

The Comparison of In Vivo Properties of Water-Soluble HPMA-Based Polymer Conjugates with Doxorubicin Prepared by Controlled RAFT or Free Radical Polymerization

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Summary

Two conjugates of anticancer drug doxorubicin (Dox) covalently bound by the hydrolytically degradable hydrazone bond to the polymer carrier based on water-soluble *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymers were synthesized and their properties were compared, namely their behavior *in vivo*. The polymer carriers differed in dispersity due to different methods of synthesis; the carrier with relatively high dispersity (HD) was prepared by free radical polymerization ($M_w = 29\,900$ g/mol, $\mathcal{D} = 1.75$) and the carrier with low dispersity (LD) by controlled radical polymerization ($M_w = 30\,000$ g/mol, $\mathcal{D} = 1.13$). Both polymer-Dox conjugates showed prolonged blood circulation and tumor accumulation of the drug in comparison with the free drug; e.g. the tumor-to-blood ratio for the polymer-bound Dox was 3-5 times higher. The LD polymer-Dox conjugate exhibited moderately higher tumor accumulation than the HD one at a dose of 1×15 mg Dox (eq.)/kg. Also, their anti-tumor activity did not differ when injected at this dose. However, the increase of the dose to 1×25 mg Dox (eq.)/kg resulted in the enhanced therapeutic activity of the conjugates, especially of the LD one with 100 % of long-term survivals. The dispersity of polymer drug carriers influenced the tumor accumulation rate, which affected the overall anti-cancer activity of polymer-drug conjugates.

Key words

HPMA copolymers • RAFT polymerization • Drug delivery system • Controlled release • Doxorubicin

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Introduction

Developing the highly efficient cancer therapy with reduced adverse side effects has become a desirable goal for numerous research teams over the last few decades. The majority of the cytostatic agents used in clinical practice are toxic for the tumor tissue and to a certain extent for the healthy tissues, too. Therefore, the administered dose limited by the toxicity towards the healthy tissues may be insufficient to kill the tumor cells. The drug delivery system, e.g. based on polymer drug carriers, transporting the cytostatic drug in an inactive form during blood circulation and delivering it to the tumor tissue is supposed to improve the cancer therapy outcome.

The higher anticancer activity of polymer-drug conjugates is induced by the polymer carrier accumulation in solid tumors due to the Enhanced Permeability and Retention (EPR) effect and by prolonged blood circulation guaranteeing sustained concentration in plasma (Maeda and Matsumura 1989). The accumulation rate of polymer carriers in solid tumors highly increases with the molecular weight (M_w) of polymer carriers (Seymour *et al.* 1995). However, the high molecular weight polymer carriers exceeding the renal excretion threshold should be biodegradable to prevent the polymer carrier retention in the body after the drug delivery.

N-(2-hydroxypropyl)methacrylamide (HPMA)-based copolymers with the cytostatic agent, doxorubicin

(Dox), bound by the biodegradable pH-sensitive hydrazone bond showed prolonged drug circulation and tumor accumulation, resulting in superior antitumor activity with reduced overall toxicity when compared to the free Dox (Mrkvan *et al.* 2005, Šírová *et al.* 2010). The same polymer carrier enabled the pH-sensitive attachment of various drugs; e.g. anticancer agents - pirarubicin (Nakamura *et al.* 2014), paclitaxel or docetaxel (Etrych *et al.* 2010), mitomycin C (Kostkova *et al.* 2013), or anti-inflammatory drug dexamethasone (Krkovičová *et al.* 2009, Kostkova *et al.* 2011). Polymer conjugates containing the single drug alone or the combination of drugs showed also an excellent antitumor activity against various solid tumors in mice.

The most frequently used method for the synthesis of HPMA-based polymer carriers is classical free radical polymerization enabling the preparation of copolymers in the wide range of molecular weights and with dispersity close to 2 without polymer fractionation. However, the development of controlled radical polymerization, in particular Reversible addition-fragmentation chain transfer (RAFT) polymerization, enabled the synthesis of precisely defined HPMA-based polymer carriers with low dispersity (about 1.1-1.2) (Moad *et al.* 2008). Moreover, the RAFT polymerization technique allows the preparation of (semi)telechelic polymer precursors employed, e.g. as building blocks in the synthesis of advanced polymer structures (Pan *et al.* 2011, Chytil *et al.* 2015).

In this study, we focused on the comparison of properties of the linear polymer-Dox conjugates of the same M_w but differing in dispersity, i.e. the conjugates prepared using free radical polymerization (Etrych *et al.* 2008) or controlled radical RAFT polymerization (Chytil *et al.* 2010). Since the physicochemical characteristics were studied in detail before, here, we describe the biological properties *in vivo*, i.e. body distribution and antitumor activity in mice. The main attention is paid to the influence of dispersity of the polymer carriers on antitumor therapy.

Materials and Methods

Materials

Methacryloyl chloride, 4,4'-azobis(4-cyanopentanoic acid), 2,2'-azobis(isobutyro-nitrile) (AIBN), *N*-ethylmaleimide trifluoroacetate, 6-aminohexanoic acid, methyl 6-aminohexanoate hydrochloride, *N,N'*-dicyclohexylcarbodiimide, *tert*-butyl carbazate, trifluoroacetic

acid (TFA), 2,4,6-trinitrobenzene-1-sulfonic acid (TNBSA) were purchased from Sigma-Aldrich Inc. Doxorubicin hydrochloride (Dox.HCl) was purchased from Meiji Seika (Japan). All other chemicals and solvents were of analytical grade. Solvents were dried and purified by conventional procedures and distilled before use.

Synthesis

The synthesis and characterization of *N*-(2-hydroxypropyl)methacrylamide (HPMA), (6-methacrylamido hexanoyl)hydrazine (MA-Ahex-NHNH₂) and *N*-(*tert*-butoxycarbonyl)-2-(6-methacrylamido hexanoyl)hydrazine (MA-Ahex-NHNH-Boc) monomers and of the chain transfer agent 4-cyano-4-thiobenzoylsulfanyl-pentanoic acid have already been published (Thang *et al.* 1999, Ulbrich *et al.* 2004, Chytil *et al.* 2010).

The polymer precursor with low dispersity (**LD**)-copolymer was synthesized by RAFT polymerization of HPMA with MA-Ahex-NHNH-Boc followed by terminal dithiobenzoate group removal and deprotection of hydrazide groups as described (Chytil *et al.* 2010). For polymerization AIBN was used as an initiator, 4-cyano-4-thiobenzoylsulfanyl-pentanoic acid as the RAFT chain transfer agent (CTA), and *tert*-butylalcohol as the solvent. The molar ratio of monomers: CTA:AIBN was 600:2:1. The molar ratio of HPMA and MA-Ahex-NHNH-Boc was 9:1. The total concentration of both monomers was 0.9 M.

The polymer precursor with high dispersity (**HD**)-copolymer was prepared by free radical polymerization of HPMA and MA-Ahex-NHNH₂ as described elsewhere (Etrych *et al.* 2008).

Both polymer-Dox conjugates were prepared by the reaction of the respective polymer precursors containing free hydrazide groups with Dox.HCl in methanol in the dark (Etrych *et al.* 2002). The polymer-drug conjugates were purified from the unbound drug by gel filtration, using a Sephadex LH-20 column and methanol as the eluent.

Physicochemical characterization of copolymers and their drug conjugates

The molecular weight and dispersity of copolymers and conjugates were determined by size exclusion chromatography (SEC): Shimadzu HPLC system equipped with Superose 6 (300 x 10 mm; 13 μm), and UV-VIS (Shimadzu SPD-10AVvp), refractive index (RI) Optilab-rEX and multiangle light scattering (MALS) DAWN EOS detectors (Wyatt Technology Co.). The

eluent was 0.3 M sodium acetate buffer pH 6.5; flow-rate 0.5 ml.min⁻¹.

The content of hydrazide groups was determined by the modified TNBSA assay as described in the literature (Etrych *et al.* 2008). The total content of the polymer-bound Dox was measured by UV-VIS spectrophotometry in water (Etrych *et al.* 2008). The amount of the unbound Dox was determined by SEC from the relative area of the peak corresponding to the free drug and that corresponding to the sum of the free and polymer bound Dox using the HPLC system equipped with the TSKgel column G3000SWxl (300 x 7.8 mm; 5 µm) and UV-VIS detection at 488 nm.

Hydrodynamic radii (R_H) in a phosphate buffer (0.1 M, with 0.05 M NaCl; polymer concentration: 0.01 g·ml⁻¹) at 25 °C were measured by dynamic light scattering (DLS) with the Nano-ZS instrument Zetasizer (ZEN3600, Malvern). The intensity of scattered light was detected at an angle $\theta = 173^\circ$. The wavelength of laser was 632.8 nm. For evaluation of DLS data, the DTS(Nano) program was used. The values were the mean of at least five independent measurements.

In vitro release of Dox

The rates of Dox release from the conjugates were investigated in phosphate buffers at pH 5.0 or 7.4 (0.1 M, with 0.05 M NaCl) at 37 °C, using the polymer concentration in the incubation buffer equivalent to 0.5 mM Dox and using extraction of the released Dox into chloroform followed by HPLC analysis, as described previously (Etrych *et al.* 2002). The values are the mean of three independent experiments.

Experimental animals and tumor model

The inbred strain of mice C57BL/6 (*H-2b* haplotype), aged 8-12 weeks, was purchased from the Animal Centre of the Institute of Physiology, Academy of Sciences of the Czech Republic. All mice were housed in accordance with the approved guidelines and provided with food and water *ad libitum*. All studies were approved by the *Institutional Laboratory Animal Care and Use Committee*, and *Department Committee of the Academy*.

The murine EL4 T cell lymphoma cell line was purchased from ATCC (TIB-39) and maintained and cultivated as recommended by the provider.

Blood clearance and Dox content in tumor tissue

C57BL/6 male mice were subcutaneously inoculated with 1×10^5 EL4 lymphoma cells. When the

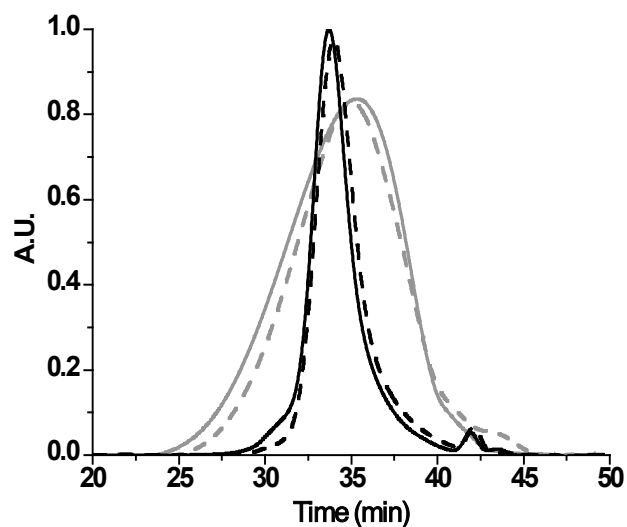
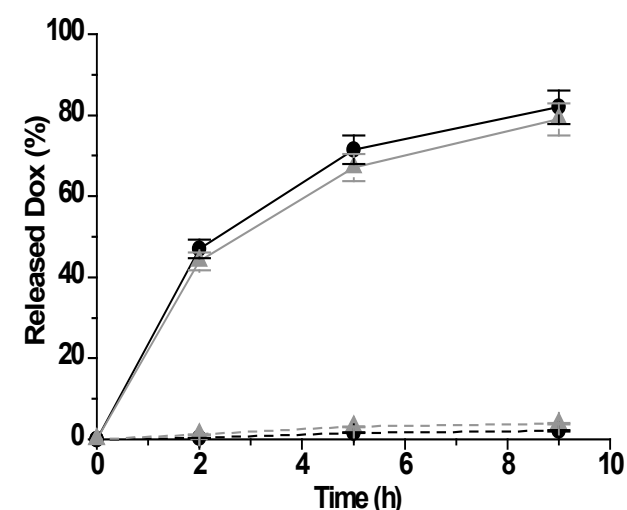
tumor reached the volume of 100-200 mm³ (after 8 days), the mice were intravenously injected either with Dox.HCl (5 mg/kg) or polymer-Dox conjugates (15 mg Dox (eq.)/kg), two mice per group/interval. Then, blood, urine, and tumor tissue samples were taken at the predetermined intervals after the injection: 0.5, 1, 3, 6, 12, 24, 48 and 72 h (blood and urine), and 12, 24, 48, and 72 h (tumors). To enable the urine collection, the mice were placed in metabolic cages, so that the urine was collected as the pooled sample from the two mice in each group. The blood samples (100 µl) were collected into heparinized tubes, and tumors were excised, weighed, and mechanically homogenized. The samples were tested for the total contents of Dox, in the case of Dox-conjugates the sum of the released and polymer-bound Dox. Determination of the total Dox content was performed after quantitative acid hydrolysis to doxorubicinone in 1 M HCl. After incubation for 1 h at 50 °C, doxorubicinone (formed aglycon of Dox) was extracted with chloroform, the organic phase was evaporated to dryness, the evaporation residue was completely dissolved in methanol and analyzed using the gradient-based HPLC Shimadzu system equipped with the reversed-phase column Chromolith Performance RP-18e (4.6 x 100 mm) and the fluorescence detector (Shimadzu RF-10Ax1) ($\lambda_{exc} = 488$ nm, $\lambda_{em} = 560$ nm) (Fraier *et al.* 1995). The calibration of the described method was conducted by injecting exact amounts of free Dox.HCl into blood, urine, and tumor samples obtained from untreated animals and then analyzed (homogenized, hydrolyzed, and extracted) as described above. The tumor-to-blood ratio was calculated as the ratio between Dox tumor content related to 1 g of the tissue and the Dox content in blood related to 1 ml of blood. Statistical analysis was performed using the Student's t-test and MS Excell software. Levels of significance are stated in the text for each analysis.

In vivo antitumor activity of the conjugates

C57BL/6 females were subcutaneously transplanted with 1×10^5 EL4 T cell lymphoma cells on the right flank on day 0. The mice in which palpable tumors reaching the volume of 100-200 mm³ developed within 8 days were intravenously treated with the conjugates diluted with PBS to 200 µl, as described in the Results. Controls were transplanted with the tumor cells and treated with PBS alone. The survival time, tumor size, and the number of long-term survivors were determined. Each experimental group consisted of eight animals.

Table 1. The characteristics of copolymers and their drug conjugates.

Sample	M_w (g/mol)	M_n (g/mol)	\bar{D}	R_H	Hydrazide content (mol%)	Dox content (w%)
LD-copolymer	30 000	26 800	1.13	4.1	7.2	-
HD-copolymer	29 900	17 000	1.75	3.9	5.6	-
LD-conjugate	38 900	33 000	1.18	4.8	n.a.	10.2
HD-conjugate	37 700	21 800	1.73	4.5	n.a.	9.8

**Fig. 1.** SEC chromatograms of polymer carriers and their drug conjugates. (— LD-copolymer, --- LD-conjugate, — HD-copolymer, --- HD-conjugate).**Fig. 2.** Drug release from the polymer-Dox conjugates incubated in phosphate buffers at 37 °C. (LD-conjugate: ●— pH 5, ●--- pH 7.4; HD-conjugate: ▲— pH 5, ▲--- pH 7.4).

Results

Synthesis of polymer precursors and their drug conjugates

Polymer precursors containing hydrazide groups were synthesized either by controlled radical polymerization (**LD-copolymer**) or by free radical polymerization (**HD-copolymer**) as described previously (Etrych *et al.* 2008, Chytil *et al.* 2010). Both copolymers had similar M_w of approximately 30 000 g/mol (see Table 1 for polymer characteristics). Nevertheless, the distribution of molecular weights differed, 1.13 and 1.75 for the **LD-** and **HD-copolymer** (Fig. 1). It means that while the **LD-copolymer** contained less than 10 % of polymer chain fractions below 20 000 g/mol, the **HD-copolymer** contained 50 % of them. On the contrary, while molecular weight of almost all polymer chains of the **LD-copolymer** was lower than 40 000 g/mol, there were 20 % of such polymer chains in the **HD-copolymer**. The content of hydrazide groups for both copolymers did not differ much and it did not influence the hydrodynamic radii, which was approximately 4 nm for both copolymers.

Dox.HCl was bound to polymer precursors by the hydrazone bond in the high yield (approx. 95 %). The M_w and the size of the conjugates increased compared to the copolymers, however, their dispersity did not change significantly. No differences in reactivity during the conjugation reaction or physico-chemical properties of prepared polymer-Dox conjugates were observed.

In vitro Dox release

The Dox was released rapidly from both conjugates under mild acidic condition in the buffer of pH 5.0 modeling the intracellular conditions of tumor cells. About 50 % of Dox was released within 2 h and after 9 h the Dox release was almost complete (Fig. 2). On the contrary, at the pH corresponding to blood, only the negligible release of Dox from the carrier was observed. No significant differences in the release rates between the conjugates were observed.

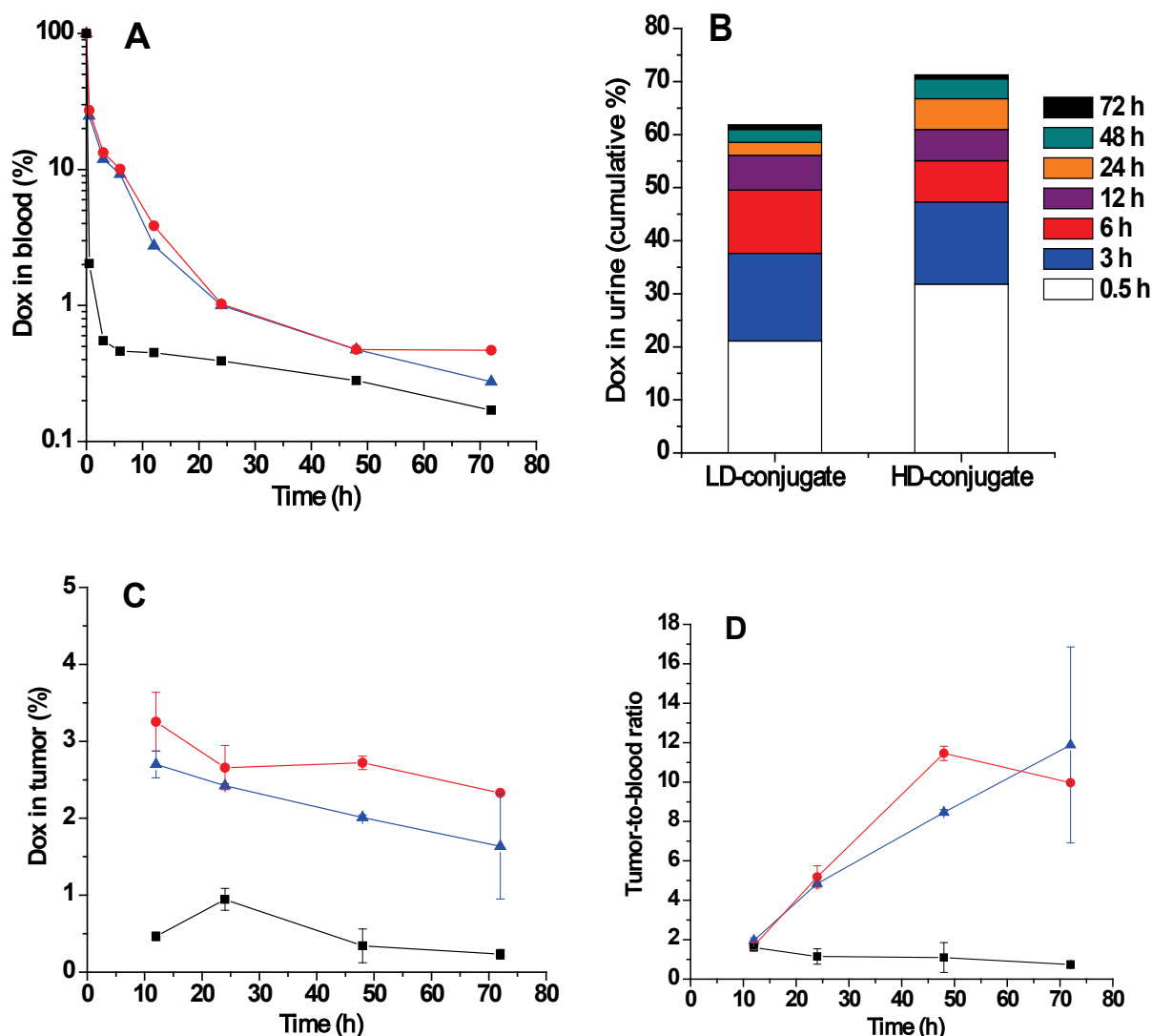


Fig. 3. Blood clearance (A), drug content in urine (B) and tumor (C) and tumor-to-blood ratio (D) in mouse bearing EL4 T-cell lymphoma; 1 x 15 mg Dox (eq.)/kg for conjugates, 1 x 5 mg Dox /kg of free drug (●— LD-conjugate, ▲— HD-conjugate, ■— Dox.HCl). In graph A and B the data shown are representative samples, in graph C and D the data shown are the mean of results from two animals.

Blood clearance and Dox content in urine and tumor tissue

The free Dox at a dose of 1 x 5 mg Dox /kg was eliminated from blood within minutes, only 2 % of the total Dox dose was found in blood after 0.5 h. However, blood clearance of the Dox-conjugates at a dose of 1 x 15 mg Dox (eq.)/kg was much slower, 25 % of the total Dox dose was remaining in blood after 0.5 h (Fig. 3A). The conjugates were cleared from blood within hours. Indeed, the Dox content in blood after 6 h was still 10 % of total Dox dose; i.e. 20 times more than for free Dox. Blood clearance of the **LD-** and **HD-conjugates** was not significantly different ($p < 0.05$). The Dox content in urine in the first interval examined (0.5 h after the

injection) was higher for the **HD-conjugate** (32 %) than for the **LD-conjugate** (21 %). Nevertheless, the difference of the Dox content in urine for both conjugates at following intervals was not significant (Fig. 3B). In sum, there was found about 10 % more of the eliminated dox in urine for the **HD-conjugate** within 72 h, showing the impact of the low-molecular-weight polymer fraction on urine elimination.

The accumulation of the free Dox and conjugates in the EL4 lymphoma tumors was compared. The **LD-** and **HD-conjugates** efficiently accumulated in the tumor tissue. About 3 % of the total Dox dose was found in tumor after 12 h for both conjugates, whereas the free Dox exhibited much lower tumor accumulation,

0.5 % of the total Dox dose in tumor after 12 h. The Dox accumulation rate for both conjugates was the highest after 12 h followed by the continuous slow decrease of the Dox content in the tumor. The Dox content of the **LD-conjugate** in tumor was slightly higher (about 20 %) than that of the **HD-conjugate** during the whole time period (Fig. 3C). While the tumor-to-blood ratio of the free Dox remained approx. 1, the tumor-to-blood ratio for both conjugates increased in the course of time (Fig. 3D) which corresponded with the decreasing blood concentration of polymer conjugates. The difference between tumor-to-blood ratios for both polymer conjugates was not significant.

In vivo antitumor activity

The *in vivo* therapeutic effect of the conjugates was evaluated in the EL4 T cell lymphoma-bearing C57BL/6 mice. At the dose of 1 x 15 mg Dox (eq.)/kg, both conjugates inhibited the tumor growth to the similar extent and cured the equal proportion of the animals (5 from 8 mice) (Fig. 4). However, at a dose of 1 x 25 mg Dox (eq.)/kg the **LD-conjugate** cured all mice, whereas the **HD-conjugate** cured only 6 of 8 treated animals. The overall survival of the treated mice for the **LD-** and **HD-conjugates** at a dose of 1 x 25 mg Dox (eq.)/kg was significantly different ($p < 0.01$).

The overall survival correlated with the tumor size after the treatment with polymer conjugates. At a dose of 1 x 15 mg Dox (eq.)/kg the tumor size decreased significantly; some tumors regressed completely after the treatment with both conjugates. The differences in the tumor size after the treatment with the **LD-** and **HD-conjugates** at a dose of 1 x 15 mg Dox (eq.)/kg were not statistically different ($p < 0.05$), and the rate of tumor growth was moderately faster in the group treated with the **HD-conjugate**. After the treatment with **LD-conjugate** at a dose of 1 x 25 mg Dox (eq.)/kg the tumors of all treated mice disappeared by day 24, and all the mice remained tumor-free whereas the treatment with the **HD-conjugate** at the same dose first led to the tumor disappearance, but by day 28 and 48, the tumors reappeared in two animals from this group (Fig. 4A).

Discussion

Synthesis of polymer precursors and their drug conjugates

The polymer backbone of HPMA-based copolymers is not biodegradable. Therefore, only

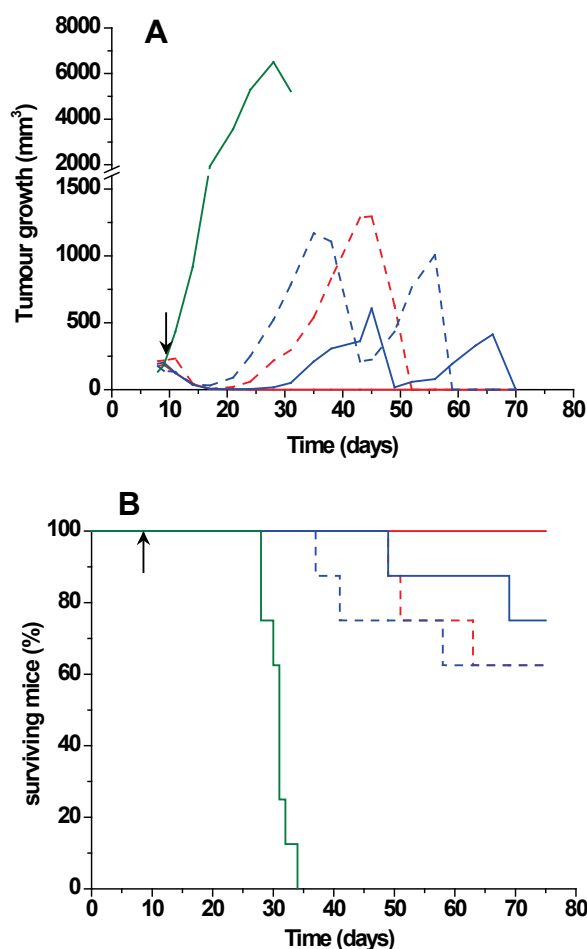


Fig. 4. *In vivo* effect of polymer–Dox conjugates on the growth of T cell lymphoma EL-4 (A), survival of mice (B) (**LD-conjugate**: — 1 x 25 mg Dox (eq.)/kg, - - - 1 x 15 mg Dox (eq.)/kg; **HD-conjugate**: — 1 x 25 mg Dox (eq.)/kg, - - - 1 x 15 mg Dox (eq.)/kg; — controls). Arrow, administration of the treatment.

polymers with molecular weights under the limit of renal filtration (M_w below 50 000 g/mol) can be used as carriers in drug delivery systems. Polymers with higher molecular weights could potentially accumulate in the body, possibly leading to side effects. M_w of the HPMA copolymer hydrazone conjugate, prepared by uncontrolled free radical polymerization and evaluated for its *in vivo* anti-cancer activity, was about 25 000–30 000 g/mol (Etrych *et al.* 2008, Šírová *et al.* 2010). Thus, the controlled RAFT polymerization conditions were optimized with the intention of obtaining copolymers with the comparable M_w (Chytil *et al.* 2010). Although the synthetic procedure consisted in polymerization, the removal of terminal dithiobenzoate group and deprotection of hydrazide groups, the almost monodispersed **LD-copolymer** was prepared. It means

that the majority of its polymer chains had the molecular weight about 30 000 g/mol. On the contrary, the content of the lower-molecular-weight fraction was quite high in the case of the **HD-copolymer** prepared by free radical polymerization. Vice versa, a significant amount of polymer chains with higher molecular weights was detected in the **HD-copolymer** sample.

In vitro Dox release

The results of the *in vitro* drug release experiments in mild acidic and neutral buffers showed that the hydrazone bond was hydrolyzed quite fast at pH below 7 during incubation of both polymer-Dox conjugates. As expected, the dispersity did not influence the hydrolysis rate. Thus, the cytostatic agent Dox can be released from both conjugates in the slightly acidic environment of the target tumor tissue or the endosomes/lysosomes of tumor cells irrespective of the conjugate dispersity.

Blood clearance and Dox content in urine and tumor tissue

The advantage of the polymer-bound Dox against the free Dox was demonstrated many times before. Also here, prolonged circulation in blood and enhanced solid tumor accumulation was showed. More importantly, the comparison of the accumulation rate for the two polymer conjugates with different dispersity was performed. We expected the **HD-conjugate** to be eliminated faster by renal filtration than the **LD-conjugate** due to its higher amount of lower-molecular-weight polymer chains. This hypothesis was supported by the relatively high Dox content from the **HD-conjugate** determined in urine in the first 30 min interval. On the contrary, the **HD-conjugate** contained also a certain amount of polymer chains of much higher molecular weight than is the renal threshold (M_w approx. 45 000 g/mol) and which could circulate much longer in the body. Overall, the slightly higher amount of the **HD-conjugate** was found in urine within 72 h showing the influence of higher dispersity of the polymer carrier on the rate of urine elimination.

However, the Dox content in blood was comparable for both conjugates and the Dox content in tumor was slightly higher, about 20 %, for the **LD-conjugate** showing moderately higher tumor accumulation. Nevertheless, the difference in the drug content either in blood or tumor was not statistically significant. Also tumor-to-blood ratio values, indicating

enhanced accumulation in solid tumor, did not significantly differ. Both parameters correspond to the applied dose of 1 x 15 mg Dox (eq.)/kg. Thus, any differences in antitumor activity probably could not be expected at this dose. However, in the case of administration of the higher dose than 1 x 15 mg Dox (eq.)/kg, which is suboptimal for the therapeutic outcome, the potential difference between the conjugates may become apparent and even statistically relevant.

In vivo antitumor activity

Based on previous experiments, the **LD** and **HD-conjugates** were administered at a dose of 1 x 15 mg Dox (eq.)/kg which is suboptimal in respect of the treatment of established EL4 lymphoma; and at a dose of 1 x 25 mg Dox (eq.)/kg of the conjugated Dox which is the optimal dose and is still far from the maximal tolerated dose (MTD) of the **HD-conjugate**, expected to be about 85 mg Dox (eq.)/kg. The treatment by the free Dox is unsatisfactory as some proportion of animals could be cured only at doses close to MTD, thus they are harmed by late toxicity of the drug. At lower Dox doses, the treated animals survived usually not longer than controls (Mrkvan *et al.* 2005, Etrych *et al.* 2008).

The similar antitumor activity of conjugates at the dose of 1 x 15 mg Dox (eq.)/kg was in accordance with the results obtained in the biodistribution experiment at the same dose. Most probably, the higher antitumor activity of the **LD-conjugate** at the dose of 1 x 25 mg Dox (eq.)/kg can be ascribed to the enhanced tumor accumulation due to the retention of polymer chains with sufficient molecular weights. Moreover, the molecular weight of the HPMA-based polymer conjugates with low dispersity prepared by RAFT polymerization can be significantly increased without exceeding the limit of renal filtration; i.e. the molecular weight of the longest polymer chains should be underneath the limit. It can be expected that such polymer-drug conjugates will be more efficient in the treatment of solid tumors and still capable of the carrier removal from the organism.

Conclusions

The linear water-soluble HPMA-based polymer-drug conjugates bearing the anticancer drug Dox bound by the pH-sensitive hydrazone bond were synthesized and characterized. Their polymer precursors had the same M_w of about 30 000 g/mol, but they differed in dispersity ($D = 1.13$ or 1.75) due to the utilization of controlled RAFT

or free radical polymerization. Considering the same drug release rate determined *in vitro*, the potential influence on biodistribution or antitumor activity *in vivo* can be attributed to the different dispersity of the carriers. Although moderately enhanced drug accumulation in EL4 T-cell lymphoma tumors was determined for the monodispersed **LD-conjugate**, the differences were not statistically significant. Thus, the therapeutic activity of both conjugates at the same suboptimal dose of 1 x 15 mg Dox (eq.)/kg did not differ (63 % of long-termed survivals). As expected, the increase of the dose to 1 x 25 mg Dox (eq.)/kg resulted in the significantly increased antitumor activity of polymer-Dox conjugates. The application of the **LD-conjugate** at the higher dose was more efficient (100 % of long-termed survivals) than that of the **HD-conjugate** (75 % of long-termed survivals). We suppose that the absence of lower-molecular-weight

polymer chains in the **LD-conjugate** facilitated the prolonged blood circulation and enhanced tumor accumulation of the **LD-conjugate**, which could result in better therapeutic activity at the higher dose. Further experiments proving such a hypothesis are under way.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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