solution was introduced into a polymerization ampule, bubbled with nitrogen, and sealed. The polymerization was carried out at 60 °C for 17 h. The polymer was isolated by precipitation into ethyl acetate and purified by reprecipitation from methanol solution into ethyl acetate. The polymer was filtered off, washed with ethyl acetate, and dried in vacuum. The yield was 1.76 g (78.5%).

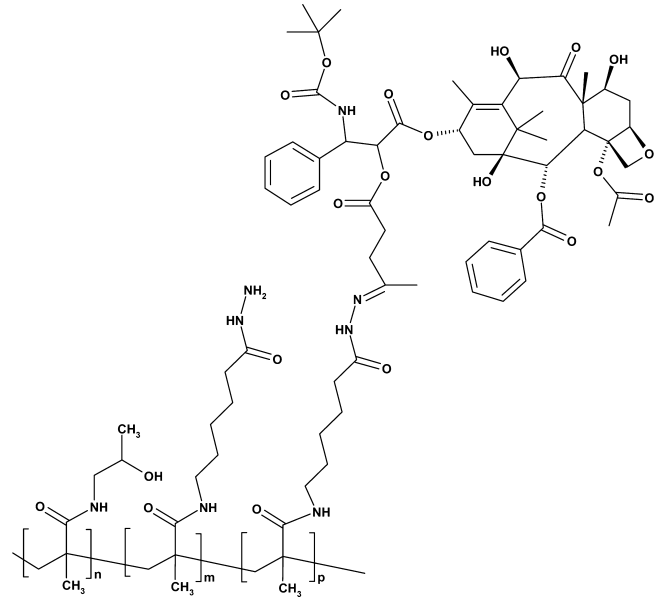

Figure 2. Schematic structure of polymer conjugate **6** with DTX-LEV derivative.

Attachment of PTX or DTX Derivatives to Polymer Precursors. Polymer−drug conjugates (conjugates **2**−**7**; Table 1, Figure 2) were prepared by the reaction of polymer precursor **1** containing hydrazide groups with derivative of PTX or DTX in methanol in the dark. The polymer precursor was dissolved in anhydrous methanol to a 10 wt % solution, and 30 or 60 mol % of PTX or DTX derivative (relative to the hydrazide groups content) was added under stirring. The reaction was performed for 2 h after addition of acetic acid (50 μL/mL of reaction mixture). The polymer conjugate was isolated, purified and fractionated by multiple gel permeation chromatography (Sephadex LH-20, column 1.5 × 60 cm, eluent methanol). The highest and the lowest molecular weight fractions were removed, methanol was evaporated and the conjugate was isolated by precipitation into diethyl ether.

Purification and Characterization of Polymers and Conjugates. All the conjugates were characterized and tested for the content of free polymer or free drug using a HPLC Shimadzu equipped with GPC columns Superose 6 or

TSKgel G3000SWxl and TLC (Kieselgel 60 F254). In addition, the content of free PTX, DTX or its derivatives was determined by HPLC Shimadzu after extraction of the respective drug from aqueous solution of the conjugate to chloroform.

The total content of derivatives of PTX or DTX in polymer conjugates was determined by HPLC Shimadzu system after complete hydrolysis of the polymer conjugates in HCl solution (pH 2) for 1 h at 37 °C and extraction of derivatives to chloroform. No evidence of by product formation during analysis was detected.

Determination of molecular weight and polydispersity of the conjugates was carried out with a HPLC Shimadzu system equipped with RI, UV and multiangle light scattering DAWN EOS (Wyatt Co., USA) detectors using (A) mobile phase 20% of 0.3 M acetate buffer (CH_3COONa/CH_3COOH; pH = 6.5; 0.5 g/L NaN_3) and 80% of methanol and TSKgel G3000SWxl column or (B) mobile phase 0.3 M acetate buffer pH 6.5 and Superose 6 column.

The content of hydrazide groups in polymer precursors was determined by a modified TNBSA assay as described.[32] Molar absorption coefficient $\varepsilon_{500} = 17\ 200\ L\ mol^{-1}\ cm^{-1}$ (λ = 500 nm) estimated for the model reaction of MA-ah-$NHNH_2$ with TNBSA was used.

The dynamic (DLS) light scattering of aqueous conjugates solutions was measured at the scattering angle 173° on a Nano-ZS, model ZEN3600 (Malvern, U.K.) zetasizer. The hydrodynamic radius (R_h) was determined by the DTS (Nano) program.

Chemical structure of the particular samples was affirmed by NMR spectrometer Bruker 600MH Avance III (5 mm NMR tubes were used): ^1H and ^{13}C NMR of 5% w/v DMSO-d_6 solution. The integrated intensities were determined with the spectrometer integration software with accuracy of ±1%. The temperature was maintained constant within ±0.2 K with BVT 3000 temperature unit. H-DMSO was used as internal standard. Typical measurement conditions for ^1H were as follows: spectral width 5 kHz, pulse width 10 μs (90° pulse), relaxation delay 10 s, 32 scans at temperature 300 K. Typical measurement conditions for ^{13}C NMR spectra were as follows: 150 MHz operating frequency, relaxation delay 10 s, 90° pulse width 8 μs, spectral width 36 kHz, 8000 scans. MS spectra were measured by LCQ Fleet spectrometer (Thermofisher Scientific).

***In Vitro* Release of Drugs from Polymer−Drug Conjugates.** The rate of PTX or DTX release, free and/or ester derivatives, from the polymer conjugates was investigated by incubation of the conjugate in phosphate buffers at pH 5.0 or 7.4 (0.1 M phosphate buffer with 0.05 M NaCl) or in human plasma at 37 °C. The concentration of the conjugate in solution was equivalent to 1.10^{-4} mM PTX or DTX. The amount of released drugs and their ester derivatives was determined by HPLC analysis after their extraction into organic solvent. Analysis was performed on an HPLC instrument (Shimadzu, Japan) using a reverse-phase column (Chromolith Performance RP-18e; 100 × 4.6 mm) with UV detection at 240 nm, an eluent of water−acetonitrile with an acetonitrile gradient of 0−100 vol %, and a flow rate of 0.5 mL/min. All drug-release data are expressed as the amounts of free drug relative to the total drug content in the conjugates. All experiments were carried out in triplicate.

Cancer Cell Lines. Murine T cell EL4 lymphoma (TIB-39) and mammary carcinoma 4T1 (CRL-2539) were purchased from the American Type Culture Collection (ATCC). The EL4 cells were cultured in RPMI-1640 medium supplemented with 4 mM L-glutamine, 1 mM Na-pyruvate, 4.5 g/L glucose, antibiotics (pen/strept, Sigma, USA), and 10% fetal calf serum (Gibco BRL). The 4T1 cells were cultivated in the same medium as EL4 additionally supplemented with 10 mM HEPES.

Mice. C57BL/6 (B/6) and BALB/c mice were obtained from the breeding colony of the Institute of Physiology ASCR, vvi (Prague, Czech Republic). Mice were used at 8−12 weeks of age, housed in accordance with approved guidelines and provided with food and water *ad libitum*. The Animal Welfare Committee of the Institute of Microbiology ASCR, approved all experiments.

***In Vivo* Tumor Models.** B/6 males were subcutaneously (sc) transplanted with 1×10^5 EL4 T cell lymphoma cells on the right shaven flank on day 0. 4T1 carcinoma (1×10^6) was sc transplanted to female BALB/c mice. The mice that developed palpable tumors reaching 6−8 mm in diameter within 8−9 days after the implantation were intravenously (iv) injected with polymer conjugates or with free drugs for controls as described in the Results and Discussion. The polymer conjugates were dissolved in 200 μL of PBS. The free drugs and their derivatives were dissolved in 40 μL of ethanol (20 μL) and Cremophor EL (Sigma) (20 mg) mixture and were further diluted up to 200 μL of PBS. The doses referred to hereinafter are expressed as mg of drug equivalent per kg. Control mice were transplanted with tumor cells and left untreated. The animals (8 mice per group) were observed three times a week for signs of tumor progression and acute toxicity. Tumor size, body weight, survival time and number of long-term survivors were determined. In order to demonstrate development of tumor resistance, the cured mice were retransplanted with the same tumor cells (1×10^5 EL4 cells or 1×10^6 4T1 cells) at a time specified in the Results and Discussion and left untreated. Tumor progression and survival time were monitored.

Results and Discussion

Recently, we have shown[32,33] that water-soluble HPMA-based copolymers containing the anticancer drug doxorubicin

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(33) Krakovičová, H.; Etrych, T.; Ulbrich, K. HPMA-based polymer conjugates with drug combination. *Eur. J. Pharm. Sci.* **2009**, *37*, 405–412.

(DOX) or a combination of DOX and the anti-inflammatory drug dexamethasone bound to the polymer via a pH-sensitive hydrazone linkage exhibit a significant therapeutic effect in murine experimental models of EL4 or 38C13 lymphomas. All these hydrazone-based conjugates are fairly stable in a phosphate buffer of pH 7.4, modeling conditions during transport in the bloodstream, but rapidly release free drugs in buffers simulating a mildly acidic environment in endosomes of target cells (pH 5−5.5). Moreover, in contrast to lysosomotropic prodrugs, these conjugates showed highly cytotoxic effects also in the erythromyeloid leukemia cell line K562 with a limited content of lysosomes where the antiproliferative activity of lysosomotropic conjugates was very low.[34]

Here, we present a study on the synthesis, physicochemical behavior and anticancer activity of new water-soluble anticancer polymer prodrugs based on HPMA copolymer conjugates with PTX and DTX derivatives. The described polymer prodrugs were designed as drug delivery systems for highly hydrophobic drugs to enable specific pH-controlled release of drugs or drug derivatives in endosomes of tumor cells or already in the tumor tissue, since the tumor microenvironment is acidic by nature.[35,36] PTX and DTX esters were attached to the polymer carrier via spacers containing pH-sensitive hydrazone bonds. The esters were prepared by esterification of the C2′ hydroxyl group with three keto acids, levulic acid, 4-(2-oxopropyl)benzoic acid or 3-(acetyl)acrylic acid. All the esters were attached to the polymer carrier via a spacer containing a 6-aminohexanoic acid residue and a hydrazone bond.

The physicochemical properties of these polymer conjugates, namely, the radius of the polymer coil in aqueous solution, molecular weight of polymer conjugates and stability of the conjugates incubated in aqueous solutions of different pH, were studied. Also, *in vivo* biological properties of polymer prodrugs were studied in detail using EL4 T cell lymphoma and 4T1 breast carcinoma experimental tumors.

Synthesis of Ester Derivatives of PTX and DTX. The polymer precursor, polymer **1**, was prepared by radical copolymerization of HPMA with a comonomer containing a free hydrazide group suitable for attachment of molecules containing a keto group via a pH-sensitive hydrazone bond. Primarily, the possibility of direct conjugation of PTX and DTX with the polymer precursor via the 9-oxo (keto) group was verified. Unfortunately, the yield of the reaction after 1 day was low (less than 0,5% at room temperature and less than 6% at 50 °C), probably due to the sterically hindered keto group. Because of this, we used an alternative strategy for the synthesis of polymer−drug conjugates based on PTX and DTX derivatives containing keto groups more suitable for conjugation with the hydrazide group-containing polymer precursor. PTX and DTX were esterified with keto acids to form derivatives with a reactive keto group and a potentially hydrolyzable ester bond. Three keto acids, two aliphatic (levulic acid and 3-(acetyl)acrylic acid) and one aromatic (4-(2-oxopropyl)benzoic acid), were selected as promising candidates for this synthetic strategy.

The levulic acid derivatives, PTX-LEV and DTX-LEV, were prepared by esterification of the primary hydroxyl group at C2′ of PTX or DTX with the carboxyl group of levulic acid. The moderately low yield of the reaction (67−71%) was caused by a competing reaction, the formation of diester derivatives of the drugs acylated on the hydroxyls at both the C2′ and C7 positions. The product was purified from free PTX, DTX, levulic acid diester and other components by preparative chromatography on silica. Approximately 95% purity of products was determined by HPLC analysis with UV detection. MS and NMR confirmed the structures.

The other derivatives, PTX-OPB and PTX-AAC, were prepared by the same method as the levulic acid derivatives. Also in this case, purification on a column with silica was necessary to obtain pure products. The yield of the reaction was around 70%. The derivatives of PTX with OPB and AAC were determined by HPLC to be 95.3% and 94% pure, respectively. The structures were confirmed by NMR and MS as mentioned above.

Synthesis of the Polymer Precursor and Polymer−Drug Conjugates. The polymer precursor **1** was prepared by radical solution copolymerization of HPMA with MA-ah-$NHNH_2$ using AIBN as initiator and methanol as solvent. The average molecular weight (M_w) of polymer **1** (27 000 g/mol) was under the limit of the renal threshold. We assume that this polymer is excretable from organisms by glomerular filtration after fulfilling its mission. The polymer precursor contained 5.7 mol % of hydrazide groups, a content sufficient for attachment of drug derivatives to the polymer precursor.

The ester derivatives of PTX or DTX were attached to the polymer **1** by a hydrazide−ketone condensation reaction in methanol in the presence of acetic acid (50 μL/mL). Attachment of the derivatives to the polymer precursor was quite rapid; within half an hour more than 90% of the derivatives were attached to the polymer precursor. The polymer conjugates purified by gel filtration contained less than 0.2 mol % of free PTX or DTX derivatives. The yield of the conjugation reaction reached 96 to 98% for all the derivatives. Polymer conjugates with PTX or DTX content ranging from 7.6 to 16.3 wt % were prepared.

The molecular weight, index of polydispersity, hydrodynamic radius and drug loading of polymer conjugates **2**−**7** is shown in Table 1. A small increase in the M_w of the polymers was observed after conjugation with the drugs, with more pronounced effects in the cases of polymer conjugates **3** and **7** containing the highest PTX and DTX loadings.

(34) Říhová, B.; Etrych, T.; Pechar, M.; Jelínková, M.; Šťastný, M.; Hovorka, O.; Kovář, M.; Ulbrich, K. Doxorubicin bound to a HPMA copolymer carrier through hydrazone bond is effective also in a cancer cell line with a limited content of lysosomes. *J. Controlled Release* **2001**, *74*, 225–232.

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(36) Wouters, B.; Weppler, G.; Koritzinsky, M.; Landuyt, W.; Nuyts, S.; Theys, J.; Chiu, R. K.; Lambin, P. Corresponding Hypoxia as a target forcombined modality treatment. *Eur. J. Cancer* **2002**, *38*, 240–257.

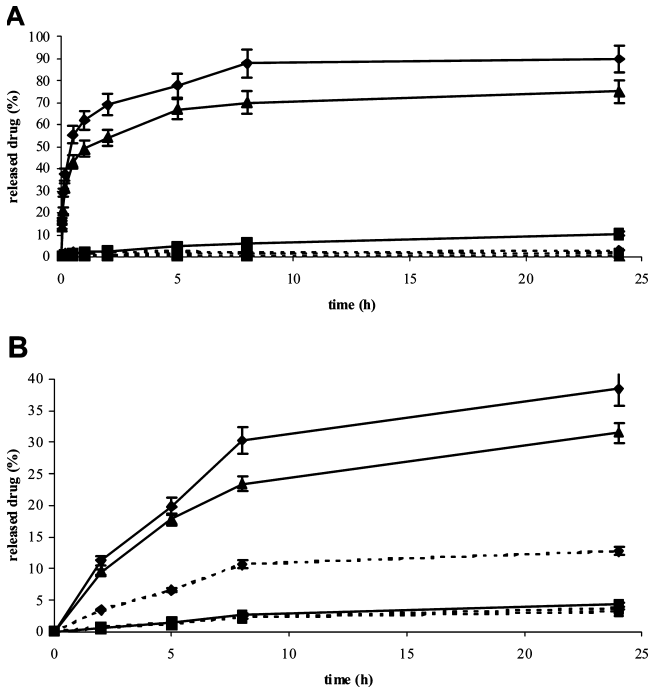

Figure 3. Release of PTX and PTX derivatives from polymer conjugates **2**, **4** and **5** incubated in phosphate buffers at pH 5 (A) and pH 7.4 (B) at 37 °C: (◆ —) conjugate **2**, PTX-LEV and PTX; (▲ —) conjugate **4**, PTX-OPB and PTX; (■ —) conjugate **5**, PTX-AAC and PTX; (◆ ---) conjugate **2**, PTX; (▲ ---) conjugate **4**, PTX; (■ ---) conjugate **5**, PTX; $n = 3$.

Nevertheless, molecular weight remained under the renal threshold found for HPMA polymers (50 kDa). The polydispersity of the polymers was not significantly influenced by the conjugation. Surprisingly, more pronounced increases in R_H not corresponding with the changes in M_w were observed, rising from 4.3 nm determined in the polymer precursor to 7.8 nm found in the conjugate with the most hydrophobic moiety PTX-OPB (see Table 1.). This indicates an ability of the hydrophobic polymer-bound moiety to generate intramolecular and intermolecular hydrophobic interactions resulting in changes of size and shape of the polymer coil in aqueous solution. Differences in physicochemical behavior of the polymer conjugates with various derivatives of PTX or DTX could be ascribed to different hydrophobic contributions of the attached drugs to such interactions.

Release of Drugs from Polymer Conjugates. The *in vitro* release profiles of PTX and its ester derivative from polymer conjugates **2** and **4** showed that the rate of release of the drug ester or free drug at pH 7.4 (37 °C) was much lower than that at pH 5 (Figure 3). After 2 h of incubation of polymer conjugate **2** at pH 7.4, 12% of PTX and PTX-LEV in total was released, while at pH 5 almost 70% of liberated PTX-LEV was determined after the same time of incubation. At pH 7.4 the hydrazone bond was cleaved faster within the first 8 h of incubation, with the rate decreasing in parallel with the decreasing concentration of hydrazone bonds in the solution. Subsequently, free PTX was released slowly by spontaneous hydrolysis of the ester bond of the PTX derivative. At pH 5 the hydrazone bond was cleaved very quickly, and ca. 90% of PTX-LEV was released within the first 8 h of incubation of polymer conjugate **2**. No evidence of hydrolysis of the ester bond in PTX-LEV was observed during 24 h of incubation at pH 5.

The *in vitro* release experiment carried out with polymer conjugate **4** showed that the rate of release of PTX and PTX-OPB is slightly lower than in polymer conjugate **2** described above at both pH values (Figure 3). Also, the release profiles obtained for polymer conjugate **4** have shown that the rate of drug release at pH 7.4 is much lower than that at pH 5. After 2 h of incubation at pH 7.4, only 9% of PTX-OPB was released, which is 20% less than the PTX-LEV released from conjugate **2**. At pH 5 around 70% of released PTX-OPB was determined after 8 h incubation, again slightly less (by 20%) than in the case of PTX-LEV. The difference in the rate of release of PTX-LEV and PTX-OPB can be explained by the presence of the aromatic ring in the spacer between the drug and polymer precursor. Its electron donating effect stabilizes not only the hydrazone bond but also the ester bond in the PTX derivative. Consequently, less free PTX was released during incubation at both pH values.

Completely different results of drug release measurements were observed for polymer conjugate **5**. The results showed that the rate of PTX or PTX-AAC release from polymer conjugate **5** was very low (up to 10% after 24 h) at both pH values (Figure 3). At pH 5 mainly PTX-AAC derivative was released, in contrast to pH 7.4, at which free PTX dominated in the incubation buffers. We suppose that the large difference in the rate of release of PTX-AAC and PTX-LEV or PTX-OPB was influenced by the presence of the double bond in the spacer between the drug and polymer precursor. The presence of the double bond in the β-position relative to the hydrazone bond leads to conjugation of the electrons in the double bond, in the carbonyl bond and at nitrogen, thus strongly increasing the stability of the hydrazone bond. A similar effect stabilizes the ester bond in the PTX-AAC derivative, and this is why less free PTX was found during incubation at both pH values.

Finally, the *in vitro* release experiment carried out with polymer conjugates **6** and **2** showed that there was no significant difference between the rate of release of DTX and DTX-LEV from conjugate **6** and PTX-LEV from conjugate **2** measured at both pH values (compare Figure 3 and Figure 4). As in the case of the other hydrazone-based conjugates, the rate of release of drugs at pH 7.4 was much lower than at pH 5. After 2 h of incubation in pH 7.4 buffer, only 11% of DTX-LEV was released, the same amount as from conjugate **2**. At pH 5 around 78% of released DTX-LEV was detected after 8 h. The loading of the drugs in the conjugates had no significant effect on the drug release profiles (see Figure SI3 in the Supporting Information). The *in vitro* release experiment carried out with polymer conjugates **6** in human plasma showed the same release profiles as observed in phosphate buffer at pH 7.4 (see Figure SI4 in the Supporting Information).

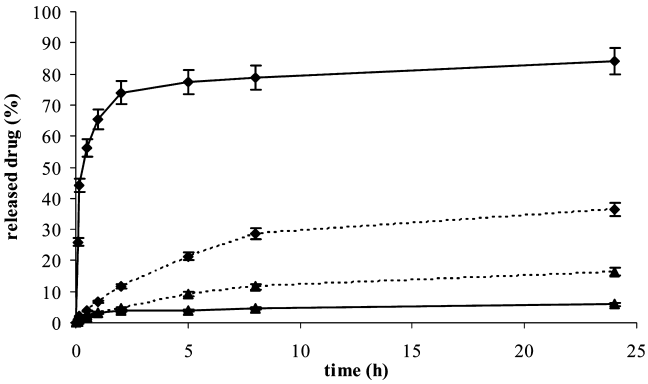

Figure 4. Release of DTX and DTX-LEV from polymer conjugate **6** incubated in phosphate buffers at 37 °C. (◆ —) DTX and DTX-LEV, pH 5; (▲ —) DTX, pH 5; (◆ ---) DTX and DTX-LEV, pH 7.4; (▲ ---) DTX, pH 7.4; $n = 3$.

A prerequisite for effective drug delivery is stability of the system during its transport in the blood and release of the drug at the site of action. Polymer conjugates **2** and **6** containing PTX-LEV or DTX-LEV better fulfill this requirement, as demonstrated by the higher difference in the stability in model solutions at pH 5 and 7.4. This was why polymer conjugates **2**, **6** and **7** were selected for further *in vivo* biological evaluation.

In Vivo **Anticancer Activity of Paclitaxel-Containing Conjugate in Breast Cancer Model.** PTX is a frequent component of chemotherapeutic protocols for the treatment of advanced breast cancer. Hence, we explored a murine model of mammary carcinoma 4T1 to test *in vivo* anticancer activity of PTX-containing conjugate **2**. A therapeutic regime of drug administration was used, i.e., the treatment started after the subcutaneous tumors developed to a palpable size of 6−8 mm in diameter. Free drugs (PTX and PTX-LEV) were dissolved in an ethanol−Cremophor EL mixture diluted with PBS. The volume of the drug solution (40 μL) turned out to be a dose-limiting parameter due to acute ethanol toxicity and limited drug solubility. The drug administration in the ethanol−Cremophor EL/PBS vehicle caused short-term ethanol intoxication, but significant flatness of mice was apparent even overnight. Due to signs of toxicity observed after the iv administration of the first dose of PTX, the second dose was injected intraperitoneally. In contrast to free drugs, HPMA-based conjugates of PTX and DTX were easily solubilized in PBS, and their solubility did not impose any limitation on the *in vivo* dosage.

The growth of 4T1 tumors was moderately decreased by free PTX and its derivative PTX-LEV and considerably retarded by the action of the conjugate **2** (Figure 5A). Body weight of the animals treated with PTX and PTX-LEV paralleled that recorded in the untreated controls. In this model, the tumor growth that normally should manifest as body weight increase was balanced with clear cachectic symptoms of the mice caused by progressive tumor growth and metastatic spread. The mice treated with conjugate **2** only showed moderate increases in body weight because no obvious cachectization occurred, implying limited side toxicity of the conjugate **2** (Figure 5B). Free PTX-treated mice (2 × 30 mg/kg, administered on days 8 and 12) died of progressive disease, and their survival was comparable with that of the untreated controls. The free PTX induced disease-free survival in only one treated mouse. The PTX-LEV derivative (2 × 30 mg/kg, days 8 and 12) was ineffective in all mice even though both the treatment doses were administered intravenously. On the contrary, the conjugate **2** (2 × 60 mg PTX equiv/kg, administration at days 8 and 12) induced complete curing of the 4T1 carcinoma in 3 of 8 treated mice. Survival of the other mice was similar to survival of the untreated controls (Figure 5C).

Notably, second transplantation of the treated mice with lethal doses of 4T1 cells without any treatment revealed resistance in all the cured animals, as they did not develop tumors and survived (Figure 5D). Thus, the complete cure was associated with the development of immunologically mediated long-lasting resistance, a phenomenon we regularly see in EL4 lymphoma-bearing mice treated with various HPMA-based conjugates of DOX bound to the polymer via hydrazone[37] or amide bonds.[38,39] The immunoprotective character of treatment with DOX-containing HPMA copolymer conjugates leading to mobilization of immune anticancer response has already been suggested in murine experimental tumors[40] and even in patient clinical trials.[41] Thus, now we demonstrate the same important feature of HPMA-based polymer prodrugs in a conjugate containing cytostatic drugs other than DOX. The resistance found in one animal cured from the 4T1 carcinoma with the free PTX could be explained by the fact that the free drug was used at suboptimal dose, producing no serious side toxicity toward the immune system. However, while the conjugate dosage could be further elevated, the dose of the free drug was the

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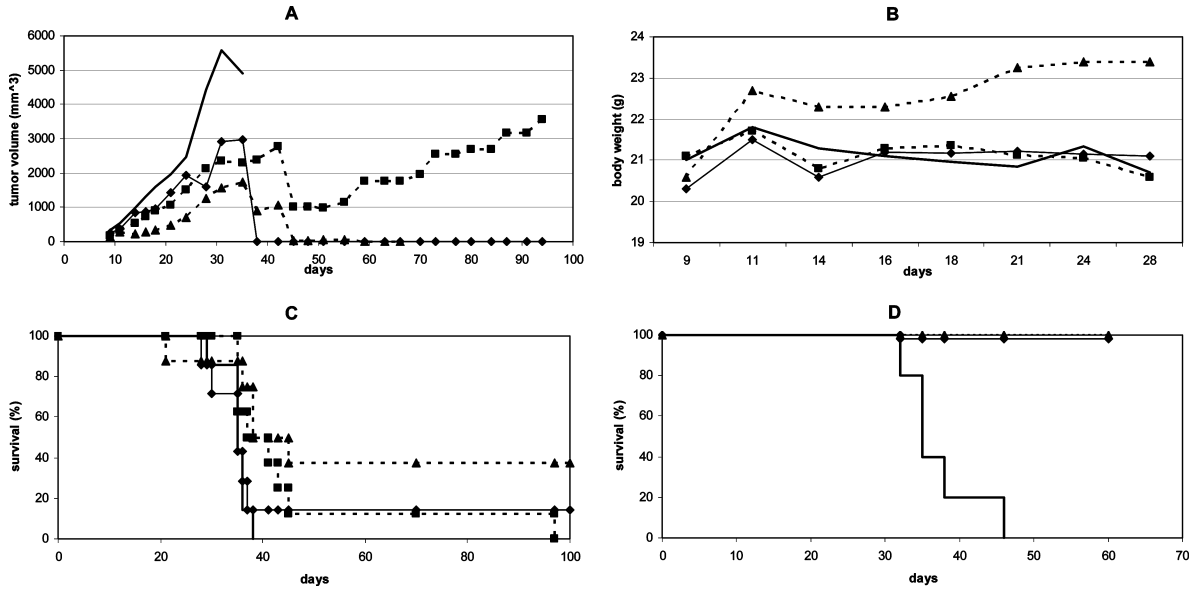

Figure 5. The antitumor efficacy, acute toxicity and survival after retransplantation of PTX, PTX-LEV and conjugate **2**-treated BALB/c mice with 4T1 breast carcinoma. Female BALB/c mice were transplanted sc with 1×10^6 4T1 cells and treated with (◆ —) PTX (2×30 mg/kg), (■ ---) PTX-LEV (2×30 mg/kg), or (▲ ---) conjugate 2 (2×60 mg/PTX equiv/kg), iv drug administration on days 8 and 12. Control mice (—) were left untreated. Tumor growth (A), body weight (B), and overall survival (C) were monitored. The surviving mice (130 days) were retransplanted with 5×10^5 4T1 cells and left untreated. Survival time (D) was monitored.

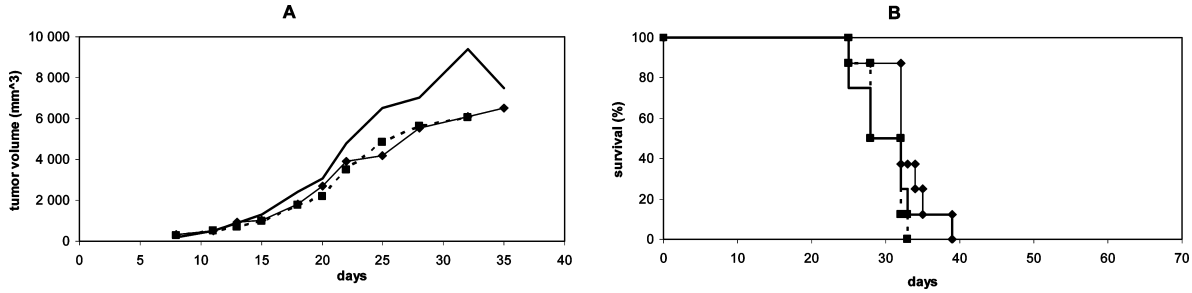

Figure 6. The antitumor efficacy of PTX-LEV and conjugate **2** in EL4 lymphoma-bearing C57BL/6 mice. C57BL/6 mice were transplanted sc with 1×10^5 EL4 cells and treated with (◆ —) PTX-LEV (2×30 mg/kg) or (■ ---) conjugate **2** (2×60 mg/PTX equiv/kg), iv drug administration on days 8 and 12. Control mice (—) were left untreated. Tumor growth (A) and overall survival (B) were monitored.

highest attainable dose due to the low solubility and limited applicable volume of the vehicle.

Antitumor Capacity of PTX- and DTX-Containing Conjugates in EL4 Lymphoma Model. Next, we tested the antitumor efficacy of conjugates **2** and **6** in an EL4 lymphoma model. We could not inject the mice with free PTX because of its low solubility. C57BL/6 mice are more robust than BALB/c mice, and thus the higher amount of drug needed was impossible to solubilize in a still tolerable volume of the ethanol−Cremophor EL mixture. Neither the PTX-LEV derivative nor the PTX-containing conjugate showed antitumor efficacy in the EL lymphoma model (Figure 6A,B).

On the contrary, conjugate **6** (containing DTX) efficiently reduced the EL4 lymphoma tumor growth (Figure 7A) and induced complete curing of one mouse (2×20 mg DTX equiv/kg iv on days 8 and 12) and even 7 mice (2×40 mg DTX equiv/kg iv on days 8 and 12) of the group of 8 mice (Figure 7C). Free DTX at a dose of 2×20 mg/kg iv administered on days 8 and 12 also reduced the tumor growth and was able to induce complete tumor regression in 4 of 8 mice. The derivative DTX-LEV at the same dosage was less active and induced complete curing of only one of the 8 treated mice (Figure 7A,C). Side toxicity evaluated as body weight change was minimal in all the treatments. Acute toxicity of the vehicle itself and of free DTX was checked in naive mice (Figure 7B) and was found to be acceptable.

All the cured mice were retransplanted with the same (lethal) dose of EL4 cells 10 weeks after the first transplantation. Five of the 7 mice cured with the higher dose of the DTX-containing conjugate **6** proved resistant in addition to the one mouse cured with the lower conjugate dose. Also, 3 of the 4 mice cured with the free DTX did not develop tumors and thus were resistant (Figure 7D). This indicates the nontoxic nature of the treatment with conjugate **6**. Due to the absence of body weight decrease recorded in mice

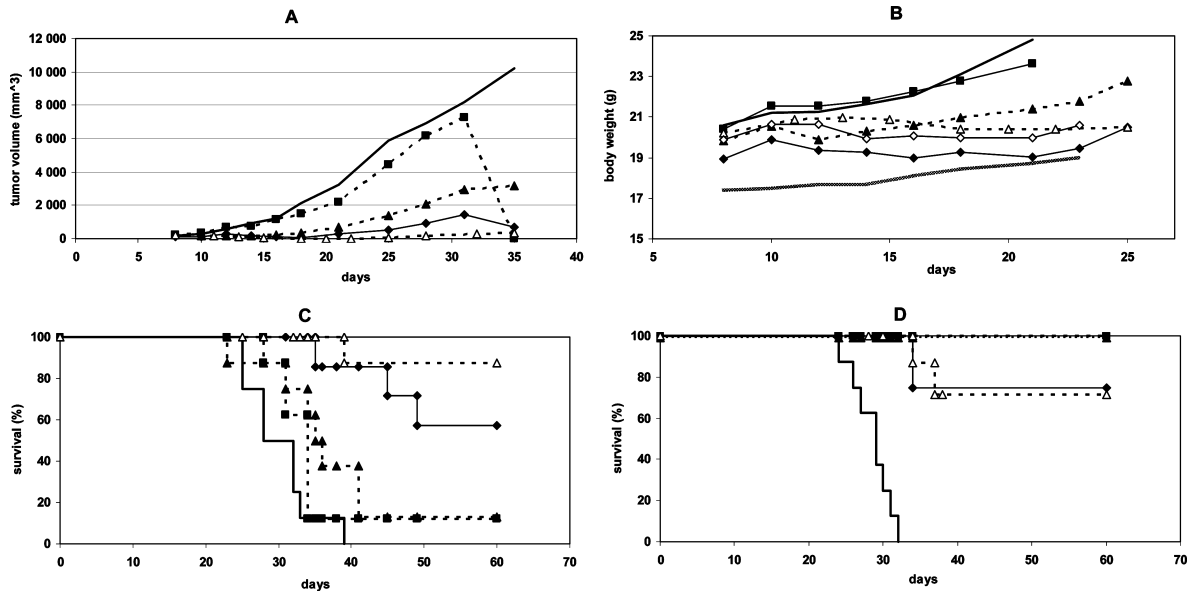

Figure 7. The antitumor efficacy, acute toxicity and survival after retransplantation of DTX, DTX-LEV and conjugate **6**-treated EL4 lymphoma-bearing C57BL/6 mice. C57BL/6 mice were transplanted sc with 1×10^5 EL4 cells and treated with (◆ —) DTX (2×20 mg/kg), (■ ---) DTX-LEV (2×20 mg/kg), (▲ ---) conjugate **6** (2×20 mg/DTX equiv/kg) or (Δ ---) conjugate **6** (2×40 mg/DTX equiv/kg), iv drug administration on days 8 and 12. Control mice (—) were left untreated. Tumor growth (A), body weight (B) ((○ ---) DTX (2×20 mg/kg), naive mice; (—) solvent 0.2 mL of ethanol + Cremophor/PBS, naive mice), and overall survival (C) were monitored. The surviving mice (70 days) were retransplanted with 1×10^5 EL4 cells and left untreated. Survival time (D) was monitored.

treated with free DTX, we assume that this treatment was not destructive toward the anticancer immune mechanisms. The MTD of DTX is 40 mg/kg over a 3 h iv infusion,[42] and thus we used a dose well below the MTD. We have shown that the EL4 lymphoma induced a moderate immune anticancer response even without treatment.[43] Hence, a treatment that is nontoxic to the immune system could lead to a complete cure followed by development of tumor resistance in a proportion of the treated animals. However, while the dose of the conjugate could be safely elevated over those we used in the experiment, the dose of the free drug could be hardly increased because it was strictly limited by the amount of drug that could be dissolved in a tolerable volume of the vehicle.

Recently, it was demonstrated that a DOX-containing HPMA-based polymer prodrug could promote tumor cell death accompanied with expression of danger stimuli, such as chaperone (calreticulin and heat shock proteins) expression on the cell surface and HMGB1 protein release from the dying cells.[43] Calreticulin stimulates engulfment of tumor-derived antigens,[44] and HMBG1 increases maturation of dendritic cells,[45] thus inducing antitumor immune response. It was shown that paclitaxel could also exert immunomodulatory activity and thus augment antitumor response.[46,47]

As the conjugates **2**, **6** and **7** release the drug in a similar manner to the DOX-containing conjugate, we can assume that the drugs could also act as inducers of immunogenic cell death. We demonstrate here that the treatment is not accompanied by acute toxicity, and is not harmful to the immune anticancer response. The hypothetical induction of immunogenic tumor cell death could be a significant added advantage of such a therapy.

Effect of HPMA Conjugates Containing Different Content of the Bound DTX. Two conjugates bearing various contents of the drug linked to the polymer backbone (conjugate **6**, 8.2 wt %, and conjugate **7**, 16.3 wt %) were tested in the EL4 lymphoma model using a suboptimal treatment dose (2×30 mg DTX equiv/kg iv, treatment on

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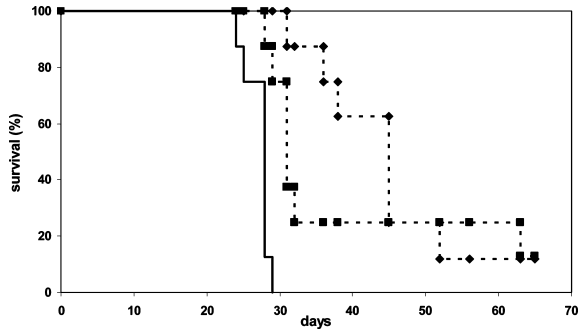

Figure 8. The antitumor efficacy of conjugates **6** and **7** in EL4 lymphoma-bearing C57BL/6 mice. C57BL/6 mice were transplanted sc with 1×10^5 EL4 cells and treated with (◆ ---) conjugate **6** (2×30 mg/DTX equiv/kg), (■ ---) conjugate **7** (2×30 mg/DTX equiv/kg), iv drug administration on days 9 and 13. Control mice (—) were left untreated.

days 8 and 12). Both conjugates induced some retardation of the tumor growth and induced complete curing in one of the 8 treated mice. However, significantly longer survival was recorded only in mice treated with the conjugate **6** that has a lower drug content (Figure 8). This could be explained by the greater hydrophobic character of conjugate **7** caused by the high drug content that probably resulted in lower bioavailability of the drug for target cells *in vivo*. Previously, we have tested similar conjugates of DOX and also observed decreased *in vivo* antitumor activity in conjugates with DOX content exceeding 10−13% when compared with those containing less DOX bound to the HPMA carrier.[48] The conjugates with the higher DOX amount had demonstrably more hydrophobic character.[31]

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Conclusions

The synthesis and properties of the linear pH-sensitive conjugates of paclitaxel and docetaxel with *N*-(2-hydroxypropyl)methacrylamide copolymer carrier designed for site-specific cancer therapy have been described. In the conjugates, derivatives of anticancer drugs paclitaxel and docetaxel were attached to the polymer carrier *via* the hydrolytically unstable hydrazone linkage. The conjugates are relatively stable at the pH of blood (7.4) and do release active drug under mildly acidic conditions (pH 5) modeling endosomal and lysosomal environments in the cells. Complete regression of established tumors accompanied by durable tumor resistance in a significant proportion of the cured animals was achieved. While the treatment of mice bearing model 4T1 mammary carcinoma or EL4 Tcell lymphoma with free drugs or their esters showed signs of acute toxicity and did not exhibit any significant antitumor effect, the treatment with HPMA copolymer conjugates was free of side toxicity in both tumor models. It was demonstrated that HPMA copolymer conjugates represent very potent polymer prodrugs suitable for effective tumor delivery and treatment.

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Supporting Information Available: Information concerning NMR spectra of newly synthesized derivatives of PTX and DTX (Figures SI1 and SI2) and two figures (SI3 and SI4) concerning *in vitro* release in phosphate buffers at pH 5 and pH 7.4 or in human plasma at 37 °C. This material is available free of charge via the Internet at http://pubs.acs.org.

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