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Research Paper

Preclinical Evaluation of Linear HPMA-Doxorubicin Conjugates with pH-Sensitive Drug Release: Efficacy, Safety, and Immunomodulating Activity in Murine Model

Milada Sirova,[1,3] Tomas Mrkvan,[1] Tomas Etrych,[2] Petr Chytil,[2] Pavel Rossmann,[1] Marketa Ibrahimova,[1] Lubomir Kovar,[1] Karel Ulbrich,[2] and Blanka Rihova[1]

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Purpose. *In vivo* efficacy and safety of HPMA-based copolymers armed with doxorubicin via a spacer containing pH-sensitive linkage that can be prepared within a broad range of attached drug contents (1) was tested in murine tumor models.
Methods. Mice bearing T cell lymphoma EL4 or B cell lymphoma 38C13 were treated with a single dose of the conjugate (15, 25, and 75 mg Dox eq./kg i.v.) in a therapeutic regime. Anti-tumor resistance of the cured animals was proved by a second challenge with a lethal dose of tumor cells without additional treatment.
Results. The content of drug bound to the polymer is an important parameter in relation to the conjugate therapeutic efficacy. The best anti-tumor effects were produced by conjugates with 10 – 13 wt% of bound doxorubicin. Free doxorubicin up to 4.6% relative to total drug content had no impact on the treatment efficacy and acute toxicity. The conjugates induced a complete cure of mice and regular treatment-dependent development of specific anti-tumor resistance. No myelosuppression or organ damage was observed.
Conclusions. A well-defined HPMA copolymer-doxorubicin conjugate with pH-sensitive drug release is a good candidate for clinical trials as it has remarkable anti-tumor efficacy and a favorable safety profile.

KEY WORDS: doxorubicin; immunomodulation; murine lymphoma; *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer conjugate; tumor resistance.

INTRODUCTION

Water-soluble synthetic polymer drug conjugates based on copolymers of *N*-(2-hydroxypropyl)methacrylamide (HPMA) represent a class of advanced anti-cancer agents (2–7). These polymer nanotherapeutics consist of a low-molecular-weight toxic molecule (e.g. cytotoxic/cytostatic drug, such as doxorubicin, daunorubicin, epirubicin, paclitaxel, mesochlorin, 5-fluorouracil, cis-platin, *etc.*) that is covalently coupled to a polymer backbone—a design ensuring favourable pharmacokinetics, biodistribution and toxicity profile. Actually, the systemic toxicity of the conjugates is negligible, as the conjugates are stable in the bloodstream. Stability at the physiological pH and high molecular weight ensure the long circulation that is crucial for preferential accumulation of the conjugates in solid tumors due to the Enhanced Permeability and Retention (EPR) effect (8). It is a generally accepted mechanism for passive tumor targeting of soluble macromolecular therapeutics ensuring a tumor-specific action of the therapy with limited systemic toxicity. A spacer coupling the drug to the polymer backbone can be designed either as a substrate for enzymatic cleavage (9), or to be susceptible to pH-dependent hydrolysis (tumor microenvironment is acidic in its nature) (10–12).

In contrast to the polymer conjugates of doxorubicin (Dox) in which the drug was bound to the polymer carrier via enzymatically cleavable bond, the pH-sensitive hydrazone linkage allows a rapid cleavage of the drug from the carrier already after transfer of the conjugate to the interstitial space of the tumor (i.e. extracellularly, but in a close proximity of target tumor cells). However, the drug is predominantly released in acidic intracellular compartments (late endosomes/lysosomes) at pH 5–6 (1,11,13). Mechanisms leading to cell death were studied, showing that the cell death is induced following doxorubicin liberation inside the cell. The pattern of gene and protein expression involved in apoptosis

[1] Laboratory of Tumor Immunology, Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i., Vídenska 1083, 142 20 Prague 4, Czech Republic.
[2] Department of Biomedicinal Polymers, Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, v.v.i., Prague, Czech Republic.
[3] To whom correspondence should be addressed. (e-mail: sirova@biomed.cas.cz)

ABBREVIATIONS: B/6, C57BL/6; CRT, calreticulin; Dox, doxorubicin; HMGB1, high mobility group box-1; HPMA, *N*-(2-hydroxypropyl)methacrylamide; PBS, phosphate buffered saline.

and cell cycle regulation induced by the hydrazone conjugate resembled that induced by free doxorubicin (14,15). Nuclear localisation of free doxorubicin released from the polymer prodrug with hydrazone bond was demonstrated (16).

We showed that HPMA-based copolymers armed with doxorubicin via a spacer containing pH-sensitive hydrazone linkage exhibit a high level of cytostatic activity in several cancer cell lines *in vitro* (2,11,13). Notably, the conjugates are also active in cells with a limited content of lysosomes (17). Linear non-targeted HPMA-based polymers with doxorubicin bound via a hydrolytically degradable pH-controlled linker possess much higher cytostatic activity *in vitro* than PK1 (FCE28068), a non-targeted linear HPMA-based conjugate with enzymatically degradable linker (14). *In vivo*, the hydrazone conjugates show tumor-specific accumulation which is reflected in a significant therapeutic efficacy in murine tumor models (2,11,18). Surprisingly, the experiments showed that the acute toxicity of the conjugate with pH-sensitive linkage of the drug is much lower than that of PK1 (2,14). Maximum tolerated dose (MTD) surpassed at least three times the value estimated for PK1, a Dox-HPMA conjugate with enzymatically cleavable bond between the drug and carrier. Thus, the hydrazone conjugate of doxorubicin demonstrated a remarkably wide therapeutic window.

Recently, we improved the methods of synthesis of these hydrazone conjugates by introducing new types of monomers, which enabled us to both simplify the synthesis and produce conjugates with well-defined structures (1). The method allows for preparing conjugates with various contents of polymer-bound doxorubicin, ranging from 5 to 23 wt%. We have analyzed and published their physicochemical properties, *in vitro* cytostatic activity, biodistribution, and preliminary therapeutic efficacy *in vivo* (1). The different physicochemical properties of the conjugates are reflected in their biological activity. Here we report a detailed study of the *in vivo* biological and therapeutic properties of these anti-cancer conjugates in murine tumor models, one of which is planned to enter regular clinical trial.

MATERIALS AND METHODS

Chemicals

1-Aminopropan-2-ol, methacryloyl chloride, 2,2′-azobis (isobutyronitrile) (AIBN), 6-aminohexanoic acid (19), methyl 6-aminohexanoate hydrochloride (ah-MeO), N,N′-dimethylformamide (DMF), N,N′-dicyclohexylcarbodiimide (DCC), leucylglycine, glycylphenylalanine, phthalaldehyde (OPA), N-ethyldiisopropylamine, dimethyl sulfoxide (DMSO), tert-butyl carbazate, hydrazine hydrate, trifluoroacetic acid (TFA) and doxorubicin hydrochloride (Dox.HCl) were purchased from Fluka (Switzerland). 2,4,6-Trinitrobenzene-1-sulfonic acid (TNBSA) was purchased from Serva (Germany).

Synthesis of Monomers

N-(2-Hydroxypropyl)methacrylamide (HPMA) was synthesized as described in (20) with the exception of using Na_2CO_3 instead of $NaHCO_3$. M.p. 70°C; elemental analysis: calcd., C 58.72, H 9.15, N 9.78; found, C 58.98, H 9.18, N 9.82.

N-(tert-butoxycarbonyl)-N′-(6-methacrylamidohexanoyl)hydrazine (Ma-ah-NHNH-Boc) was prepared by a two-step synthesis as described in (21). M.p. 110–114°C, elemental analysis: calcd. C 57.70, H 8.33, N 13.46; found C 57.96, H 8.64, N 13.25.

6-Methacrylamidohexanohydrazide (Ma-ah-$NHNH_2$) was prepared by the reaction of methyl 6-aminohexanoate hydrochloride with methacryloyl chloride followed by hydrazinolysis of the methylester as described in (1). M.p. 79 – 81°C; elemental analysis: calcd., C 56.32, H 8.98, N 19.70; found, C 56.49, H 8.63, N 19.83. 1H-NMR 300 MHz ($CDCl_3$, 297 K): 1.35 m (2H, $CH_2(CH_2)_2$-N); 1.50-1.69 m (4H, $CH_2CH_2CH_2CH_2$-N); 1.95 dd (3H, CH_3); 2.17 t (2H, ((C=O)-CH_2); 3.26 dt (2H, N-CH_2); 3.91 s (2H, NH_2); 5.30 t (1H, C=CH_2 E); 5.67 t (1H, C=CH_2 Z); 6.10 s (1H, $NHNH_2$); 7.45 s (1H, NH-CH_2). Purity of all monomers was examined by HPLC (LDC Analytical, USA) using a reversed phase column Tessek SGX C18 (15×33 mm) with UV detection at 230 nm, eluent water-methanol with methanol gradient 50–100 vol.%, flow rate 0.5 ml/min.

Synthesis of Polymer Precursors

Random copolymer of HPMA with Ma-ah-NHNH-Boc was prepared by radical polymerization in methanol (AIBN, 1 wt%; monomer concentration 14 wt%; molar ratio HPMA : Ma-ah-NHNH-Boc 93 : 7; 60°C; 23 h) as described in (21). Free hydrazide groups of polymer precursor were prepared by deprotection of Boc-hydrazide in concentrated TFA.

Statistical copolymers of HPMA with Ma-ah-$NHNH_2$ containing free hydrazide groups were prepared by radical copolymerization in methanol (AIBN, 0.6 – 1.0 wt%; monomer concentration 18 wt%; molar ratio HPMA: Ma-ah-$NHNH_2$/Ma-GFLG-$NHNH_2$ 93 : 7 or 88 : 12; 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 ampoule, 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%).

Attachment of Doxorubicin to Polymer Precursors

Polymer-Dox conjugates (conjugates 1 – 11; Table I) were prepared by the reaction of corresponding polymer precursors containing hydrazide groups with Dox.HCl in methanol in the dark as described (11). The polymer-drug conjugates were purified by removing low-molecular-weight impurities (Dox or its degradation products) by gel filtration using a Sephadex LH-20 column and methanol as eluent.

Purification and Characterization of Polymers and Conjugates

All the conjugates were characterized and tested for the content of the free polymer or free drug using a HPLC Shimadzu equipped with GPC columns Superose™ 6 or Superose™ 12 and TLC (Kieselgel 60 F254). In addition, the

Table I. Characteristics of polymer-Dox conjugates

Polymer conjugate	M_w	M_w/M_n	Dox (wt%)	free Dox (%)
1	19,500	1.65	5.1	0.11
2	25,000	1.80	9.9	0.20
3	20,800	1.73	13.2	0.16
4	24,200	1.84	16.7	0.32
5	25,300	1.75	22.5	0.35
6	25,000	1.80	9.9	1.3
7	25,000	1.80	10.0	2.3
8	25,000	1.80	10.3	4.6
9	19,500	1.71	8.7	0.23
10	25,400	1.66	8.9	0.17
11	29,600	1.72	8.5	0.20

content of free Dox was determined by HPLC Shimadzu after extraction of Dox from aqueous solution of the conjugate to chloroform or by GPC in aqueous methanol solution (Shimadzu HPLC system equipped with GPC column TSKgel G3000SWxl (300×7.8 mm; 5 μm); 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; flow rate 0.5 ml/min) with UV-VIS detection (Shimadzu SPD-10AVvp) (λ=488 nm) from the area of the peaks corresponding to the free and polymer-bound DOX.

The total content of Dox in polymer conjugates was determined spectrophotometrically on Helios α (Thermochrom) spectrophotometer. Molar absorption coefficients of the free drug (ε488=11 500 L mol^{-1} cm^{-1} (water)) and modified Dox (Ma-ah-NHN=Dox: ε488 (water)=9 800 L mol^{-1} cm^{-1}) were used for calculating the Dox content.

Determination of molecular weight and polydispersity of the conjugates were carried out with a HPLC Shimadzu system equipped with RI, UV and multiangle light scattering DAWN EOS (Wyatt Co., USA) detectors using 0.3 M acetate buffer pH 6.5 and Superose™ 12 or Superose™ 6 column.

The content of hydrazide-terminated side chains in polymer precursors was determined by a modified TNBSA assay as described (11). Molar absorption coefficient ε500 = 17 200 L mol^{-1} cm^{-1} (λ =500 nm) estimated for the model reaction of MA-AH-$NHNH_2$ or MA-GFLG-$NHNH_2$ with TNBSA was used.

Cancer Cell Lines

Murine T cell EL4 lymphoma was purchased from the American Type Culture Collection (ATCC, USA) and 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. The B cell lymphoma cell line 38C13 (22) was a kind gift of Prof. J. Kovar, 3rd Faculty of Medicine, Charles University, Prague, Czech Republic. The 38C13 cells were cultivated in RPMI-1640 medium supplemented with 2 mM L-glutamine, 1 mM Na-pyruvate, 0.05 mM 2-mercaptoethanol, 10 mM HEPES, antibiotics (pen/strept, Sigma, USA), and 10% fetal calf serum.

Mice

C57BL/6 (B/6) mice were obtained from the breeding colony of the Institute of Physiology ASCR, v.v.i. (Prague, Czech Republic). C3H/N mice were from Anlab Ltd. (Czech Republic). Mice were used at 8–12 weeks of age, housed in accordance with approved guidelines and provided food and water *ad libitum*. The Animal Welfare Committee of the Institute of Microbiology, ASCR, v.v.i. approved all experiments.

In Vivo Tumor Models

B/6 males were subcutaneously transplanted with 1×10^5 EL4 T cell lymphoma cells on the right shaven flank on day 0. 38C13 B cell lymphoma was transplanted to female C3H/N mice in the same way (1×10^5 s.c.). The mice that developed palpable tumors reaching 4–9 mm in diameter within 8–9 days after the implantation were intravenously treated with polymer conjugates diluted in PBS, as described in Results. The doses referred to hereinafter are expressed as the doxorubicin equivalent per kg (mg Dox eq./kg). Control mice were transplanted with the tumor cells and left untreated. The animals were observed three times a week for signs of tumor progression and acute toxicity. The tumor size, body weight, survival time and number of long-term survivors were determined. Experimental groups contained 8 mice. For the histological analysis, the mice were sacrificed on day 16 post tumor transplantation, and samples of organs and tissues were harvested, fixed in 5% formalin, embedded in paraffin, and the sections stained with hematoxylin/eosin. Liver, heart, kidney, lung, spleen, vertebral bone marrow and subcutaneously localized tumor focus were examined.

RESULTS

Dose-Dependent Anti-Tumor Efficacy and Acute Toxicity of Linear HPMA Copolymer-Bound Doxorubicin

Anti-tumor efficacy and acute toxicity of conjugate 2 were tested in a murine syngeneic tumor model of EL4 T cell lymphoma in B/6 mice (Fig. 1). A single i.v. dose of 75 mg Dox eq./kg cured all the mice in the experimental group and proved to still be safe. Marginal decrease in the body weight not exceeding 10% was recorded on days 5 and 7 post treatment, and all the mice fully recovered within 2 weeks. A lower dose of 25 mg Dox eq./kg conjugate 2 induced a complete cure in 6 of 8 mice per group (75%), and 15 mg Dox eq./kg cured 5 of 8 mice (62.5%). Again, the change of body weight as a measure of acute toxicity was negligible in the two lower doses. The treated mice that finally succumbed to tumor growth had a significantly prolonged survival as compared with untreated controls (Fig. 1).

Efficacy and Acute Toxicity of Conjugates Relative to Polymer-Bound Doxorubicin Content

Conjugates with various content of doxorubicin ranging from 5.1 to 13.2 wt% were synthesized, and their anti-tumor potential was tested in the EL4 lymphoma model. B/6 mice were injected with 1×25 mg Dox eq./kg of the hydrazone conjugates containing 5.1 wt% (conjugate 1), 9.9 wt%

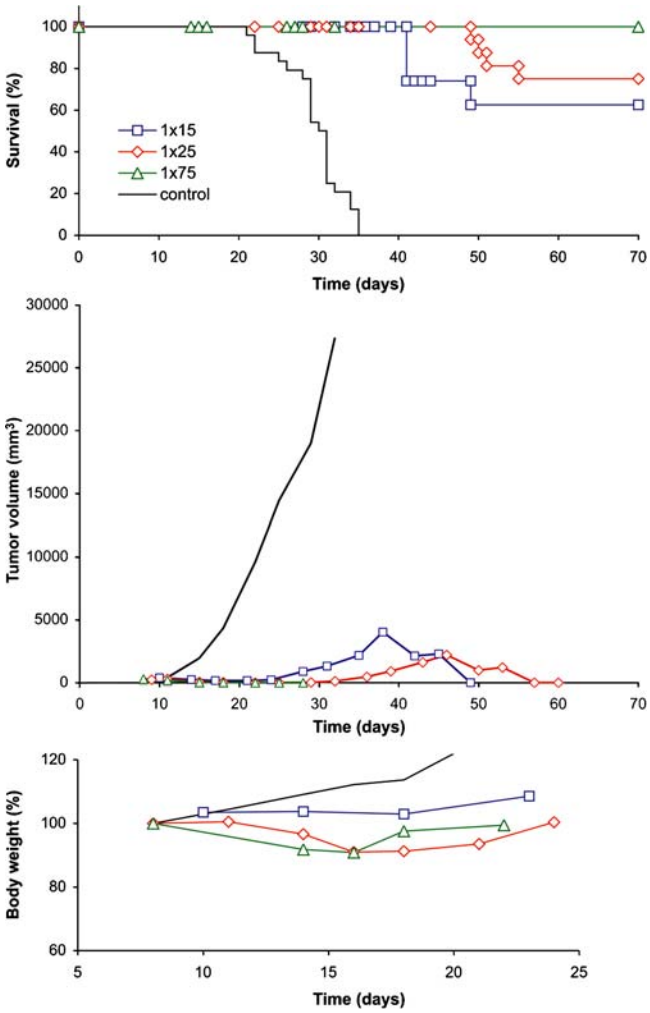

Fig. 1. The anti-tumor efficacy and acute toxicity of conjugate 2 in EL4 lymphoma-bearing C57BL/6 mice. B/6 mice were transplanted s.c. with 1×10^5 EL4 lymphoma cells and treated with single i.v. dose of the conjugate 2 (1×75, 1×25, and 1×15 mg Dox eq./kg) on day 9. Control mice were left untreated. Overall survival, tumor growth, and body weight were monitored. The figure summarizes data from 3 independent experiments.

(conjugate 2) or 13.2 wt% (conjugate 3) of doxorubicin. It is of note that a suboptimal dose for conjugate 2 was chosen in order to assess whether the content of polymer-bound doxorubicin has any effect on the anti-tumor efficacy. The conjugate 1 with the lowest content of doxorubicin induced a complete tumor regression in 3 of 8 mice (37.5%) and prolonged the survival of the other mice in the group. Conjugate 2 showed better anti-tumor effect than conjugate 1 and cured 62.5% of animals. The mice treated with conjugate 3, having the highest content of doxorubicin, were all cured (Fig. 2A). No significant decrease of body weight (less than 10%) was recorded in the treated mice, suggesting a favourable safety profile of the conjugates. These results imply that a correlation exists between the increased content of the drug in the conjugate and an improved therapeutic outcome.

To prove this, we introduced another two conjugates containing an even higher content of doxorubicin in the conjugate, i.e. 16.7 wt% (conjugate 4) and 22.5 wt% (conjugate 5). In order to document any potential increase in the therapeutic efficacy, we decreased the dose to 15 mg Dox eq./kg, as the most effective conjugate in the previous experiment (conjugate 3; Fig. 2A), which was used here for reference, cured all treated mice at a dose 25 mg Dox eq./kg. Surprisingly, the nanotherapeutics with the highest load of the drug (conjugate 5; 22.5 wt%) were significantly less effective compared to the conjugates containing 16.7 (conjugate 4) and 13.2 wt% (conjugate 3) of doxorubicin. No significant acute toxicity of the conjugates was recorded at this dosage (Fig. 2B).

Effect of Unbound Doxorubicin in Conjugate Preparations on Treatment and Toxicity

A part of the preclinical development of the prodrug was to document that contaminating unbound doxorubicin that would be administered within the clinically used formulation could not affect the outcome of the treatment. Four conjugates differing in the content of the unbound doxorubicin present in the final formulation were compared (conjugates 6–8, and conjugate 2). The samples were prepared by adding an estimated amount of free drug to conjugate 2. Thus, the content of bound doxorubicin was around 10 wt% (see above), and the amount of unbound doxorubicin ranged from 0.2% to 4.6% relative to total doxorubicin content (see Table I). The therapeutic efficacy was tested in the model of EL4 lymphoma. The treatment with a single i.v. dose of 25 mg Dox eq./kg of conjugates 6–8 cured 7 out of 8 mice in each experimental group, implying that the presence of unbound doxorubicin up to 4.6% did not affect the treatment (Fig. 3). Body weight change of the mice was monitored to assess the acute toxicity of the conjugates. None of the conjugates induced significant decrease in the body weight of the animals (not shown), i.e., no acute toxicity was seen.

Effect of Mw of the Polymer Conjugates on the Treatment Efficacy

Three conjugates uniform in the amount of doxorubicin (around 8.7 wt%) but differing in their Mw – 19,500, 25,400 or 29,600, respectively, were synthesized (Table I). The therapeutic efficacy of the conjugates was tested in the model of EL4 lymphoma. The mice were given a single i.v. dose of 25 mg Dox eq./kg of the conjugates on day 8, and the progression of the tumor growth was monitored. In this experiment, the treatment cured 7 of 8 mice (conjugate 9) or even all mice in the group (conjugates 10 and 11), and thus the effect of Mw on the efficacy could not be fully estimated (not shown). To detect smaller differences between these conjugates, we repeated the experiment using another tumor model of 38C13 B cell lymphoma, which is more difficult to cure. This time the treatment with a single dose of 25 mg Dox eq./kg of the conjugates cured approximately half of the mice in each treated group (Fig. 4).

Histological Analysis of Organs of Treated Mice

Histological analysis of selected organs of mice treated with a high dose of conjugate 2 (75 mg Dox eq./kg, i.v.) was

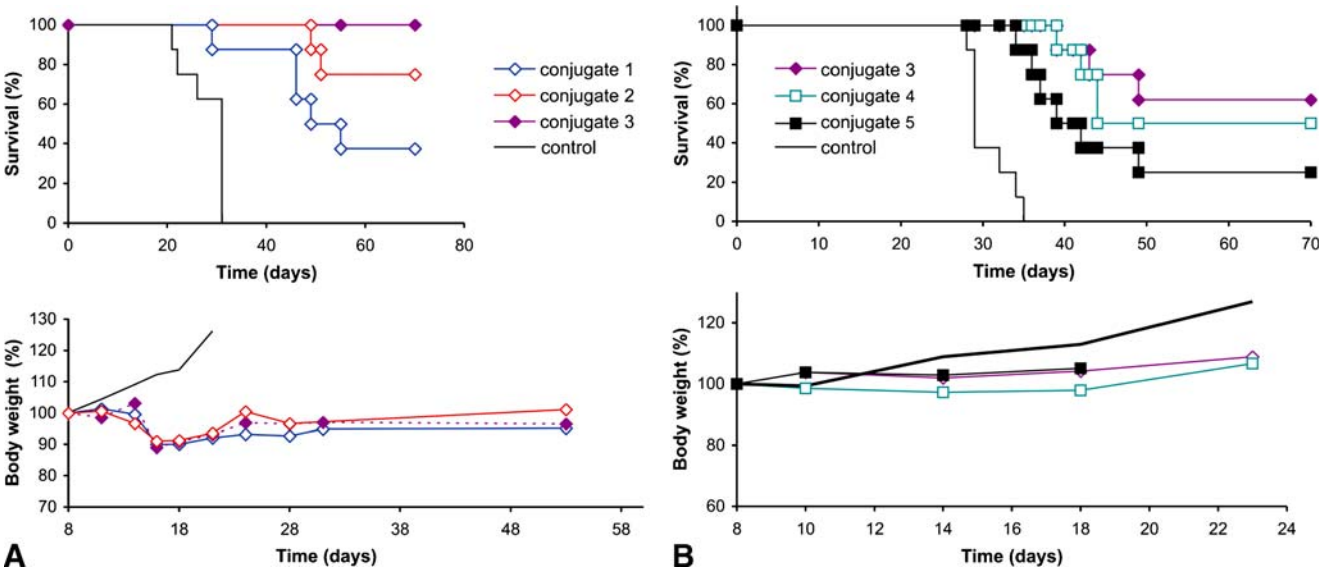

Fig. 2. The effect of the conjugates with different content of bound doxorubicin on survival time and body weight of mice. Mice were inoculated with 1×10^5 EL4 lymphoma cells s.c. **A** Mice treated with conjugates 1, 2, and 3 were injected i.v. with 1×25 mg Dox eq./kg of the conjugates. **B** Mice treated with conjugates 4, 5, and 3 for comparison were injected i.v. with 1×15 mg Dox eq./kg B. The conjugates were administered on day 8 post tumor transplantation. Each experimental group contained 8 mice.

carried out on day 8 post treatment. Heart, kidney, and lung showed no abnormalities. Liver tissue was almost normal, with solitary hepatocyte mitoses indicating minimal increase in regenerative activity presumably caused by the treatment. Spleens of the treated mice had fully preserved architecture with extramedullar hemopoesis, normal follicles and no cell clusters suspect of tumor. Notably, vertebral bone marrow presented with only weakly tenuous cellularity and no atypical cellular elements (Fig. 5A). The subcutaneously located site of tumor implantation revealed necrosis with collateral oedema and intimate granuloma formation, containing infrequent macrophages. No focus of growing tumor was found (Fig. 5B). Solitary elements resembling tumor cells with large light nuclei and prominent nucleoli were detected in the peripheral part of the tumor implantation area that could eventually be a source of relapsing tumor.

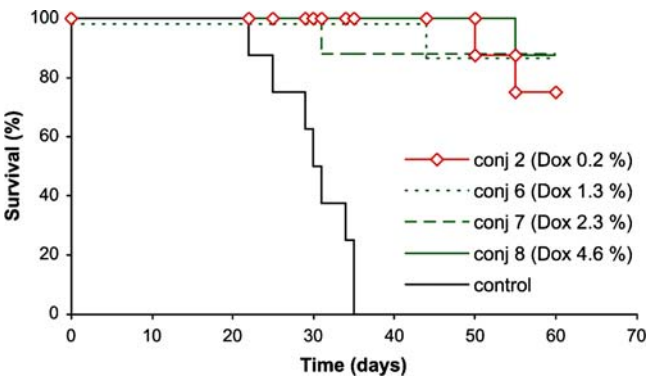

Fig. 3. The effect of the conjugates with impurities in the form of free doxorubicin on overall survival of mice. Mice were transplanted with 1×10^5 EL4 lymphoma cells s.c. and treated on day 9 with the conjugates containing the same amount of bound doxorubicin (~ 10 wt%) but various amounts of free drug (Table I). The dose of the conjugates was 1×25 mg Dox eq./kg injected i.v. Control mice were without treatment.

Tumor Resistance in the Cured Mice

To demonstrate the potential development of resistance following the conjugate 2 treatments, we re-transplanted the long-term survivors cured from the EL4 lymphoma (doses 1×15, 1×25, and 1×75 mg Dox eq./kg given on day 9) s.c. with 1×10^5 EL4 cells, and left untreated. The mice cured with the lowest dose did not develop any tumor and remained healthy for at least 90 days following the re-transplantation, thus showing perfect tumor resistance. 83% of the mice cured with 1×25 mg Dox eq./kg resisted the re-transplantation, and almost 30% of those cured with the highest dose of the conjugate (Fig. 6). These data are in good concordance with our previous paper showing the anti-tumor immunity triggered by treatment with a similar conjugate (23), where

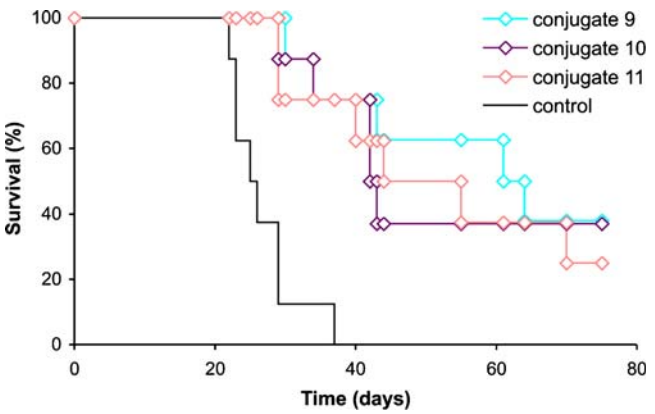

Fig. 4. The effect of Mw of the conjugates on the overall survival of C3H/N mice bearing 38C13 lymphoma. Mice were inoculated with 1×10^5 38C13 lymphoma cells s.c. and treated i.v. with a single dose of the conjugates 9, 10, and 11 on day 7 (1×25 mg Dox eq./kg). Control mice were left untreated. Tumor growth (not shown) and overall survival of the mice was monitored. Each experimental group contained 8 mice.

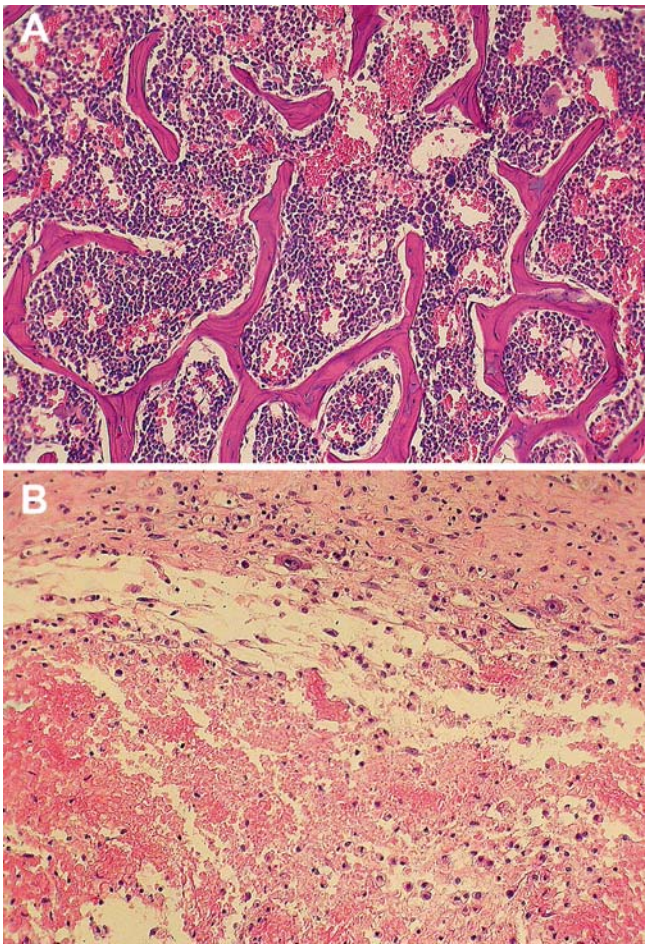

Fig. 5. Histological analysis of organs and tumor residue of mice with EL4 lymphoma treated with 1×75 mg Dox eq./kg. **A** Fully preserved hematopoiesis in the vertebral bone marrow. **B** Residue of the tumor with necrosis and hemorrhage and scattered macrophages (bottom). Collateral non-specific inflammatory reaction with initial fibroplasia. Two large elements with prominent nucleoli resembling possible persisting tumor cells are present.

the proportion of the resistant animals was 100%, 90%, and 40% following the treatment with 3×25, 1×25 and 1×75 mg Dox eq./kg, respectively.

DISCUSSION

Polymer conjugates that bind the anti-cancer drugs with pH-labile bond have already been described, either with *N*-cis-aconityl spacer (24,25) or with hydrazone linkage (10,11,26). Preliminarily, anti-tumor activity of pH-sensitive HPMA-based copolymers armed with doxorubicin was already documented *in vitro* and *in vivo* (2,11,13,17,21,23,27).

We have already published a study on chemotherapy of mouse lymphoma EL4 by linear HPMA copolymer conjugate with doxorubicin bound via a pH-sensitive linker (23). Doses of 1×25, 3×25 and 1×75 mg Dox eq./kg i.v. that were used induced a significant proportion of complete cures and triggered anti-tumor immunity that had an inverse relationship to the treatment dosage. Here we have used a conjugate of similar parameters in terms of bound and free doxorubicin content, and polydispersity, as an internal standard (conjugate 2; Table I). The anti-tumor activity of conjugate 2 in the EL4 lymphoma model proved to be in perfect concordance with the already published data (23), pointing to the reproducibility of the prodrug synthesis procedure. The treatment-related acute toxicity of conjugate 2 was negligible even at the highest dose of 75 mg Dox eq./kg. Maximum tolerated dose (MTD) of this hydrazone conjugate is at least 130 mg Dox/kg (Mrkvan T, personal observation) and 45 mg/kg in PK1, whilst only 3×6 mg/kg or 1×10 mg for free doxorubicin. Indeed, treatment of lymphoma EL4 with free doxorubicin at its MTD (3x6 mg/kg) resulted in 75% of mice with completely regressed tumors, but on second tumor challenge all the mice died because of delayed toxicity of the drug (23). The limited side toxicity of the hydrazone conjugate reflects its pharmacokinetics that are characteristic of polymer prodrugs. The conjugate showed prolonged blood circulation, tumor accumulation peaking at 48 hours post administration, and tumor-to-blood ratio increasing with time, implying that the prodrug is passively accumulated in tumor tissue by EPR effect (1,18).

The examination of anti-tumor capacity of the conjugates related to their drug content showed an increase in the activity of conjugates containing 10–13 wt% of bound doxorubicin, and a significant decrease of anti-tumor effect in conjugates with drug content exceeding 13 wt%. We hypothesize that this phenomenon might result from changes in hydrodynamic behaviour of the respective conjugate polymer coils in aqueous solutions. The increase in the activity of polymer conjugates correlating with the increase in the drug content (up to 13 wt% of the drug) could most probably be due to the elevated transport of the drug to tumor cells by fluid-phase pinocytosis. There is a limit to the number of polymer molecules pinocytosed by the cell in a certain period of time. With the higher drug content in each polymer molecule, an increased quantity of the drug is transported to the cell and released here in endosomes and lysosomes by pH-controlled hydrolysis. Moreover, as it was reported with a drug model bound to HPMA copolymer carrier through amide bond, a graded content of the substituent conferring the conjugate enhanced hydrophobicity, resulting in increased capacity for adsorptive pinocytosis

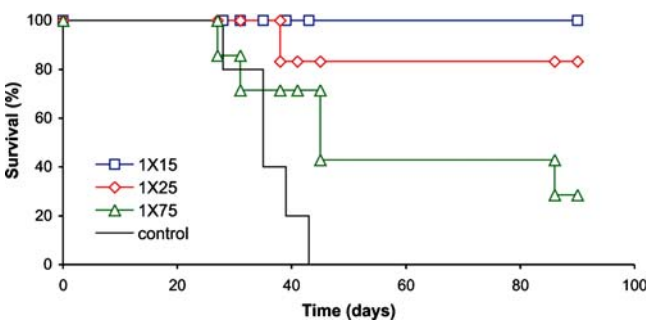

Fig. 6. Re-transplantation of long-term survivors cured from the EL4 lymphoma with different doses of conjugate 2. The EL4 lymphoma-bearig mice were cured with 1×15, 1×25, and 1×75 mg Dox eq./kg injected i.v. on day 8 post tumor transplantation. 73 days later, the surviving mice were re-transplanted with 1×10^5 EL4 cells and left untreated. Tumor growth and survival time was monitored. Summary data from two independent experiments are shown.

(28). We assume that a similar non-specific adsorption occurs also in the conjugates with Dox bound via a pH-sensitive hydrazone linkage and contributes to efficacy of the treatment, since our recent results showed that the HPMA-based Dox conjugates with higher doxorubicin content are molecularly dissolved with R_H~5 nm but show hydrophobic character of polymer chain domains, which becomes more prominent with increasing drug loading (1).

The relation of drug content and solubility of the conjugate was studied in HPMA copolymers bearing a model drug, p-nitroaniline, attached to the polymer via amide link and biodegradable oligopeptide spacer (29). With increasing amount of the hydrophobic spacer and model drug, the hydrophobicity of the polymer conjugate increased, and at 6.9%mol of p-nitroaniline content the conjugates started to form high-molecular-weight aggregates in aqueous solutions at 37°C. Accordingly, increasing content of hydrophobic doxorubicin in hydrazone conjugates results in increasing amount of hydrophobic domains, and at the Dox content significantly exceeding 13 wt%, the copolymer, due to elevated hydrophobic interactions, self-assembles into intermolecular associates with R_H~1000 nm (light scattering). The rate of pinocytosis of the bulky associate by the target cancer cells is significantly lowered if compared with a single molecule; this results in a lower efficiency of the treatment.

As the proportion of drug released from HPMA copolymer carriers *in vitro* is comparable in conjugates containing 10.1 and 22.5 wt% of Dox (1), the decreasing *in vivo* efficacy seen in the conjugates with the doxorubicin content exceeding 13 wt% could only be explained by a difference in availability of the drug for the target cells *in vitro* and *in vivo*. Regardless of the actual cause of different efficacy of conjugates with a range of drug contents, the experiments indisputably showed that the best therapeutic outcomes with minimal adverse toxicity were delivered by conjugates with doxorubicin content ranging from 10 to 13 wt%. The previous study (1) revealed more favourable hydrophilic character and better solubility of conjugate 2 (10 wt%); this conjugate has therefore been chosen as a candidate for planned clinical trial. Thus, further detailed study of treatment outcomes, such as histological analysis of organs and development of tumor resistance, was performed with this conjugate.

All nanotherapeutics prepared in a routine way contained a small amount of unreacted parent drug, irrespective of the elaborate purification. Free doxorubicin was usually present in amounts close to the detection limit of the analytical method used. Anyway, it was needful to check the range of concentrations of free drug that could be present in the specimen preparations that does not affect the treatment efficacy or acute toxicity. Thus the most important conclusion from our experiment was that the presence of unbound doxorubicin up to about 4–5% relative to total drug content did not influence the efficacy of the treatment and, notably, did not cause treatment-related acute toxicity. This amount of unbound drug is, in fact, 10–20 times higher than that present in the conjugate preparations (usually 0.1 to 0.2%; Table I).

The conjugates with various contents of polymer-bound doxorubicin differed not only in the contents of the drug, but also their molecular weight was in the range from 19,500 to 30,000. This range is well below the renal excretion threshold, which is about 40,000 for HPMA copolymer (30), to ensure ultimate renal elimination of the non-biodegradable polymer. A possible influence of the Mw on the therapeutic efficacy was examined here in a separate experiment. Not surprisingly, there was no significant difference in the survival of mice among the treated groups, implying that the Mw within the range of 20,000 to 30,000 does not have any significant impact on the therapeutic efficacy of the prodrug.

The histological analysis of organs and tissues of the mice treated with conjugate 2 first documented the efficacy of treatment, as we did not see any visible signs of tumor growth either in the primary localization or in organs to which EL4 lymphoma spreads in the form of micrometastases, such as spleen or bone marrow (31). Second, the safety of the treatment was apparent. No signs of significant acute toxicity were seen. Notably, the high-dose treatment (75 mg Dox eq./kg) did not cause any toxic damage to heart, kidney, spleen, liver or bone marrow. It is important to stress that doxorubicin, administered in free form, produces numerous side effects and toxic lesions, namely cardiotoxicity, which is the therapy-limiting damage (32,33). Similarly to other cytotoxic drugs targeting proliferating cell populations, doxorubicin has significant gastrointestinal toxicity and causes severe myelosuppression. The remarkable safety of HPMA-copolymer-bound cytotoxic drugs has been proved and published in several papers (34–38). The conjugation of doxorubicin to the HPMA copolymer via amide bond significantly reduces all aspects of doxorubicin-associated non-specific toxicity, including myelotoxicity (35), immunotoxicity (39), and cardiotoxicity (40–42). Evaluation of PK1 (FCE28068) in Phase I clinical study provided proof of the principle that polymer conjugation decreases doxorubicin dose-limiting toxicities (42). These data were corroborated by the results of a pilot clinical study in breast cancer patients, in whom administration of HPMA-doxorubicin conjugate containing human immunoglobulin did not provoke myelosuppression, cardiotoxicity and other drug-related toxicities despite the fact that heavily doxorubicin-pretreated patients were enrolled (43,44).

The linear HPMA-based conjugates with hydrolytically activated cleavage of doxorubicin revealed a remarkable therapeutic activity against murine syngeneic EL4 and 38C13 lymphomas. The dosage enabled us to induce even a complete cure of EL4 lymphoma-bearing mice with a single and safe dose of the conjugate. Immunoprotective character of the treatment with doxorubicin conjugates based on HPMA copolymer has already been suggested; it leads to mobilization of the host immune anti-tumor defence mechanisms, both in experiment (6,39,45) and in clinical trial (43,44). As we published earlier, a remarkable phenomenon associated with HPMA copolymer conjugates is the development of treatment-related tumor-specific resistance, as demonstrated by re-transplantation of the cured mice with a lethal dose of the viable tumor cells but without any other treatment (6,23,43,45,46). The tumor resistance has been shown to develop during the treatment with different types of HPMA-based doxorubicin conjugates, either with pH-sensitive linkage of the drug (23) or with amide bond between the polymer and the drug (46,47).

The resistance induction is dependent upon a substantial available amount of tumor-derived antigens (48). This could be the explanation of the fact that the higher treatment dose induced a lower level of tumor resistance and *vice versa*. The

availability of the tumor antigens for the immune system is higher if eradication of the tumor is slower, as regularly seen in treatments using lower dosage of the doxorubicin conjugate. This inverse relationship between the dose of the treatment and proportion of resistant mice was demonstrated in our papers concerning different types of HPMA-based conjugates in the model of EL4 lymphoma (23,46) and BCL1 leukemia.

The development of resistance and tumor-specific memory is complex, most probably involving several mechanisms. Immunologically active tumor cell death induced by some anti-cancer drugs, including doxorubicin, is currently a widely debated phenomenon (49–51). Doxorubicin has the capacity to induce immunogenic death of tumor cells, i.e. apoptosis, that is not immunologically inert, as originally believed, but serves as an immunogenic signal to trigger specific anti-tumor immunity. In that situation, the dying tumor cells express calreticulin (CRT) on their surface and release high mobility group box-1 (HMGB1) protein. The doxorubicin-killed CRT^+ tumor cells are a source of the antigen. It was shown that in addition to CRT expression, which provokes phagocytosis of the CRT^+ dying cells, secretion of HMGB1 protein is a signal to induce maturation of dendritic cells to trigger specific anti-tumor immune responses. Our data suggest that the HPMA-conjugates with pH-controlled prodrug activation can induce CRT expression and HMGB1 protein release corresponding to the free doxorubicin (7). Thus, this could be at least one of mechanisms responsible for the induction of anti-cancer immunity and development of the anti-tumor resistance regularly seen in the treatment with HPMA-based doxorubicin conjugates with pH-sensitive drug linkage. Notably, the treatment is not accompanied by acute toxicity, as shown here, or late toxicity (23), both of which are well-known to appear following administration of the free doxorubicin. By virtue of that, the protected immune system retains its capacity to respond to the immunogenic signals induced by the conjugate treatment. In our opinion, the treatment-induced development of the tumor resistance is the most valuable characteristic of the therapies involving doxorubicin bound to the HPMA copolymer.

CONCLUSIONS

In summary, it was shown that the HPMA-based copolymers armed with the anticancer drug doxorubicin via a spacer containing pH-sensitive linkage prepared by a novel simple synthesis can be produced with a broad range of contents of attached drug. We demonstrate that the doxorubicin content significantly affects the therapeutic outcome. The most potent anti-cancer activity was delivered by conjugates with doxorubicin content falling within 10 and 13 wt%. The conjugates also showed negligible treatment-related acute toxicity. The increase of drug content above this level resulted in a decrease of treatment efficacy. The data suggest that an increase in doxorubicin content above 13 wt% is probably reflected by skewing the physical (solution) properties of the conjugates towards more hydrophobic nature, which results in lower efficacy. Regarding the contents of unbound doxorubicin that could be potentially present in the final formulation of the prodrug, neither significant acute toxicity nor any change in the anti-tumor effect was detected up to levels of 4.6% of unbound drug relative to total drug content. We also proved that Mw of the conjugates in a range from 20,000 to 30,000 had no impact on the efficacy of the treatment. Autopsy of mice treated with a high dose of the conjugate (75 mg Dox eq./kg) further documents the remarkable safety of the treatment. Moreover, the treatment induced in a significant proportion of mice a complete EL4 lymphoma tumor regression associated with development of anti-tumor resistance that prevents second tumor attack. The described nanotherapeutics can be manufactured on a large scale, and the conjugate that showed the best therapeutic index (conjugate 2) is about to enter phase I of a clinical trial.

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