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Synergistic Action of Doxorubicin Bound to the Polymeric Carrier Based on *N*-(2-Hydroxypropyl)methacrylamide Copolymers through an Amide or Hydrazone Bond

B. Říhová,*,† T. Etrych,‡ M. Šírová,† L. Kovář,† O. Hovorka,† M. Kovář,† A. Benda,§ and K. Ulbrich‡

Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i., Videnska 1083, 142 20 Prague 4, Czech Republic, Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, v.v.i., Heyrovsky Sq. 2, 162 06 Prague 6, Czech Republic, and J. Heyrovsky Institute of Physical Chemistry, Academy of Sciences of the Czech Republic, v.v.i., Dolejskova 2155/3, 182 23 Prague 8, Czech Republic

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Abstract: The cytostatic effects of polymeric conjugates based on *N*-(2-hydroxypropyl)methacrylamide copolymers (PHPMA) and containing doxorubicin bound through amide and hydrazone bonds (mixed conjugates) were compared with the cytostatic effects of monoconjugates containing drug bound through an amide or hydrazone bond. One group of mixed conjugates was formed from two comonomers containing doxorubicin bound to the methacryloyl group through a spacer and an amide (DOX^{AM}) or hydrazone (DOX^{HYD}) bond via copolymerization with HPMA. A second group of mixed conjugates was formed from two different interconnected HPMA copolymers, one containing DOX^{AM} and the other DOX^{HYD}, forming a high-molecular-weight branched structure. The third mixed polymeric system was a simple mixture of monoconjugates DOX^{AM}−PHPMA and DOX^{HYD}−PHPMA. Simultaneous treatment with all mixed forms of the polymeric derivatives of doxorubicin significantly increased antitumor efficacy after application of monoconjugates, suggesting a synergizing effect that could be used in designing new doxorubicin-containing therapeutic systems.

Keywords: HPMA; branched polymers; EL4 T cell lymphoma; calreticulin; nu/nu mice; acute toxicity; retransplantation; doxorubicin; anticancer immunity

Introduction

Despite significant advances in treatment and intensive research, the prognosis for patients with advanced cancer remains poor. Therefore, the need remains for improved anticancer therapy that effectively and specifically targets cancer cells while minimizing toxic side effects. In this respect, the concept of combination therapy appears to be a promising modern modality. The idea was already proven for "polymeric therapeutics" [1] using a *N*-(2-hydroxypropyl)-methacrylamide (HPMA) copolymer carrier to which doxorubicin (a typical chemotherapeutic drug) and mesochlorin e_6 monoethylenediamine (representative of photodynamic therapy, PDT) were bound, and synergistic antitumor effects were reported.[2−4] In addition, a combination therapy with

* Corresponding author. Mailing address: Academy of Sciences of the Czech Republic, v.v.i., Institute of Microbiology, Department of Immunology and Gnotobiology, Videnska 1083, 142 20 Prague 4, Czech Republic. Tel: +420 241062345. Fax: +420 241721143. E-mail: rihova@biomed.cas.cz.

† Institute of Microbiology.

‡ Institute of Macromolecular Chemistry.

§ J. Heyrovsky Institute of Physical Chemistry.

(1) Duncan, R.; Dimitrijevic, S.; Evagorou, E. G. The role of polymer conjugates in the diagnosis and treatment of cancer. *STP Pharma Sci.* **1996**, *6*, 237–263.

(2) Krinick, N. L.; Sun, Y.; Joyner, D.; Spikes, J. D.; Straight, R. C.; Kopeček, J. A. polymeric drug delivery system for the simultaneous delivery of drugs activatable by enzymes and/or light. *J. Biomater. Sci., Polym. Ed.* **1994**, *5*, 303–324.

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polymeric therapeutics based on HPMA was described for hormone-dependent cancer by Vicent et al.[5]

Drugs can be bound to their polymeric carriers through different covalent bonds that are responsible for tumor site-specific drug release. Doxorubicin has been routinely attached to HPMA copolymer (PHPMA) either through a proteolytically cleavable amide bond (DOX^{AM}−PHPMA)[6] or through a hydrolytically cleavable hydrazone bond (DOX^{HYD}−PHPMA).[7] The *in vitro* cytotoxic and cytostatic activities and the *in vivo* anticancer activity of such monoconjugates have been reported from numerous laboratories.[6,8−13] Polymeric therapeutics composed of HPMA copolymers and doxorubicin are still worth studying because they represent a safe and efficient drug targeting system, and questions persist about their mechanisms of action. DOX^{AM}−PHPMA, also known as PK1 or FCE 28068, was designed by attaching a drug to a polymeric backbone via an amide bond that is cleavable by lysosomal enzymes.[6,14] Subsequently, DOX^{HYD}−PHPMA was developed, employing a pH-sensitive hydrazone bond between doxorubicin and an HPMA copolymer; this modification was also effective in cells with a limited amount of lysosomes.[7,15,16] Up to this point, the fact that doxorubicin is released from the DOX^{HYD}−PHPMA conjugate intracellularly at sites with low pH, i.e., endosomes and lysosomes, has not been questioned. From those sites, doxorubicin penetrates the cytoplasm and accumulates in the nuclei, where it induces apoptosis.[17,18] The mechanism of action of the amide counterpart DOX^{AM}−PHPMA is still being studied extensively, but it has yet to be elucidated. The hypothesis, which is endorsed by Kopeček and Duncan,[6,12] focuses on the original idea underlying the design of the conjugate in which doxorubicin is bound to the polymeric carrier through a proteolytically cleavable oligopeptide sequence −GlyPheLeuGly. It is hypothesized that the drug is released via the action of lysosomal proteolytic enzymes; it then penetrates the lysosomal membrane and is accumulated in the nuclei after transportation through the cytoplasm.

Despite intensive effort, we were unable to prove *in vitro* the direct correlation between cytotoxicity and the rate of drug release from the polymeric carrier.[19] Due to some technical problems connected with spontaneous release of extremely fluorescently active derivative D*,[17,20−22] we were

(3) Peterson, C. M.; Lu, J. M.; Sun, Y.; Peterson, C. A.; Shiah, J. G.; Straight, R. C.; Kopecek, J. Combination chemotherapy and photodynamic therapy with *N*-(2-hydroxypropyl)methacrylamide copolymer-bound anticancer drugs inhibit human ovarian carcinoma heterotransplanted in nude mice. *Cancer Res.* **1996**, *56*, 3980–3985.

(4) Hongrapipat, J.; Kopečková, P.; Liu, J.; Prakongpan, S.; Kopeček, J. Combination chemotherapy and photodynamic therapy with Fab′ fragment targeted HPMA copolymer conjugates in human ovarian carcinoma cells. *Mol. Pharmaceutics* **2008**, *5*, 696–709.

(5) Vicent, M. J.; Greco, F.; Nicholson, R. I.; Paul, A.; Griffiths, P. C. Duncan. Polymer therapeutics designed for a combination therapy of hormone-dependent cancer. *Agnew. Chem., Int. Ed.* **2005**, *44*, 4061–4066.

(6) Kopeček, J.; Kopečková, P.; Minko, T.; Lu, Z. HPMA copolymer-anticancer drug conjugates: design, activity, and mechanism of action. *Eur. J. Pharm. Biopharm.* **2000**, *50*, 61–81.

(7) Etrych, T.; Jelínková, M.; Říhová, B.; Ulbrich, K. New HPMA copolymers containing doxorubicin bound via pH sensitive linkage: synthesis and preliminary in vitro and in vivo biological properties. *J. Controlled Release* **2001**, *73*, 89–102.

(8) Říhová, B; Kopeček, J. Biological properties of targetable poly N-(2-hydroxypropyl)methacrylamide-antibody conjugates. *J. Controlled Release* **1985**, *2*, 289–310.

(9) Říhová, B.; Kopečková, P.; Strohalm, J.; Rossmann, P.; Větvička, V.; Kopeček. J. Antibody-directed affinity therapy applied to the immune system: In vivo effectiveness and limited toxicity of daunomycin conjugated to HPMA copolymers and targeting antibody. *Clin. Immunol. Immunopathol.* **1988**, *46*, 100–114.

(10) Duncan, R.; Kopečková, P.; Strohalm, J.; Hume, I. C.; Lloyd, J. B.; Kopeček, J. Anticancer agents coupled to *N*-(2-hydroxypropyl)methacrylamide copolymers. II. Evaluation of daunomycin conjugates *in vivo* against L1210 leukaemia. *Br. J. Cancer.* **1988**, *57*, 147–156.

(11) Minko, T; Kopečková, P; Kopeček, J. Mechanisms of anticancer action of HPMA copolymer-bound doxorubicin. *Macromol. Symp.* **2001**, *172*, 35–47.

(12) Duncan, R. N-(2-Hydroxypropyl)methacrylamide copolymer conjugates. In *Polymeric Drug Delivery Systems*; Kwon, G. S., Ed.; Drugs and the Pharmaceutical Sciences 148; Taylor & Francis: Boca Raton, 2007; pp 1−92.

(13) Kovář, M.; Tomala, J.; Chmelová, H.; Kovář, L.; Mrkvan, T.; Josková, R.; Zákostelecká, Z.; Etrych, Z.; Strohalm, J.; Ulbrich, L.; Šírová, M.; Říhová, B. Overcoming immunoescape mechanisms of BCL1 leukemia and induction of $CD8^+$ T cell-mediated BCL1-specific resistance in mice cured by targeted polymer-bound doxorubicin. *Cancer Res.* **2008**, *68*, 9875–9883.

(14) Duncan, R. Drug-polymer conjugates: potential for improved chemotherapy. *Anticancer Drugs* **1992**, *3*, 175–210.

(15) 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.

(16) Mrkvan, T.; Šírová, M.; Etrych, T.; Chytil, P.; Strohalm, J.; Plocová, D.; Ulbrich, K. Chemotherapy based on HPMA copolymer conjugates with pH-controlled release of doxorubicin triggers anti-tumor immunity. *J. Controlled Release* **2005**, *110*, 119–129.

(17) Hovorka, O.; Etrych, T.; Šubr, V.; Strohalm, J.; Ulbrich, K.; Říhová, B. HPMA based macromolecular therapeutics: Internalization, intracellular pathway and cell death depend on the character of covalent bond between the drug and the peptidic spacer and also on spacer composition. *J. Drug Targeting* **2006**, *14*, 391–403.

(18) Kovář, L.; Strohalm, J.; Chytil, P.; Mrkvan, T.; Kovář, M.; Hovorka, O.; Ulbrich, K.; Rihova, B. The same drug but a different mechanism of action: comparison of free doxorubicin with two different *N*-(2-hydroxypropyl)methacrylamide copolymer-bound doxorubicin conjugates in EL-4 cancer cell line. *Bioconjugate Chem.* **2007**, *18*, 894–902.

(19) Jelínková, M.; Strohalm, J.; Plocová, D.; Šubr, V.; Šťastný, M.; Ulbrich, K.; Říhová, B. Targeting of human and mouse T-lymphocytes by monoclonal antibody-HPMA copolymer-doxorubicin conjugates directed against different T-cell surface antigens. *J. Controlled Release* **1998**, *52*, 253–270.

(20) Fiallo, M.; Laigle, A.; Borrel, M. N.; Garnier-Suillerot, A. Accumulation of degradation products of doxorubicin and pirarubnicin formed in cell culture medium within sensitive and resistant cells. *Biochem. Pharmacol.* **1993**, *45*, 659–665.

unable to unequivocally document nuclear accumulation of the drug. The signs of both apoptosis and necrosis are detectable in cells exposed to the DOX^{AM}−PHPMA conjugate.[17,23]

Kovář and co-workers[18] analyzed death signals triggered in EL4 cancer cells upon exposure to the free drug or to doxorubicin bound to an HPMA copolymer carrier via a proteolytically/enzymatically or hydrolytically cleavable bond. It was reported that the two conjugates differ in pro-apoptotic JNK and pro-survival ERK phosphorylation, pointing to a similarity shared only between the free drug and the conjugate containing doxorubicin bound via a hydrazone bond. Similarly, an activation of caspase 3 and decreased binding activity of the p50 subunit of NFκB was observed only in cells treated with free doxorubicin or conjugate DOX^{HYD}−PHPMA; no such effects were seen in cells incubated with DOX^{AM}−PHPMA.

Recent studies have reported that translocation and cancer cell surface expression of calreticulin (CRT) as a sign of "immunogenic cancer cell death" [24] were detected only in cells incubated with free or hydrazone-bound drug.[25] These findings support the idea that different intracellular killing events follow the intracellular accumulation and distribution of DOX^{AM} and PHPMA conjugates containing DOX^{HYD}.

Assuming a different mechanism of action of two drug−PHPMA monoconjugates, we address the question of their possible combination effects if injected in the form of mixed conjugates or in a mixture of monoconjugates.

Experimental Section

Materials. Hydrazine monohydrate, methacryloyl chloride, 1-aminopropan-2-ol, 4-nitrophenol, 6-aminohexanoic acid (ah), methyl-6-aminohexanoate hydrochloride (ah-MeO), glycyl-L-phenylalanine, L-leucylglycine, 2,2′-azobis(isobutyronitrile) (AIBN), 2-(dimethylamino)ethyl methacrylate, *N*-ethyldiisopropylamine, dimethylformamide (DMF), phthalaldehyde (OPA), *N*,*N*′-dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole (HOBT), dimethyl sulfoxide (DMSO) and doxorubicin hydrochloride (DOX·HCl) were purchased from Fluka Chemie AG. 2,4,6-Trinitrobenzene-1-sulfonic acid was purchased from SERVA Feinbiochemica Heidelberg. All other reagents and solvents were of analytical grade.

Synthesis and Characterization of Monomers. *N*-(2-Hydroxypropyl)methacrylamide (HPMA) was synthesized according to known procedures[26] using Na_2CO_3 as a base in the methacryloylation reaction (mp 70 °C; elemental analysis (calcd/found), 58.80/58.98% C; 9.16/9.18% H; 9.79/9.82% N). 6-Methacrylamidohexanohydrazide (Ma-ah-$NHNH_2$) was prepared in a two-step synthesis:[27] mp 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. 6-Methacrylamidohexanohydrazone-doxorubicin (Ma-ah-NHN═DOX) was prepared by the reaction of Ma-ah-$NHNH_2$ with DOX·HCl:[27] mp 172−175 °C; TLC on silica gel 60 F_{254} (methanol:chloroform:acetic acid 2:8:1), one spot at $R_f = 0.9$; MALDI-TOF MS, 762.2 (M + Na). *N*-Methacryloylglycyl-DL-phenylalanyl-L-leucylglycine 4-nitrophenyl ester (Ma-GFLG-ONp) was synthesized as previously reported:[28,29] mp 134−136 °C; amino acid analysis, Gly/L-Phe/D-Phe/L-Leu = 2.05/0.54/0.47/1.00; elemental analysis (calcd/found), C 59.89/59.21%; H 6.07/6.25%; N 12.04/12.32%; HPLC showed two peaks of equal areas at 14.41 min (L-Phe peptide) and 14.71 min (D-Phe peptide). *N*-Methacryloylglycyl-DL-phenylalanylleucylglycyldoxorubicin (Ma-GFLG−DOX) was prepared by the reaction of an equivalent amount of Ma-GFLG-ONp and DOX·HCl in DMF at 4 °C as previously reported:[26] TLC, two spots at $R_f = 0.46$ and 0.4 corresponding to the D-Phe and L-Phe isomers (chloroform−methanol 8:1); HPLC showed a single peak at 18.22 min (UV detection at 230 and 484 nm); amino acid analysis, Gly/L-Phe/D-Phe/L-Leu 2.06:0.56:0.45:1.00.

The purity of each monomer was determined by HPLC (LDC Analytical, USA) using a reversed-phase column Chromolith RP-18e (100 × 4.6 mm) with UV detection at 230 nm, water−methanol as the eluent with a gradient of

(21) Říhová, B.; Strohalm, J.; Hovorka, O.; Šubr, V.; Etrych, T.; Chytil, P.; Pola, R.; Plocová, D.; Bouček, J.; Ulbrich, K. Doxorubicin release is not a prerequisite for the in vitro cytotoxicity of HPMA-based pharmaceuticals: *in vitro* effect of extra drug-free GlyPhe-LeuGly sequences. *J. Controlled Release* **2008**, *127*, 110–120.

(22) Říhová, B.; Hovorka, O.; Kovář, L.; Kovář, M.; Mrkvan, T.; Šírová, M.; Šubr, V.; Ulbrich, K. HPMA-anticancer drug conjugates. In *Macromolecular Anticancer Therapeutics*; Reddy, L. H., Couvreur, P., Eds.; Humana Press: Totowa, NJ, 2010; pp 87−132.

(23) Demoy, M.; Minko, T.; Kopečková, P.; Kopeček, J. Time- and concentration-dependent apoptosis and necrosis induced by free and HPMA copolymer-bound doxorubicin in human ovarian carcinoma cells. *J. Controlled Release* **2000**, *69*, 185–196.

(24) Obeid, M.; Tesniere, A.; Ghiringhelli, F.; Fimia, G. M.; Apetoh, L.; Perfettini, J.L.; Castedo, M.; Mignot, G.; Panaretakis, T; Casares, N.; Metivier, D.; Larochette, N.; van Endert, P.; Cioccosanti, F.; Piacentini, M.; Zitvogel, L.; Kroemer, G. Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat. Med.* **2007**, *13*, 54–61.

(25) Říhová, B.; Kovář, L.; Kovář, M.; Hovorka, O. Cytotoxicity and immunostimulation: double attack on cancer cells with polymeric therapeutics. *Trends Biotechnol.* **2009**, *27*, 11–17.

(26) Ulbrich, k.; Šubr, V.; Strohalm, J.; Plocová, D.; Jelínková, M.; Říhová, B. Polymeric drugs based on conjugates of synthetic and natural macromolecules: I. Synthesis and physico-chemical characterisation. *J. Controlled Release* **2000**, *64*, 63–79.

(27) Etrych, T.; Mrkvan, T.; Chytil, P.; Koňák, Č.; Říhová, B.; Ulbrich, K. Polymer prodrugs based on HPMA conjugates with pH-controlled activation of doxorubicin: I. Synthesis, physicochemical characterisation and preliminary biological evaluation. *J. Appl. Polym. Sci.* **2008**, *109*, 3050–3061.

(28) Rejmanová, P.; Labský, J.; Kopeček, J. Aminolyses of monomeric and polymeric 4-nitrophenyl esters of N-methacryloylated amino acids. *Makromol. Chem.* **1977**, *178*, 2159–2168.

(29) Říhová, B.; Bilej, M.; Větvička, V.; Ulbrich, K.; Strohalm, J.; Kopeček, J.; Duncan, R.; et al. Biocompatibility of N-(2-hydroxypropyl) methacrylamide copolymers containing adriamycin. Immunogenicity, and effect on haematopoietic stem cells in bone marrow in vivo and mouse splenocytes and human peripheral blood lymphocytes in vitro. *Biomaterials* **1989**, *10*, 335–42.

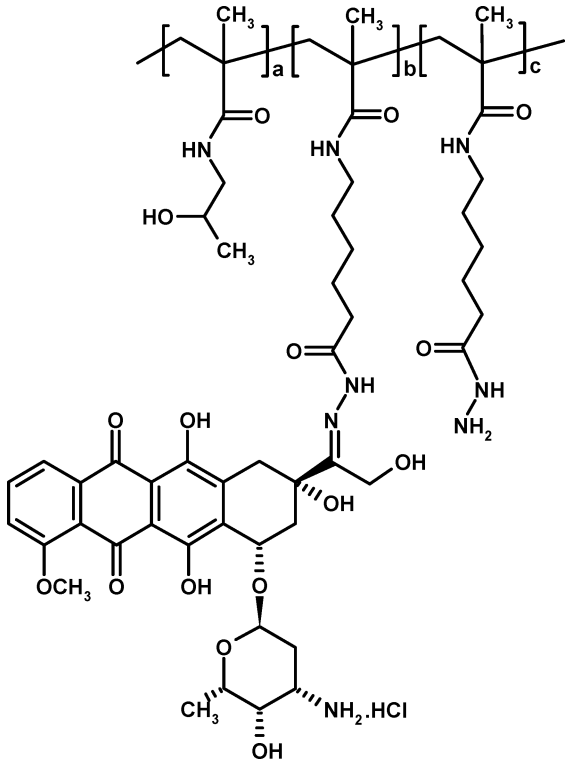


Figure 1. Structure of polymer conjugate DOX^{HYD}–PHPMA containing DOX attached via hydrazone bond (conjugates **3** or **4**).

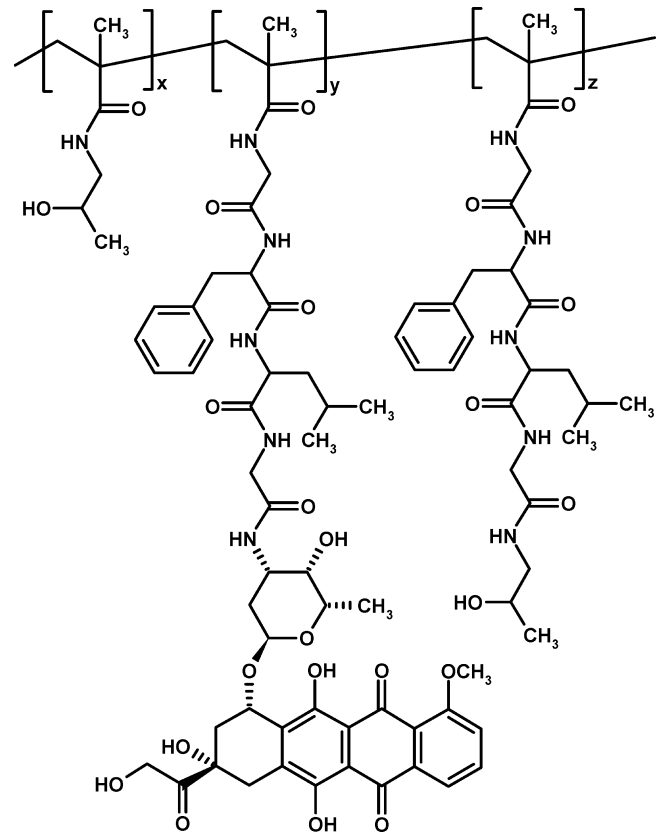


Figure 2. Structure of polymer conjugate DOX^{AM}–PHPMA containing DOX attached via amide bond (conjugate **5**).

50−100 vol % methanol, and a flow rate of 0.5 mL/min. The amino acid analysis was performed using an amino acid analyzer (LDC Analytical, USA) with precolumn OPA derivatization.

Synthesis of Polymer Precursors. Copolymer **1** (poly(HPMA-*co*-Ma-ah-$NHNH_2$)) was 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; 60 °C; 17 h) as described in ref 27. The content of hydrazide groups in each precursor and its respective conjugate was determined by a modified TNBSA assay as described.[7]

Polymer precursor **2** (poly(HPMA-*co*-Ma-GFLG-ONp) was prepared by radical precipitation polymerization.[26] The content of 4-nitrophenoxy group (ONp) was determined by UV spectrophotometry (ε = 9 500 L mol^{-1} cm^{-1}, λ = 274 nm, DMSO). The molecular weight of each polymer was determined with a FPLC Pharmacia system equipped with RI, UV and multiangle light scattering DAWN DSP-F (Wyatt Co., USA) detectors using a 0.3 M acetate buffer (pH 6.5) and a Superose 6 column.

Synthesis and Characterization of Polymer−DOX Conjugates. Polymer conjugates **3** and **4** (poly(HPMA-*co*-Ma-ah-NHN═DOX), DOX^{HYD}−PHPMA, for structure see Figure 1) containing DOX attached via a pH-sensitive hydrazone bond was prepared by the reaction of polymer precursor **1** with DOX·HCl in methanol in the dark as described.[7] Polymer conjugate **5** (poly(HPMA-co-Ma-GFLG-DOX), DOXAM-PHPMA), structure shown in Figure 2) containing DOX attached *via* an enzymatically degradable oligopeptide sequence was prepared by the reaction of poly(HPMA-*co*-Ma-GFLG-ONp) with DOX·HCl in DMSO in the presence of triethylamine as described.[26]

Random copolymer conjugate **6** (DOX^{AM}−DOX^{HYD}–PHPMA, for structure see Figure 3) was prepared by solution radical copolymerization in methanol initiated with AIBN. HPMA (334 g, 3.34 mmol), Ma-ah-NHN═DOX (33 mg, 0.45 mmol) and Ma-GFLG−DOX (44 mg, 0.45 mmol) were dissolved in methanol (3.5 mL). The solution was introduced into a polymerization ampule and bubbled with nitrogen, and the ampule was sealed. The polymerization was carried out at 60 °C for 22 h. The polymer was isolated by precipitation into an acetone/diethyl ether mixture (1:1) and purified by reprecipitation from a methanolic solution. The polymer was filtered off, washed with diethyl ether, and dried in vacuum. The yield was 0.33 g (77%).

Mixtures of polymer conjugates (DOX^{HYD}−PHPMA/DOX^{AM}−PHPMA), polymer preparations **7**−**11**, were prepared by dissolution of the proper amount of polymer conjugates **4** and **5** in methanol, followed by mixing of the solutions and separation of the polymer mixture by precipitation into ethyl acetate. Branched polymer conjugates (DOX^{AM}−DOX^{HYD}−branchPHPMA, structure shown in Figure 4) **13**−**16** were prepared by conjugation of polymer **4**, which contains DOX attached via a hydrazone bond, with polymer **5**, which contains DOX attached via an amide bond, in methanol in the presence of acetic acid. Procedure for

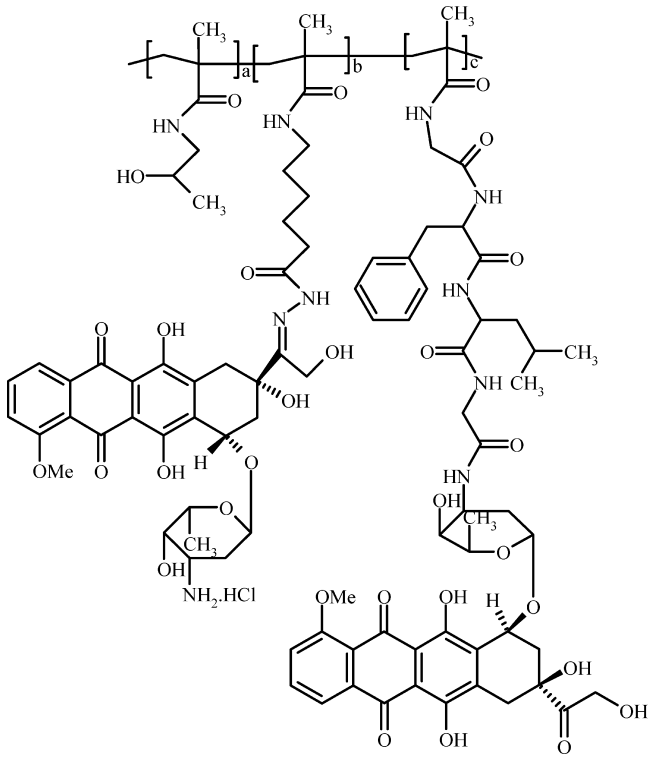


Figure 3. Structure of linear random copolymer DOX^{AM}−DOX^{HYD}−PHPMA (conjugate **6**) containing DOX attached via hydrazone or amide bond both randomly distributed along the HPMA copolymer backbone.

preparation (polymer **13**): 100 mg of polymer **5** and 75 mg of polymer **4** were dissolved in 2 mL of methanol and mixed together. After 1 min of stirring, 80 μL of acetic acid was added and the solution was stirred for 5 h. Next, impurities with low molecular weight were removed by gel filtration using a LH-20 column with methanol elution. The corresponding polymer fraction was collected, and polymer conjugate **13** was precipitated into ethyl acetate, filtered, washed with ethyl acetate and dried in vacuum. Polymer conjugates **14**, **15** and **16** were prepared by the same procedure described above with a variation of the reaction time and the ratio of polymers **4** and **5**. The variations are as follows: polymer conjugate **14** (100 mg of polymer **5** and 75 mg of polymer **4**, 20 h), polymer conjugate **15** (100 mg of polymer **5** and 150 mg of polymer **4**, 24 h) and polymer conjugate **16** (100 mg of polymer **5** and 150 mg of polymer **4**, 16 h).

The polymer−drug conjugates were freed of low-molecular-weight impurities (such as DOX, 4-nitrophenol) by gel filtration using a Sephadex LH-20 column with methanol elution, and they were tested for the content of the free drug using HPLC analysis after extraction of free DOX into chloroform from an aqueous polymer solution. The content of DOX in the conjugates was measured spectrophotometrically in water using $\varepsilon = 11\ 500$ L mol^{-1} cm^{-1} at $\lambda = 488$ nm).

In Vitro **Degradation of the Branched Polymer Conjugates.** The branched polymer conjugates **13**−**16** were incubated in saline−sodium phosphate buffers (0.1 M sodium phosphate; 0.05 M NaCl; 1 mM EDTA; pH 5 or 7.4) at 37 °C at a final polymer concentration of 10 mg/mL. At fixed time intervals, aliquots of the incubation mixture were desalted by gel filtration using a PD-10 column with distilled water elution. After freeze-drying, the molecular weights of the samples were evaluated with a FPLC Pharmacia system equipped with RI, UV and multiangle light scattering DAWN DSP-F (Wyatt Co., USA) detectors using a 0.3 M acetate buffer (pH 6.5) and a Superose 6 column.

Cancer Cell Lines, Cell Cultures and Cytostatic Activity *in Vitro*. Ten different cancer cell lines of mouse (T-cell lymphoma EL4, B cell lymphoma 38C13, fibroblasts 3T3, B cell leukemia BCL1, melanoma B16F10 and breast carcinoma 4T1) and human (metastazing colorectal carcinoma SW 620, B cell lymphoma Raji, T cell leukemia Jurkat and spinocellular carcinoma FaDu) origin were used for *in vitro* studies. Mouse T-cell lymphoma EL4 was used for *in vivo* studies. All cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD). Cell cultures and cytostatic activity measurement were performed as previously described.[21]

Tumor Models *in Vivo*. *Acute Model.* C57BL/6 (B/6; H-2^b) females were subcutaneously transplanted once with a lethal dose (1×10^5) of EL4 T cell lymphoma cells, i.e., a dose that induces tumor growth in all controls. The mice that developed palpable tumors reaching 5 to 8 mm^3 in diameter within 8 to 9 days after the implantation were intravenously treated with polymer conjugates diluted in PBS, as described in Results and Discussion. Those surviving at least 60 days without any signs of a tumor were considered as long-term survivors (LTS), and they were retransplanted with a lethal dose of the same tumor cells and left without treatment to determine the therapy-induced tumor resistance.

Chronic Model. C57BL/6 females were injected sc 6 times (every other day) with a low dose (1×10^4) of EL4 T cell lymphoma. None of them developed a palpable tumor until the sixth injection. Then, some of them developed a tumor. In those cases, when the tumor reached the size of about 8 mm^3, the treatment was started as described in Results and Discussion. Nontreated mice served as a "chronic" control.

Generally, 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 at least 8 mice.

Conventional mice were obtained from the breeding colony of the Institute of Physiology, Academy of Sciences of the Czech Republic, v.v.i., and female nu/nu CD-1 mice were obtained from Anlab (Czech Republic). The mice were 2 to 3 months old at the start of experiments and were given food and water ad libitum. All experiments were approved by the Animal Welfare Committee of the Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i.

Fluorescence Lifetime Microscopy. Fluorescence lifetime imaging (FLIM) was carried out as described in a previous study.[30] Time-resolved confocal fluorescence imaging and spectroscopy with single molecule sensitivity and sub-

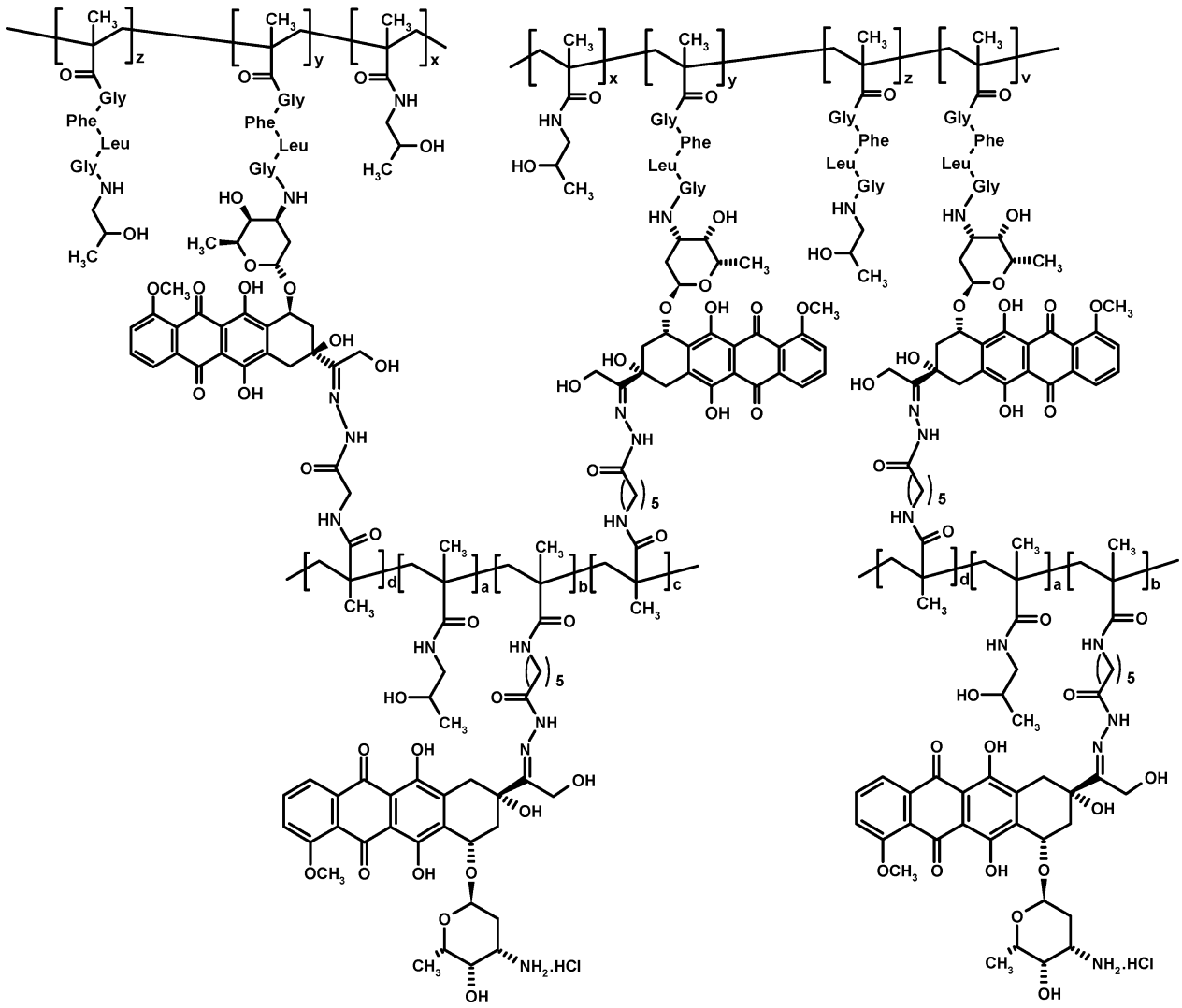


Figure 4. Scheme of the structure of the branched polymer conjugates DOX^{AM}−DOX^{HYD}−branchPHPMA (conjugates **13**−**16**).

micrometer resolution were performed on a MicroTime 200 inverted epifluorescence scanning confocal microscope (Picoquant, Germany). The configuration contained the following: a pulsed diode laser (LDH-P-C-470, 470 nm; Picoquant) providing 80 ps at up to a 40 MHz repetition rate; a proper filter set (Z470/10 clean up filter, z470rdc dichroic mirror, and HQ585/70 band-pass filter) (Chroma Technology, VT); a water immersion objective (1.2 NA, 60×) (Olympus, Japan); and a SPAD (single-photon avalanche diode) detector (MPD, Italy). Data acquisition was performed in TTTR mode (time-tagged time-resolved), which is an advanced mode of TCSPC (time correlated single photon counting). The data were acquired and analyzed with SymphoTime200 software (Picoquant). The FLIM images were analyzed by an amplitude FLIM approach. The total fluorescence decay from the complete image was tail-fitted by two or three exponentials. The obtained lifetimes were fixed and amplitudes for the fixed decay times were obtained for every pixel of the image. An RGB image was then created from the amplitudes. The average lifetime used in the histograms is an intensity-weighted average of all the components.

Calreticulin Detection on the Surface of EL4 Cells. 5×10^5 EL4 tumor cells were incubated for 24 h with or without the indicated amount of DOX·HCL, samples **4**, **5**, **7**, **8** and **9** in 5% CO_2, washed with FACS solution (PBS/2% FTS/2 mM EDTA/0,05% NaN_3) and then incubated for 20 min at 4 °C with rabbit anti-mouse calreticulin antibody (1:100, Stressgen, USA). Next, unbound antibodies were washed out and cells were incubated with goat anti-rabbit Alexa Fluor 647 secondary antibody (Invitrogen, USA). Finally, labeled cells were analyzed by FACS (LSRII, BD, San Jose, CA) with the use of FowJo software (Tree Star, San Carlos, CA).

Statistical Analysis. All *in vivo* experiments were repeated at least once, and similar results were obtained. Student's *t* test was used for evaluating statistical significance. $P < 0.05$

(30) Wahl, M.; Koberling, F.; Patting, M.; Rahn, H.; Erdmann, R. Time-resolved confocal Fluorescence imaging and spectroscopy system with single molecule sensitivity and sub-micrometer resolution. *Curr. Pharm. Biotechnol.* **2004**, *5*, 299–308.

Table 1. Characteristics of Polymer Precursors and Monoconjugates

polymer precursor	prepared from	M_w	M_w/M_n	reactive group (mol %)	DOX (wt %)
1		27000	1.80	5.7/HYD	
2		39000	1.52	5.1/ONp	
3	**1**	24500	1.78		7.5[a]
4	**1**	27000	1.80		9.9[a]
5	**6**	46000	1.96		7.4[b]

[a] DOX attached via pH-labile hydrazone bond (DOX^{HYD}). [b] DOX attached via amide bond to oligopeptide GFLG spacer (DOX^{AM}).

was considered to be a significant value. Results of representative experiments are shown.

Results and Discussion

Synthesis of Mixed Conjugates. The polymer precursors **1** and **2** containing hydrazide or ONp groups were prepared by radical copolymerization of HPMA with a monomer bearing hydrazide or ONp groups; the reaction was initiated with AIBN. Molecular weights, polydispersity and the content of reactive groups in the copolymers are given in Table 1. Attachment of DOX to the polymer precursors was performed using procedures described earlier.[7,26] The attachment of DOX had no significant influence on the molecular weight, polydispersity, and aqueous solution properties of the polymer conjugates.

The polymer conjugate **6**, which contained DOX attached by two different spacers that were both randomly distributed along the polymer chain, was prepared by direct radical solution copolymerization of HPMA with DOX-containing monomers Ma-ah-NHN═DOX and Ma-GFLG−DOX; the reaction was initiated with AIBN. Polymer conjugate **6** (Table 2) showed a well-defined structure consisting only of HPMA and drug-bearing monomer units. Moreover, this method enables the synthesis of polymer conjugates with a suitable amount of DOX bound in both forms, DOX^{AM} and DOX^{HYD}. The molecular weight of polymer conjugate **6** was slightly higher than that of conjugates **3** or **4** with DOX^{HYD}, but it was similar to that of polymer **5** with DOX^{AM}. The molecular weights of the conjugates remained below the limit of renal threshold estimated for HPMA-based polymer conjugates (to be ∼50000 g/mol).[31]

Mixtures of polymer conjugates, polymer preparations **7**−**11**, were prepared from homogeneous methanolic solutions of both conjugates **4** and **5** by coprecipitation. Molecular weights, polydispersity and the content and ratio of DOX in conjugates are given in Table 2. The mixing of polymer conjugates did not change their molecular weight or polydispersity significantly; both characteristics remained between the values found for single polymer conjugates.

Branched polymer conjugates DOX^{AM}−DOX^{HYD}−branchPHPMA (**13**−**16**) were prepared from polymer conjugates **4** and **5**, which contained functional groups that enabled the formation of interchain linkers containing hydrazone bonds, a hydrolytically degradable spacer. Polymer conjugates **4** and **5** contained either hydrazide groups (polymer **4**) remaining after attachment of DOX or a keto group on C13 of the amide-bonded DOX (polymer **5**). Reaction of the remaining hydrazide groups with keto groups on DOX, which was carried out in methanol in the presence of acetic acid, led to the formation of branched polymer conjugates with high molecular weights (Figure 4). The molecular weights of the branched polymer conjugates could be controlled by changing the reaction conditions, specifically the time of the reaction and the content of the hydrazide groups remaining free in conjugate **4** after its conjugation with DOX.

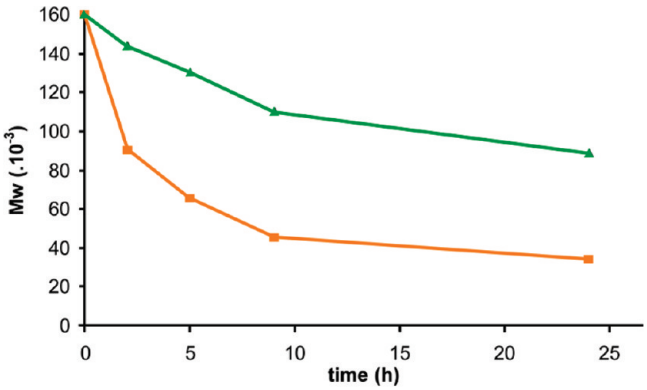
In Vitro **Degradation of Branched Polymer Carriers.** Previously, we described the passive accumulation of the HPMA copolymers in solid tumors (EPR effect) in mice;[32] the accumulation significantly increased with the increasing molecular weight of the polymer. However, polymers with molecular weights above the renal threshold should contain biodegradable linkages susceptible to intracellular or extracellular degradation, enabling degradation of that polymer to short polymer fragments that can be excreted from the organism. An increase in the molecular weight of a polymer could be facilitated with various strategies; one such strategy would involve the branching of shorter multivalent polymer chains.

The branched polymer conjugates DOX^{AM}−DOX^{HYD}−branchPHPMA (**13**−**16**) are composed of the shorter poly-

Table 2. Characteristics of Polymer Conjugates with DOX^{AM} and DOX^{HYD}

polymer	structure	M_w	M_w/M_n	DOX (wt %)	ratio of DOX (AM:HYD)
6	DOX^{AM}−DOX^{HYD}−PHPMA	44500	2.22	8.9	1:1
7[a]	DOX^{HYD}−PHPMA/DOX^{AM}−PHPMA	44500	1.99	7.6	2:1
8[a]	DOX^{HYD}−PHPMA/DOX^{AM}−PHPMA	43000	1.92	8.5	1:1
9[a]	DOX^{HYD}−PHPMA/DOX^{AM}−PHPMA	42500	2.10	9.4	1:2
10[a]	DOX^{HYD}−PHPMA/DOX^{AM}−PHPMA	42000	1.81	10.6	1:3
11[a]	DOX^{HYD}−PHPMA/DOX^{AM}−PHPMA	41000	1.85	10.9	1:4
12[a]	DOX^{HYD}−PHPMA/DOX^{AM}−PHPMA	37000	2.05	12.0	1:6
13	DOX^{AM}−DOX^{HYD}−branchPHPMA	115000	2.95	8.7	1:1
14	DOX^{AM}−DOX^{HYD}−branchPHPMA	195000	3.91	8.6	1:1
15	DOX^{AM}−DOX^{HYD}−branchPHPMA	240000	4.82	9.5	1:2
16	DOX^{AM}−DOX^{HYD}−branchPHPMA	160000	3.72	8.9	1:2

[a] Mixture of polymer conjugates **4** and **5**.



(■) pH 5; (▲) pH 7.4. Polymer concentration was 10 mg/mL.

Figure 5. Degradation of the polymer conjugate **16** in phosphate buffers pH 5 and pH 7.4 at 37 °C.

mers **4** and **5** linked together via hydrolytically degradable spacers containing hydrazone bonds. Hydrolysis of the conjugates was studied in phosphate buffers (pH 5 and 7.4), simulating the conditions found in the endosomes of target cells or in the blood circulation. After incubation in a buffer at pH 7.4 and 37 °C for 24 h, the molecular weight of polymer conjugates decreased to approximately one-half of the original value; a rapid decrease in molecular weight was observed within 24 h after incubation in a buffer of pH 5 (37 °C), simulating conditions found in the endosomes of target cells. The molecular weights of polymer conjugates rapidly decreased below the limit of renal threshold within the first 9 h of incubation (Figure 5). These results allow us to hypothesize that the branched conjugates will be sufficiently stable in blood circulation and will be disintegrated into excrete able fragments in the mildly acidic environment of tumor tissue or tumor cells.

Intracellular Localization of Monoconjugates and Mixed Conjugates. 3T3 fibroblasts (1×10^6 per well) were incubated for 24 h in a mixture of free doxorubicin hydrochloride (1.0 μg/mL) and either isolated derivative 7,8-dehydro-9,10-desacetyldoxorubicinone (D*) (0.1 μg/mL)[20,33] or a mixture of monoconjugates, sample **11** (5 μg/mL). Figure 6 shows the lifetime distributions identifying the cellular localization of free drug and the doxorubicin-containing mixture of monoconjugates. Free doxorubicin (released from conjugate), which has a lifetime of about 1.5 ns, is accumulated in nuclear DNA and inside the lumen of endosomes and lysosomes (dark blue color code, Figure 7).[17] A prolonged lifetime distribution peak is caused by the colocalization of free DOX together with both polymeric forms of the drug inside the endocytic compartment. The average lifetime is 2.3 ns[33] (blue/green color in Figure 7). Simultaneous accumulation of the DOX^{AM}−PHPMA and degradation product D* was observed inside membrane compartments (green to yellow areas). The red color localized in the plasma membrane represents an extremely weak autofluorescence of phospholipids and glycolipids in the membrane. This can be clearly seen from the confocal picture; in the appropriate areas, almost no signal was detected.

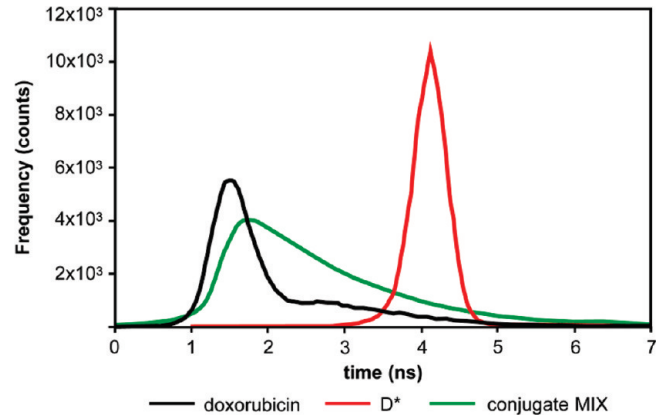


Figure 6. *In vitro* lifetime distributions of free doxorubicin, D* and sample **11** as quantified by SymphoTime software (PicoQuant).

Cytostatic Effect of Monoconjugates and Mixed Conjugates *in Vitro*. First, we compared the *in vitro* antiproliferative capacity of conjugates containing doxorubicin bound to the polymeric carrier either through an amide bond (DOX^{AM}−PHPMA, sample **5**) or through a pH-sensitive hydrazone bond (DOX^{HYD}−PHPMA, sample **4**). The efficacy of the mixed conjugates, i.e., samples containing both polymeric forms of doxorubicin, was compared with the activity of the monoconjugates alone (DOX^{HYD}−PHPMA, sample **4** and DOX^{AM}−PHPMA, sample **5**). The mixed conjugates were synthesized by the following methods: direct copolymerization reaction (DOX^{AM}−DOX^{HYD}−PHPMA, sample **6**); interconnecting polymeric chains (DOX^{AM}−DOX^{HYD}−branchPHPMA, samples **13**, **14** and **15**); and a simple mixture of two different monoconjugates (samples **7**, **8** and **9**). Table 3 shows that the inhibitory activity of mixed conjugates, IC_{50}, corresponds to the presence of hydrazone-bound doxorubicin. Furthermore, no additional effect was observed *in vitro* with the two-drug formulations.

Antitumor Effect *in Vivo* in Conventional Mice. *Comparison of Three Different Physical-Chemical Forms of Mixed Conjugates.* 10 mg of DOX equiv/kg of body weight was given once on the eighth day after cancer cell transplantation in the following forms: the random conjugate DOX^{AM}−DOX^{HYD}−PHPMA (sample **6**); the branched conjugate DOX^{AM}−DOX^{HYD}−branchPHPMA (sample **13**); and mixture of monoconjugates DOX^{HYD}−PHPMA/DOX^{AM}−

(31) Seymour, L. W.; Miyamoto, Y.; Maeda, H.; Brereton, M.; Strohalm, J.; Ulbrich, K.; Duncan, R. Influence of molecular weight on passive tumour accumulation of a soluble macromolecular drug carrier. *Eur. J. Cancer* **1995**, *31A*, 766–770.

(32) Pimm, M. V.; Perkins, A. C.; Strohalm, J.; Ulbrich, K.; Duncan, R. Gamma scintigraphy of the biodistribution of 123I-labelled N-(2-hydroxypropyl)-methacrylamide copolymer-doxorubicin conjugates in mice with transplanted melanoma and mammary carcinoma. *J. Drug Targeting* **1996**, *3*, 375–383.

(33) Hovorka, O.; Šubr, V.; Cimburek, Z.; Větvička, D.; Kovář, L.; Strohalm, J.; Strohalm, M.; Benda, A.; Ulbrich, K.; Říhová, B. Spectral analysis of doxorubicin accumulation and indirect quantification of its DNA intercalation, manuscript in preparation.

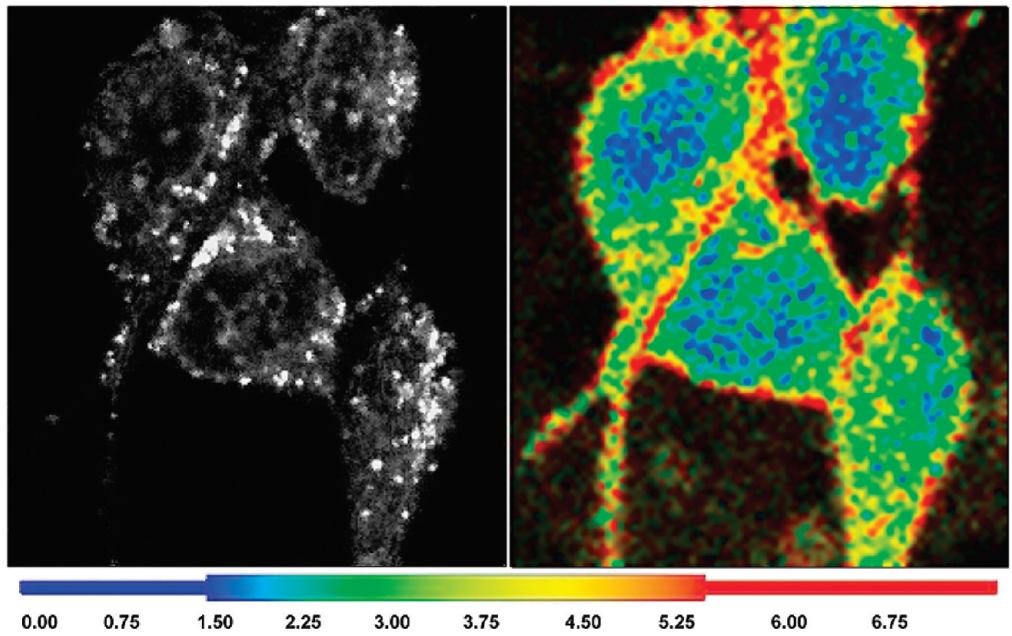


Figure 7. Confocal fluorescent image (left panel) and FLIM image (right panel, without the intensity component) in false colors, based on the lifetime distribution as analyzed in Figure 6.

Table 3. Cytostatic Effect of Monoconjugates and Mixed Conjugates *in Vitro* in Various Cancer Cell Lines

	IC_{50} (μg/mL)									
sample	EL4	38C13	3T3	BCL1	B16F10	4T1	SW620	Raji	Jurkat	FaDu
4	0.16	0.012	0.02	0.092	0.03	0.19	0.015	0.009	0.59	0.008
5	>40.0	1.796	2.05	0.626	1.56	19.43	2.549	0.224	14.0	1.469
6	0.61	0.024	0.07	0.110	0.04	0.82	0.042	0.039	0.56	0.221
7	0.51	0.035	0.08	0.084	0.03	0.91	0.085	0.041	0.38	0.067
8	0.27	0.018	0.05	0.075	0.05	0.92	0.009	0.012	0.44	0.032
9	0.39	0.024	0.03	0.042	0.06	0.76	0.049	0.011	0.43	0.115
13	0.74	0.091	nd[a]	nd	nd	nd	0.074	nd	nd	nd
14	1.60	0.127	nd	nd	nd	nd	0.097	nd	nd	nd
15	0.58	0.013	0.03	0.059	0.09	nd	0.053	0.027	0.50	0.082
DOX	0.01	0.001	0.01	0.001	0.02	0.04	0.008	0.001	0.06	0.005

[a] nd = Not determined.

PHPMA (sample **8**). In all three samples, the ratio of DOX^{AM} and DOX^{HYD} was 1:1. The *in vivo* effect was tested using experimental mouse T cell lymphoma EL4, which kills all control (nontreated) mice in an average of 30 days. Figure 8 documents the relatively low efficacy of a random conjugate (sample **6**, DOX^{AM}−DOX^{HYD}−PHPMA, Figure 3), which was able to cure only about 12.5% of tumor-suffering mice. In contrast, the same dose of DOX^{HYD}−PHPMA/DOX^{AM}−PHPMA (a mixture of monoconjugates, sample **8**) and DOX^{AM}−DOX^{HYD}−branchPHPMA (sample **13**, Figure 4) cured 90% of experimental animals.

It was rather surprising to see that DOX^{AM}−DOX^{HYD}−PHPMA (sample **6,** Figure 3), which was constructed from two different comonomers, is significantly less effective when compared with the mixed branched conjugate formed from two different polymeric chains, one carrying DOX^{AM} and one carrying DOX^{HYD} (sample **13**). The activity of the latter mixed system was almost the same as a simple mixture of two different monoconjugates, which was undoubtedly the best one in all of the experiments.

Comparison of Antitumor Effect of Mixed Conjugates with That of Monoconjugates. The random copolymer conjugate DOX^{AM}−DOX^{HYD}−PHPMA (sample **6**), which had the lowest efficacy seen in previous experiments, was used to document the tentative superior antitumor effect of mixed conjugates above monoconjugates. The results, which are presented in Figures 9A and 9B, show that the highest tumor-killing activity of copolymer DOX^{AM}−DOX^{HYD}−PHPMA occurred when it was given at doses equal to 25 mg of DOX equiv/kg of body weight (100% of cured mice) or 15 mg DOX of equiv/kg of body weight (more than 60% of cured mice). If a rather low dose (10 mg of DOX equiv/kg) was used for the treatment, no difference and no synergizing effect was detected between

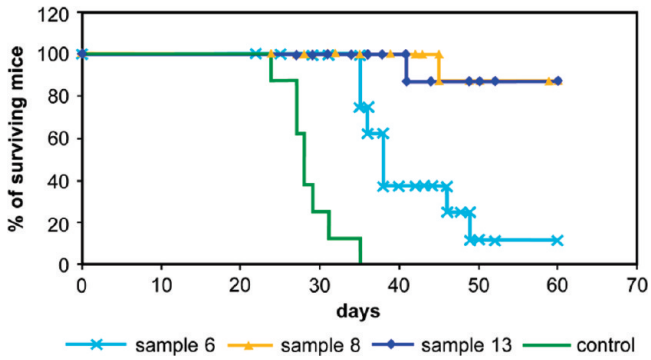


Figure 8. Antitumor efficacy of different physicochemical forms of mixed copolymers. B/6 mice were subcutaneously transplanted with 1×10^5 EL4 cancer cells and at day 8 injected with 10 mg of DOX equiv/kg of random copolymer DOX^{AM}–DOX^{HYD}–PHPMA (sample **6**, Figure 3), DOX^{AM}–DOX^{HYD}–branchPHPMA (sample **13**, Figure 4) or with a mixture DOX^{HYD}–PHPMA/DOX^{AM}–PHPMA (sample **8**). Data are representative of three independent experiments. Statistical significance $P < 0.01$.

the random copolymer and both types of monoconjugates; rather, all of them cured about 10% of tumor-bearing mice (Figure 9C).

While no clear complementary effect of DOX^{AM} and DOX^{HYD} was observed *in vitro, in vivo* experiments unambiguously showed a superior activity of the combined mixed systems. To our understanding, this proves a different mechanism of action for the two doxorubicin derivatives or at least that they behave differently in living organisms. If their *in vivo* activities are the same, the repeatedly documented synergistic effect of mixed systems could hardly be seen, especially if applied in very low doses. Of course, it was challenging to explain this result. In an effort to confirm or disprove the differences in cellular and intracellular transport as one of many factors that might be involved in a synergizing effect, we attempted to determine the rate of intracellular accumulation of mixed conjugates in comparison to monoconjugates. However, it was extremely difficult to determine the rate of intracellular accumulation of the drug with certainty because of the release of fluorescence-active doxorubicin derivative D*, especially from DOX^{AM}.[20] In the absence of this information, we propose that the combinatorial effect is a result of the nucleotoxic effect of doxorubicin released from the DOX^{HYD}–PHPMA conjugate, which resembles the activity of the free drug and the large membrane toxicity of the DOX^{AM}–PHPMA conjugate.[17,25,34]

The Effect of DOX^{AM}:DOX^{HYD} Ratios on the Pharmacological Activity of Mixed Conjugates. The simple mixture of monoconjugates with different DOX^{AM}:DOX^{HYD} ratios (2:1, 1:1 and 1:2, samples **7**, **8**, **9**) was used to see if both forms of doxorubicin contribute equally to the final pharmacological effect or if one of them is superior.

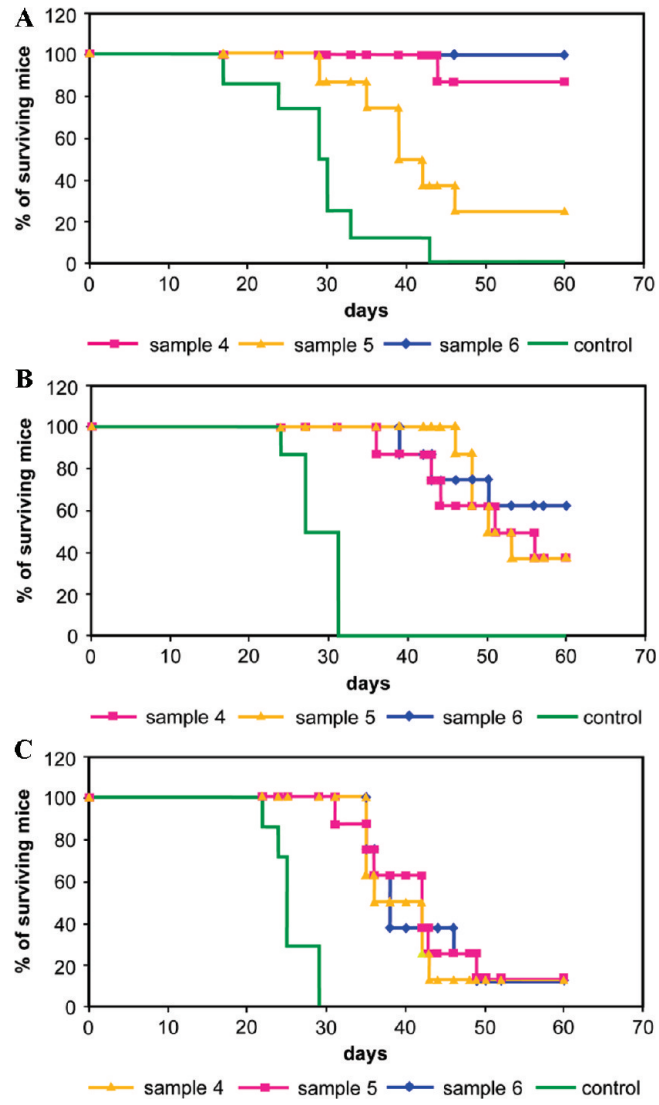


Figure 9. The comparison of antitumor activity of monocopolymers and mixed copolymers. B/6 mice were subcutaneously transplanted with 1×10^5 EL4 cancer cells and at day 9 injected with different doses of random copolymer DOX^{AM}–DOX^{HYD}–PHPMA (sample **6**), monoconjugate DOX^{AM}–PHPMA (sample **5**) or DOX^{HYD}–PHPMA (sample **4**). (A) = 25 mg of DOX equiv/kg; (B) = 15 mg of DOX equiv/kg; (C) = 10 mg of DOX equiv/kg. Data are representative of three independent experiments. Statistical significance $P < 0.05$.

The data obtained after injection with 7.5 mg of DOX equiv/kg and 5 mg of DOX equiv/kg of body weight are recorded in Figures 10A and 10B. It can be seen that a mixture with a 2-fold prevalence of derivative with hydrazone-bound doxorubicin (ratio 1: 2) is the best one, and very low doses cured almost 80% or 50% of diseased mice, respectively.

The Effect of Molecular Weight. We have tested high-molecular-weight mixed conjugates in the form of branched polymer conjugates to determine the involvement of molecular weight and an enhanced permeation and retention (EPR) effect[35] in their antitumor activity. The following samples

(34) Hovorka, O.; Šťastný, M.; Etrych, T.; Šubr, V.; Strohalm, J.; Ulbrich, K.; Říhová, B. Differences in the intracellular fate of free and polymer-bound doxorubicin. *J. Controlled Release* **2002**, *80*, 101–117.

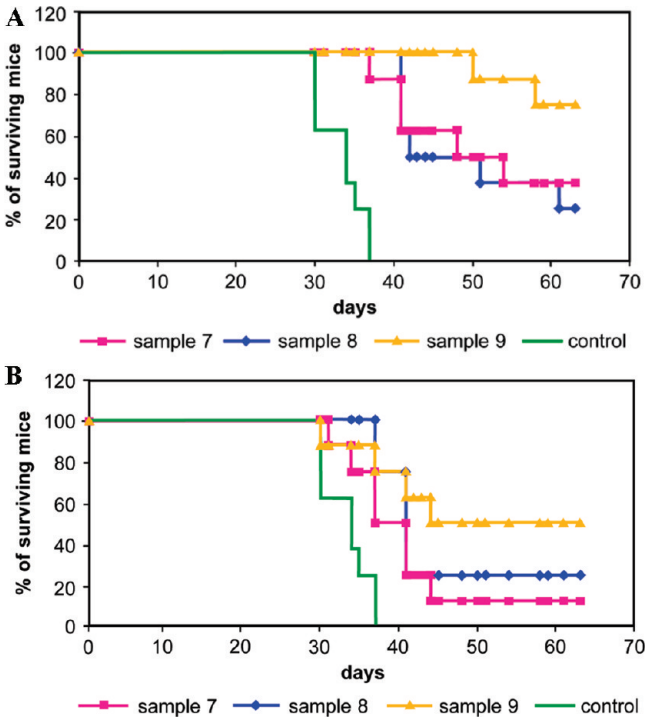


Figure 10. The involvement of DOX^{AM}:DOX^{HYD} ratio in the efficacy of the treatment. B/6 mice were subcutaneously transplanted with 1×10^5 EL4 cancer cells and at day 8 injected with different doses of a simple mixture of monoconjugates DOX^{AM}−PHPMA (sample **5**) and DOX^{HYD}−PHPMA (sample **3**) with different DOX^{AM}:DOX^{HYD} ratios (2:1, 1:1, 1:2). (A) = 7.5 mg of DOX equiv/kg; (B) = 5 mg of DOX equiv/kg. Data are representative of three independent experiments. Statistical significance $P < 0.05$.

were compared: (a) DOX^{AM}−DOX^{HYD}−branchPHPMA with DOX^{AM}:DOX^{HYD} ratio 1:1 (molecular weight 115 kDa, sample **13**; and 195 kDa, sample **14**) and (b) DOX^{AM}−DOX^{HYD}−branchPHPMA with DOX^{AM}:DOX^{HYD} ratio 1:2 where the molecular weights were 160 kDa (sample **16**) and 240 kDa (sample **15**). The conjugates were injected in doses of 15 mg of DOX equiv/kg and 10 mg of DOX equiv/kg on day 8 into B/6 mice bearing palpable (size about 5−8 mm^3) EL4 T cell lymphoma. The differences in survival of the experimental mice were not that remarkable (Figures 11A and 11B), suggesting that the dose is more critical to efficacy than the molecular weight, provided that it exceeds a certain level needed for the implementation of the EPR effect.

Acute Toxicity of Mixed Conjugates. Ensuring safety is imperative when developing new systems because they may be eventually used in human patients. Acute toxicity, which was demonstrated as a significant weight loss, was monitored in the days following treatment and was observed only after application of the highest dose, i.e., 25 mg of DOX equiv/kg of branched copolymer DOX^{AM}−DOX^{HYD}−branch-PHPMA (sample **15**) and random copolymer DOX^{AM}−

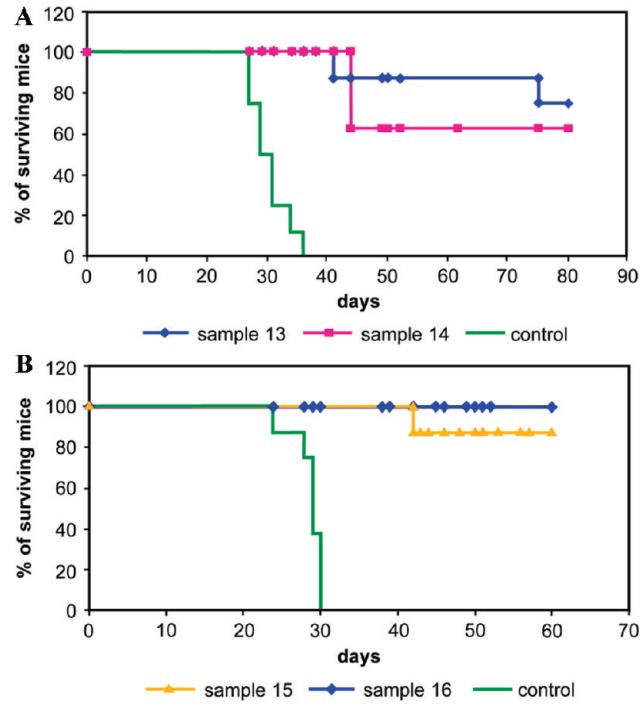


Figure 11. The effect of molecular weight of conjugates on their antitumor efficacy. B/6 mice were subcutaneously transplanted with 1×10^5 EL4 cancer cells and at day 8 injected with different doses of branched polymer conjugates DOX^{AM}−DOX^{HYD}−branchPHPMA (samples **13**, **14**, **15**, **16**, Figure 4). (A) = 10 mg of DOX equiv/kg; (B) = 15 mg of DOX equiv/kg. Data are representative of two independent experiments. Statistical significance $P < 0.05$.

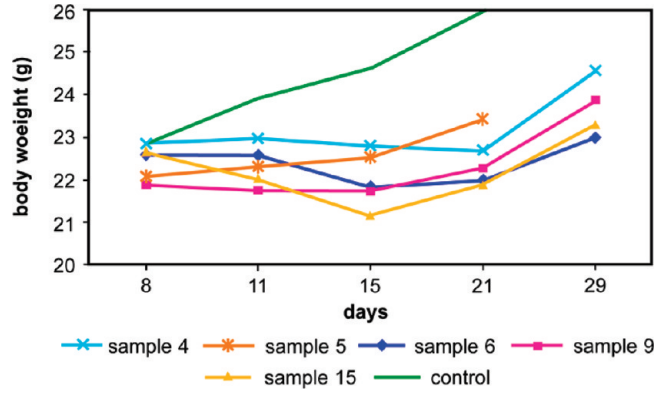


Figure 12. Acute toxicity of mixed copolymers. B/6 mice were subcutaneously transplanted with 1×10^5 EL4 cancer cells and at day 9 injected with 25 mg of DOX equiv/kg of monoconjugates DOX^{AM}−PHPMA (sample **5**), DOX^{HYD}−PHPMA (sample **4**), random DOX^{AM}−DOX^{HYD}−PHPMA (sample **6**), mixture of monoconjugates (sample **9**) and branched copolymer DOX^{AM}−DOX^{HYD}−PHPMA (sample **15**). The body weight was checked on days 8, 11, 15, 21, and 29 after the treatment. Data are representative of three independent experiments. Statistical significance $P < 0.01$.

DOX^{HYD}−PHPMA (sample **6**) (Figure 12). It culminated at day 7 following the treatment and was only marginal (less

(35) Maeda, H.; Matsumura, Y. EPR effect based drug delivery design and clinical outlook for enhanced cancer chemotherapy. *Adv. Drug Delivery Rev.* **2010**, doi 10.1016/j.addr.2010.05.001.

than 10%). As the application of other tested samples and lower doses (15 mg of DOX equiv/kg, 10 mg of DOX equiv/kg and 7.5 mg of DOX equiv/kg) were not accompanied with a significant weight loss, the mixed systems were considered to be safe for their recipient.

The Involvement of the Immune System in the Outcome of the Therapy. We repeatedly reported a dual cytostatic and immunomobilizing/immunostimulating *in vivo* effect of HPMA-based copolymers containing doxorubicin.[16,25,36−40] The observation that the treatment with a cytotoxic drug regularly triggers a systemic anticancer response that protects mice from a second cancer attack is remarkable. Such treatment-inducible "autovaccination" is dose and time-dependent; more aggressive treatment, which facilitates very rapid elimination of tumor cells, induces low or even undetectable tumor resistance, whereas a slower eradication of tumor mass induces tumor resistance that is strong and could protect up to 100% of cancer-bearing animals.[25] However, only monoconjugates have been tested thus far.

Retransplantation. First, conventional mice were injected with monoconjugates DOX^{AM}−PHPMA (sample **5**), DOX^{HYD}−PHPMA (sample **4**), mixed random conjugate DOX^{AM}−DOX^{HYD}−PHPMA (sample **6**) and either branched conjugate DOX^{AM}−DOX^{HYD}−branchPHPMA (sample **15**) or a mixture of copolymers DOX^{HYD}−PHPMA/DOX^{AM}−PHPMA (sample **9**). The dose was 15 mg of DOX equiv/kg. The best result was noted with DOX^{AM}−DOX^{HYD}−branch-PHPMA and with a mixture of polymers DOX^{HYD}−PHPMA/DOX^{AM}−PHPMA, which produced 90% of long-term survivors (LTS). Random copolymer DOX^{AM}−DOX^{HYD}−PHPMA was less effective (60% LTS), and only 40% of surviving mice were recorded after therapy with single drug containing conjugates DOX^{AM}−PHPMA (sample **5**) and DOX^{HYD}−PHPMA (sample **4**) (Figure 13A).

After more than two months, LTS cured from the primary thymoma EL4 were retransplanted with a lethal dose (1×10^5cells) of the same cancer cells and left without treatment.

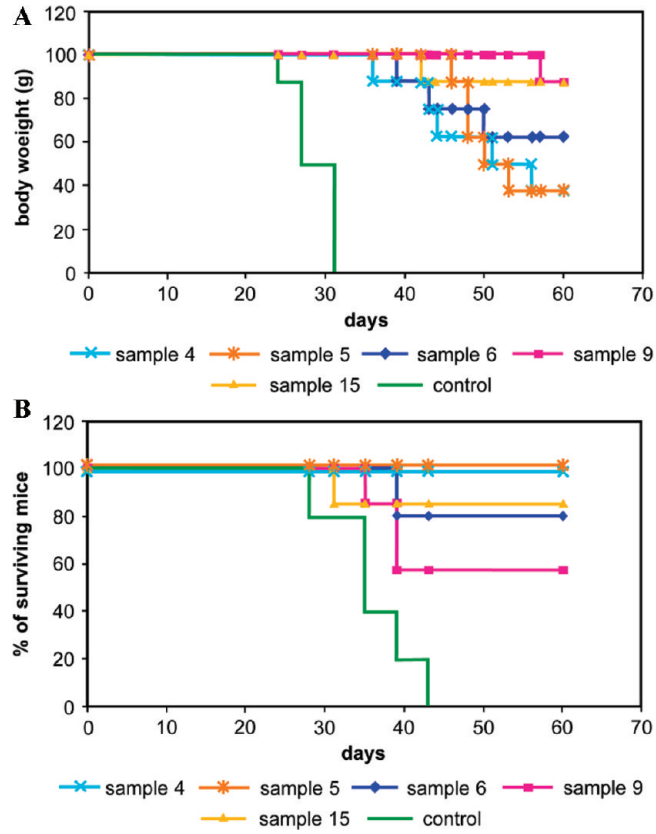


Figure 13. The treatment-dependent systemic tumor resistance. B/6 mice were subcutaneously transplanted with 1×10^5 EL4 cancer cells and at day 8 treated with the same samples as described on Figure 12 (dose 15 mg of DOX equiv/kg). The resistance of long-term survivors was verified by the subcutaneous injection of lethal dose (1×10^5) of EL4 cancer cells. After tumor transplantation, the mice were left without treatment. (A) Primary response. (B) Retransplantation. Data are representative of two independent experiments. Statistical significance $P < 0.05$.

The result, which is presented in Figure 13B, confirms the rule we have reported previously using different mouse cancer models,[25] namely, that more efficient treatment leads to a lower systemic cancer resistance and vice versa. Conjugates DOX^{AM}−PHPMA and DOX^{HYD}−PHPMA, which have a rather low primary pharmacological activity, protected 100% of retransplanted LTS against tumor growth. In contrast, only 60% of mice cured with a mixture of copolymers DOX^{HYD}−PHPMA/DOX^{AM}−PHPMA exhibited an effective resistance to a second attack of cancer.

The Reaction of Immunodeficient Athymic nu/nu Mice. The participation of the immune system in the cancer-bearing host in the outcome of HPMA copolymer-based cancer therapy was tested by comparing the response of conventional and immunodeprived nu/nu (athymic) mice suffering from the absence of T cell-dependent adaptive immunity. Samples of DOX^{HYD}−PHPMA/DOX^{AM}−PHPMA with different DOX^{AM}:DOX^{HYD} ratios (1:3, sample **10**; 1:4, sample **11** and 1:6, sample **12**) were used at a dose of 10 mg of DOX equiv/kg. Mice were injected iv on day 9 after

(36) Říhová, B.; Strohalm, J.; Hoste, K.; Jelínková, M.; Hovorka, O.; Kovář, M.; Plocová, D.; Šírová, M.; Šťastný, M.; Schacht, E.; Ulbrich, K. Immunoprotective therapy with targeted anticancer drugs. *Macromol. Symp.* **2001**, *172*, 21–28.
(37) Říhová, B.; Strohalm, J.; Kubáčková, K.; Jelínková, M.; Hovorka, O.; Kovář, M.; Plocová, D.; Šírová, M.; Šťastný, M.; Rozprimová, L.; Ulbrich, K. Acquired and specific immunological mechanisms co-responsible for efficacy of polymer-bound drugs. *J. Controlled Release* **2002**, *78*, 97–114.
(38) Říhová, B.; Strohalm, J.; Prausová, J.; Kubáčková, K.; Jelínková, M.; Rozprimová, L.; Šírová, M.; Plocová, D.; Etrych, T.; Šubr, V.; Mrkvan, T.; Kovář, M.; Ulbrich, K. Cytostatic and immunomobilizing activities of polymer-bound drugs: experimental and first clinical data. *J. Controlled Release* **2003**, *91*, 1–16.
(39) Šírová, M.; Strohalm, J.; Šubr, V.; Plocová, D.; Mrkvan, T.; Ulbrich, K.; Říhová, B. Treatment with HPMA copolymer-based doxorubicin conjugate containing human immunoglobulin induces long-lasting systemic anti-tumor immunity in mice. *Cancer Immunol. Immunother.* **2007**, *56*, 35–47.
(40) Říhová, B.; Kovář, M. Immunogenicity and immunomodulatory properties of HPMA-based polymers. *Adv. Drug Delivery Rev.* **2010**, *62*, 184–191.

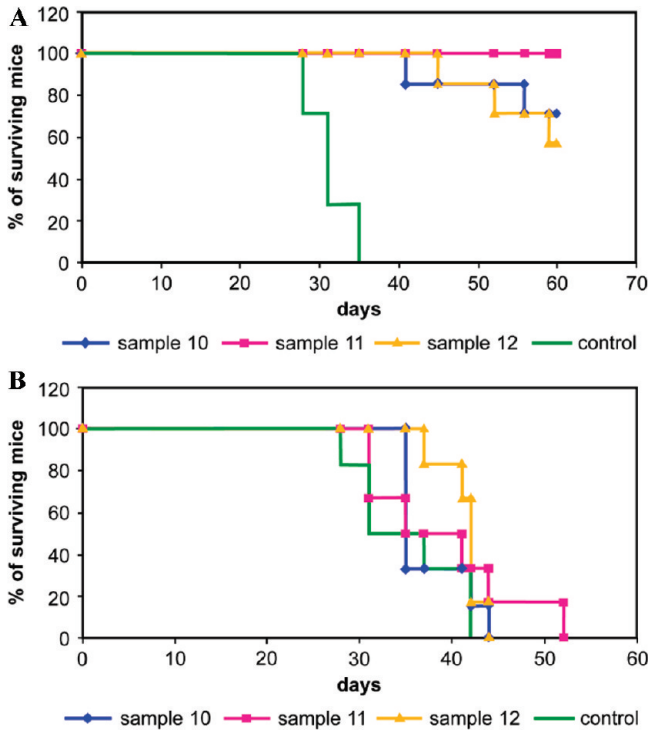


Figure 14. The comparison of treatment response of conventional and immunocompromised athymic nude (nu/nu) mice. Conventional B/6 mice or immunodeprived nu/nu CD-1 mice were subcutaneously transplanted with 1×10^5 EL4 cancer cells and at day 8 treated with 10 mg of DOX equiv/kg of mixed conjugates DOX^{HYD}−PHPMA/DOX^{AM}−PHPMA (samples **10**, **11**, **12**). (A) The response of the conventional mice. (B) The response of the nu/nu mice. Data are representative of two independent experiments. Statistical significance $P < 0.05$.

transplantation of cancer cells EL4 (conventional mice with 1×10^5 cells; nu/nu mice with 5×10^5 cells) when the tumor was palpable and reached a size of approximately 5 to 8 mm^3. The best result, i.e., 100% of LTS mice, was obtained if conventional mice were treated with 10 mg of DOX equiv/kg of conjugate **11** where the ratio of DOX^{AM}:DOX^{HYD} was 1:4. The mixed conjugates with ratios of 1:3 (sample **10**) and 1:6 (sample **12**) were also remarkably efficient as they cured 70% and 60% of cancer-suffering animals, respectively (Figure 14A). On the other hand, no cured animals were recorded in the cohort of nude mice after treatment with either conjugate (Figure 14B), suggesting a critical contribution of T-cell mediated functional immune response to the final outcome of the treatment. A slight prolongation of life in diseased mice was observed after the treatment with sample **11** but was not statistically significant. As proven by neutralization Winn's test, cytotoxic T lymphocytes ($CD8^+$) are the chief mediators of the observed tumor resistance.[39] The fact that nu/nu mice are incurable is evidence of the active involvement of the immune system in experimental cancer therapy with HPMA-bound doxorubicin.

Acute Model versus Chronic Model. Numerous studies involving mice tumor models and human cancers have shown

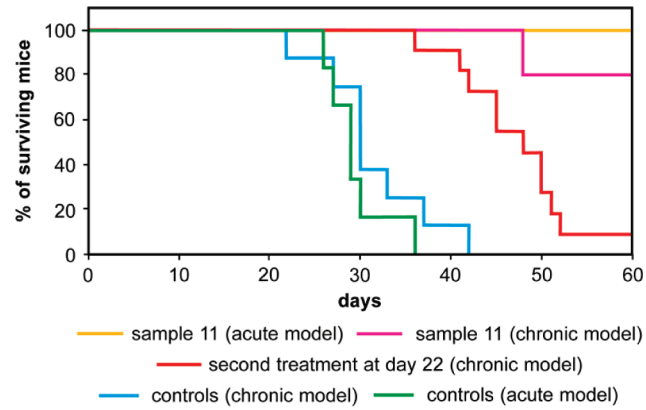


Figure 15. The antitumor efficacy of mixed conjugates in mice suffering from acute or chronic tumor model. B/6 mice representing an acute cancer model were sc injected once with 1×10^5 EL4 cancer cells and treated at day 8 with 10 mg of DOX equiv/kg of a simple mixture DOX^{HYD}−PHPMA/DOX^{AM}−PHPMA of monoconjugates (sample **11**). B/6 mice representing a chronic cancer model were injected 6 times (every other day) with a low dose (1×10^4) of EL4 cancer cells, and those with a tumor size of about 8 mm^3 were injected (usually at day 14 after cancer cell transplantation) with 10 mg of DOX equiv/kg of a simple mixture DOX^{HYD}−PHPMA/DOX^{AM}−PHPMA of monoconjugates (sample **11**). Data are representative of two independent experiments. Statistical significance $P < 0.05$.

that not only can the immune system protect the host against the development of primary tumor but, under certain conditions, it can also promote tumor growth leading to more aggressive disease.[41] To date, routinely used experimental animal models involve the application of cancer cells into healthy animals with no previous experience with a tumor growth. Evidence now exists that the equilibrium phase of the cancer immunoediting process, i.e., constant contact between the tumor and the host's immune system, may last for years before the emergence of clinically detectable malignant disease.[42] This is the main reason that we compared treatment response in mice with acute and chronic cancer.

Two experimental groups of C57BL/6 mice were created: one suffering from acute tumor growth and one suffering from chronic tumor growth. When the tumor reached a size of about 8 mm^3, the mice were iv injected with 10 mg of DOX equiv/kg of DOX^{HYD}−PHPMA/DOX^{AM}−PHPMA (sample **11**). In addition, on day 22 some mice (eight mice per one experimental group) with chronic tumors were reinjected with a dose of 25 mg of DOX equiv/kg of the same mixture. The data presented in Figure 15 show that mice with acute tumors exhibited a better therapeutic

(41) Zitvogel, L.; Apetoh, L.; Ghiringhelli, F.; André, F.; Tesniere, A.; Kroemer, Q. The anticancer immune response: indispensable for therapeutic success. *J. Clin. Invest.* **2008**, *118*, 1991–2001.
(42) Sengupte, N.; MacFie, T. S.; MacDonald, T. T.; Pennington, D.; Silver, A. R. Cancer immunoediting and "spontaneous" tumor regression. *Pathol. Res. Pract.* **2010**, *206*, 1–8.

response; 100% of those mice could be cured while only 80% of LTS were recorded in the group of mice with chronic tumors. Their reinjection with a rather high dose of DOX^{HYD}−PHPMA/DOX^{AM}−PHPMA did not save their lives as we predicted, and 90% of them died within two months. These results suggest that a nonexhausted immune system plays an important role in the host's protection against tumor development.

We have obtained additional data from a new generation of HPMA-based conjugates in which only 40% or fewer of mice with chronic cancer responded to the treatment that cured 100% of animals suffering from acute cancer [nonpublished data]. This emphasizes the necessity of including a chronic tumor model in the evaluation of newly developed anticancer agents to avoid a false overestimation of the results.

Translocation of Chaperon Calreticulin (CRT) to the Surface of EL4 T Cell Thymoma after Exposure to Simple Conjugates and Their Combinations. In the past few years, new data has been published proving an immunogenicity of cancer cells after treatment with some drugs, a so-called "immunogenic cancer-cell death".[24,41] These findings could be used, at least in part, for an explanation of the systemic cancer resistance regularly observed after treatment with HPMA-based copolymers. We also reopen the question seeking to know the mechanisms of action in DOX^{AM}−PHPMA and DOX^{HYD}−PHPMA conjugates. Obeid et al.[24] reported that the chaperon calreticulin (CRT) rapidly translocates, together with the bisulfide isomerase ERp57, from the endoplasmic reticulum (ER) to the plasma-membrane surface of dying cancer cells exposed to doxorubicin. CRT-tagged cells are then rapidly recognized by dendritic cells of the immune system, which triggers the specific anticancer response. Based on such new data and the widely reported immunostimulating/immunomobilizing effect of all doxorubicin-containing PHPMA conjugates,[16,22,36−40] we have tested CRT and ERp57 translocation in numerous cancer cell lines exposed to DOX^{AM}−PHPMA and DOX^{HYD}−PHPMA. Rather surprisingly, CRT translocation and its cancer cell surface expression were detected only if cancer cells were exposed to free doxorubicin, DOX^{HYD}−PHPMA (sample **4**) or to mixtures containing DOX^{HYD} (samples **7**, **8** and **9**)

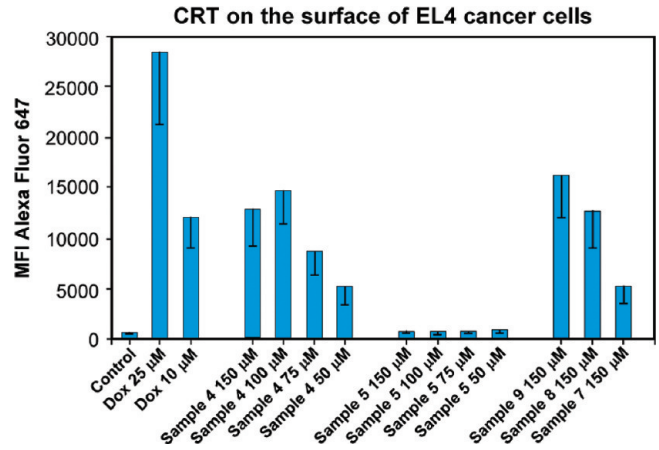


Figure 16. Expression of calreticulin (CRT) on the surface of EL4 T cell lymphoma. Cancer cells EL4 were exposed *in vitro* for 24 h to parent drug, i.e. doxorubicin, to DOX^{AM}−PHPMA (sample **5**), DOX^{HYD}−PHPMA (sample **4**) or to their mixtures DOX^{HYD}−PHPMA/DOX^{AM}−PHPMA (**7**, **8** and **9**) differing in DOX^{AM}:DOX^{HYD} ratio (1:2, 1:1 and 2:1). A high expression of CRT on the surface of EL4 cancer cells is seen only if they are exposed to free parent doxorubicin or to conjugates containing doxorubicin bound to the polymeric carrier through a hydrazone bond. Interestingly enough, no CRT expression was detected if cancer cells were exposed to DOX^{AM}−PHPMA where doxorubicin is bound to the polymer through amide bond.

(Figure 16), in which polymer-bound doxorubicin behaves like its free counterpart inside the target cells. CRT was not seen on the surface of cancer cells exposed to DOX^{AM}−PHPMA conjugates, which supports the concept that different intracellular events are activated after cell exposure to DOX^{AM}−PHPMA and DOX^{HYD}−PHPMA.

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