Starlike vs. Classic Macromolecular Prodrugs: Two Different Antibody-Targeted HPMA Copolymers of Doxorubicin Studied *in Vitro* and *in Vivo* as Potential Anticancer Drugs

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Purpose. Two different monoclonal antibody-targeted HPMA copolymer-doxorubicin conjugates, classic and starlike, were synthesized to be used for site-specific cancer therapy. The anti–mouse Thy-1.2 (IgG3) and two anti–human CD71/A (IgG1) and CD71/B (IgG2a) monoclonal antibodies were used as targeting structures.

Methods. Their binding and cytotoxic activity *in vitro*, body distribution, and anticancer activity *in vivo* were evaluated.

Results. The results of flow cytometric analysis showed comparable binding of classic and starlike conjugates to the target cells. The *in vitro* cytotoxic effect was 10-fold higher if cancer cells were exposed to the starlike conjugate compared to the classic one. Biodistribution studies showed that the starlike conjugate remained in a relatively high concentration in blood, whereas the classic conjugate was found in a 6.5-times lower amount. In contrast to the low antitumor activity of free doxorubicin and nontargeted HPMA copolymer–doxorubicin conjugate, both anti–Thy-1.2 targeted conjugates (classic and starlike) cured all mice bearing T-cell lymphoma EL4. On the other hand, starlike conjugates containing anti-CD71/A or anti-CD71/B monoclonals as targeting structures were more effective against human colorectal cancer SW 620 than the classic one.

Conclusions. We have shown that the starlike conjugates are more effective systems for targeted drug delivery and cancer treatment than classic conjugates.

KEY WORDS: antibody-targeted doxorubicin; HPMA copolymer; polymer drug carrier; starlike conjugate; drug delivery.

INTRODUCTION

Antibody-targeted macromolecular therapeutics went through many years of development, and the first product has now been used for the treatment of human solid cancer (1). Such targeted polymeric drugs are based on N-(2-hydroxypropyl) methacrylamide (HPMA) copolymeric carrier to which doxorubicin (DOX) and antibodies are attached through a GlyPheLeuGly spacer via an enzymatically degradable bond (2). The HPMA copolymeric carrier changes the pharmacokinetics and pharmacodynamics of conjugated cytostatics, reduces their nonspecific toxicity, and decreases the immunogenicity of targeting antibodies (3). The antibodies further increase the therapeutic index of macromolecular drugs in the treatment of experimental mouse tumors, e.g., T-cell lymphoma EL4 (4), B-cell lymphoma 38C13 (5), and B-cell leukemia BCL1 (6). In addition, the newest data suggest that antibody-targeted HPMA copolymer-DOX conjugates not only kill cancer cells but also protect and mobilize the immune system (7).

Two types of antibody-targeted HPMA copolymer-DOX conjugates (mAb-P-DOX; P stands for HPMA copolymer), classic and starlike, have been synthesized. The classic conjugate is based on a HPMA copolymeric backbone containing biodegradable GlyPheLeuGly side chains (spacers), terminating in either the targeting antibody or the drug. In this system, the targeting and drug moieties are randomly distributed along the polymer chain (2). In the starlike conjugate, several HPMA copolymeric chains bearing DOX attached by a biodegradable GlyPheLeuGly spacer are linked to the central antibody molecule via an amide bond situated at the end of each backbone chain. The conjugate thus represents a well-defined system in which the central antibody molecule is surrounded by 30 to 40 copolymer chains covalently attached to the lysine ε-amino groups of the antibody (8). No cross-linking or branching, often observed in classic conjugates, was found, and the distribution of molecular weight of the starlike conjugates was narrow (9).

We tested three monoclonal antibodies (mAbs), anti–mouse Thy-1.2 and two anti–human CD71, as targeting structures. Anti–Thy-1.2 mAbs identify tumor-associated antigen (Thy-1.2) expressed on mouse T-cell lymphoma EL4, and anti-CD71 mAbs recognize transferrin receptor (CD71) expressed on human colorectal carcinoma SW 620. We compared two different mAbs, anti-CD71/A (MEM-75, IgG1 isotype) and CD71/B (MEM-105, IgG2a isotype), with respect to targeting efficacy.

In this study we have compared the binding capacity, antiproliferative activity, body distribution, and anticancer efficacy of classic and starlike conjugates targeted with various monoclonal antibodies.

MATERIALS AND METHODS

Synthesis and Characterization of HPMA Copolymer–DOX Conjugates

The nontargeted HPMA copolymer–DOX conjugate (PK1) as well as the monoclonal antibody-targeted HPMA copolymer-bound DOX, both classic and starlike, were prepared by multiple-step synthesis as previously described (8–11).

In the classic conjugate, DOX was bound to the polymeric precursor, P-GFLG-ONp (P stands for HPMA copolymer) by aminolysis of a part of ONp groups. The remaining ONp groups were aminolysed with 1-aminopropane-2-ol (PK1) or used for subsequent conjugation with an antibody. Polymeric precursor of the starlike conjugate was prepared by radical copolymerization of HPMA with the MA-GFLG-DOX in the presence of 3-mercaptopropionic acid as a chain-transfer agent. The terminal carboxylic group of this HPMA copolymer was activated by the reaction with N-

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hydroxysuccinimide and N,N′-dicyclohexylcarbodiimide in N,N′-dimethylformamide.

In the final step, two different HPMA copolymer–DOX conjugates with antibodies were synthesized. In the case of classic conjugate, a polymeric precursor was dissolved in distilled water, and a solution of antibody was added. The pH of the reaction mixture was adjusted to 8, and the course of the reaction was controlled by the consumption of a borate solution (pH-stat Radiometer). The starlike polymer precursor was introduced into an antibody solution in phosphate buffer, and the pH was slowly increased from 7.3 to 7.8. Both resulting conjugates were purified by gel filtration on a Sephacryl 300 column (Pharmacia) followed by ultrafiltration using YM-30 (Millipore) membrane. Polymer conjugates were characterized by UV spectrophotometry (DOX content), size exclusion chromatography using FPLC Pharmacia equipped with RI, UV, and multiangle light scattering DAWN DSP-F (Wyatt Co., USA) detectors using Superose™ 12 or Superose™ 6 columns (M_w and M_n), amino acid analysis (antibody content), and electrophoresis; Table I. Both conjugates were stored frozen at –20°C.

Monoclonal Antibodies

Monoclonal antibody (clone 1aG4/C5) directed against mouse Thy-1.2 antigen (IgG3 isotype) was kindly provided by Dr. P. Dráber (Institute of Molecular Genetics AS CR, Prague). Two anti-CD71 monoclonal antibodies directed against human transferrin receptor were kindly provided by Prof. V. Hořejší (Institute of Molecular Genetics AS CR, Prague). Anti-CD71/A mAb (clone MEM-75) was of the IgG1 isotype, and anti-CD71/B (clone MEM-105) of the IgG2a isotype. All mAbs were purified from mouse ascitic fluid by a combination of twofold 45% saturated ammonium sulfate precipitation and protein A affinity chromatography (BioRad). The concentration was determined by spectrophotometry at 280 nm, and the purity was checked by SDS-PAGE.

Cell Lines

Mouse T-cell lymphoma EL4 (Thy-1.2$^+$) and human colorectal carcinoma SW 620 (TfR$^+$) were obtained from American Type Culture Collection. The EL4 line is relatively resistant to free DOX (12). EL4 and SW 620 cells were cultivated in RPMI 1640 (Sigma) with 4 mM L-glutamine, 1.5 mg/ml sodium bicarbonate, 4.5 mg/ml glucose, 1.0 mM sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin (Sigma), and 10% fetal bovine serum (GibcoBRL).

Antibody-Binding Assay: Flow Cytometric Analysis

Harvested cells from exponentially growing cell culture were centrifuged at 300 × *g* for 5 min and resuspended to a concentration of 10^6 cells/0.1 ml in cold wash buffer (phosphate-buffered saline with 0.1% NaN_3 and 1% BSA). Then 0.1 ml of diluted mAb–targeted conjugates (resulting concentration was 10 μg mAb/ml) was added and incubated for 30 min at 4°C. The cells were then washed three times with a cold wash buffer and incubated for 30 min at 4°C in the dark with fluorescein-labeled second-step antibody (swine anti–mouse IgG, Sevac). Incubation of the cells with the secondary antibody alone was used as a control. Finally, the washed cells were resuspended in 0.5 ml of wash buffer. At least 5000 cells were accumulated for each measurement. Dead cells and debris were excluded from analysis by a combination of gates set on forward/site scatter and cell staining with propidium iodide. Flow cytometric analysis was performed on a FACS (Fluorescence-Activated Cell Sorter) equipped with CellQuest version 3.1 software (Becton Dickinson).

Cytotoxicity Assay by [^{3}H]Thymidine Incorporation

Cytotoxicity assay was performed using [^{3}H]thymidine incorporation. NUNCLON 96-well tissue culture plates (NUNC) were seeded with 5 × 10^4 EL4 or 1 × 10^4 SW 620 cells. Different concentrations of samples or free doxorubicin (0.004–80 μM) were added to the wells to reach a final volume of 0.25 ml. Triplicate wells were used for each test condition. The EL4 or SW 620 cells were incubated with the samples at 37°C for 48 h and 72 h, respectively. After cultivation, each well was pulsed with 1 μCi (37 kBq) of ^{3}H-thymidine (Amersham Corp.) for 5–6 h. Cells were then collected onto glass fiber filters (Filtermat, Wallac) using a cell harvester (Tomtec). When the mixture was dried, a sheet of solid scintillator, Meltilex (Wallac), was placed together with the filter containing 96 samples in a sample bag and run together through a heat sealer (Microsealer, Wallac). Samples were measured in MicroBeta Trilux (Wallac).

Table I. Characteristics of HPMA Copolymer Conjugates

No.	Structure	M_w (kDa)	M_w/M_n	Content of DOX (%wt.)	Content of mAb (%wt.)
Classical conjugates					
1	Anti-Thy-1.2-GFLG-P*-GFLG-DOX	860	~5.0	4.1	20.6
2	Anti-CD71/A-GFLG-P-GFLG-DOX	750	~5.0	3.8	25.7
3	Anti-CD71/B-GFLG-P-GFLG-DOX	800	~5.0	3.6	30.7
4	P-GFLG-DOX (PK1)	21	1.6	5.3	—
5	NH_2-Tyr-P-GFLG-DOX	20	1.7	3.7	Tyr = 0.5
Starlike conjugates					
6	Anti-Thy-1.2-GFLG-P-GFLG-DOX	300	2.2	3.8	44.7
7	Anti-CD71/A-GFLG-P-GFLG-DOX	320	2.0	3.5	37.9
8	Anti-CD71/B-GFLG-P-GFLG-DOX	350	2.4	2.9	48.2

* P stands for HPMA copolymer; M_w, molecular weight of the conjugates; M_w/M_n, polydispersity index; DOX (%wt.) and mAb (%wt.), weight percentage of doxorubicin or monoclonal antibody in the conjugates.

Experimental Animals

Inbred strains of Balb/c and C57BL/10 mice, aged 8 weeks, were purchased from the Animal Center of the Institute of Physiology, Academy of Sciences of the Czech Republic. Immunodeficient nu/nu CD-1 mice were bought from AnLab Ltd., Prague. All the mice were housed in accordance with approved guidelines and were provided with food and water *ad libitum*. The experimental designs were in accordance with the Czech Republic Act for Experimental Work with Animals (Decrees No. 311/97, 117/87, and Act No. 246/92), which is fully compatible with the corresponding European Community Acts.

Blood Clearance

Samples of free DOX, classic, starlike or PK1 conjugate containing 0.1 mg DOX were injected into the lateral tail vein of each female Balb/c mouse (n = 5). Blood samples were taken at different time intervals (10 min, 0.5, 3, 6, 12, 24, and 48 h) after the injection. The blood was mixed with 2 N HCl in a 1:1 ratio and incubated for 1 h at 50°C. The amount of total DOX was determined by HPLC after its extraction into chloroform as described by Etrych *et al.* (13). The calibration was carried out using DOX·HCl.

Body Distribution

Anti–Thy-1.2 mAbs of targeted conjugates and terminal tyrosine of nontargeted PK1 conjugate were radiolabeled with [^{125}I]iodide (Amersham) using the Chloramine T method. Briefly, 0.2 ml polymer at a concentration of 5 mg/ml PBS and 10 μl of Na^{125}I (1 mCi) were mixed. Reaction was started by 0.1 ml chloramine T (4 mg/ml). After 10 min the labeled polymer was purified of free ^{125}I by means of size exclusion chromatography on a PD10 desalting column (Sephacryl G-25, Pharmacia). Radiolabeled samples with 1 μCi activity were mixed with nonlabeled "cold" polymer (2 mg/0.2 ml PBS) and injected i.v. into the female Balb/c mice (n = 5). The animals were killed by cervical dislocation 0.5, 3, and 24 h after injection, and the radioactivity of blood, liver, kidney, and spleen was assessed. The radioactivity of 0.25 ml of the collected blood was recalculated to a 2-ml volume. The radioactivity of individual tissues was expressed as percentage of the injected radioactivity.

Anticancer Activity

Male C57BL/10 mice (n = 5) were inoculated s.c. with 10^5 T-cell lymphoma EL4 on day 0. Controls develop tumor within 12 days at 100% frequency (5/5). The conjugates or free DOX were injected i.p. on days 1, 3, 5, 7, and 9 at a total dose equivalent to 25 mg DOX/kg. Female athymic nu/nu mice (n = 8) were inoculated s.c. with 10^7 cells of human SW 620 colorectal cancer on day 0. When the tumors reached a size of about 20 mm^2, the treatment was started, usually on days 3, 5, 7, 9, and 11 at a dose equivalent to 25 mg DOX/kg. The free DOX was injected in both cases in the maximum tolerated dose of 12.5 mg DOX/kg. Tumor size and survival of experimental animals were checked every other day.

RESULTS

In Vitro Binding Capacity of Antibody-Targeted Conjugates

Flow cytometric analysis was used to evaluate the binding of mAb-P-DOX conjugates to the cancer cells. Suspension of EL4 or SW 620 cells was incubated in the first step either with free antibody (anti–Thy-1.2, anti-CD71/A, and anti-CD71/B mAbs) or with an antibody-targeted conjugate (samples 1, 2, 3, 6, 7, and 8) and in the second step with a fluorescein-labeled secondary antibody. The results of the measurement were expressed as a mean fluorescence intensity (MFI, Table II). Flow cytometric analysis showed that the binding capacity of free anti–Thy-1.2 mAb (MFI = 39.23) was two times higher than that of either free anti-CD71/A (MFI = 15.18) or anti-CD71/B (MFI = 19.10) mAbs. The binding efficacy of the anti–Thy-1.2 antibody (MFI = 39.23) after its conjugation to the HPMA copolymer (samples 1 and 6) was partly affected (MFI = 11.84 and 11.46, respectively), but the binding was still strong enough to allow an efficient targeting of the conjugates. The conjugates targeted with anti-CD71 mAbs (samples 2, 3, 7, and 8) showed a similar binding capacity to the original free mAbs. The only modest decrease from MFI = 19.10 in unconjugated anti-CD71/B mAb to MFI = 11.89 was detected in sample 8 (starlike conjugate with anti-CD71/B). There was no significant difference in the mean fluorescence intensity of two different anti-CD71/A and anti-CD71/B mAbs.

Table II. Binding Studies of Antibody-Targeted Conjugates by Flow Cytometric Analysis*

	Mean fluorescence intensity (MFI) of original antibody and antibody-targeted HPMA copolymeric conjugates after binding to target			
	Control	Anti–Thy-1.2 to EL4 cells	Anti-CD71/A to SW 620 cells	Anti-CD17/B to SW 620 cells
Original free antibody	2.66	39.23	15.18	19.10
Classical conjugate	2.75	11.84	13.12	16.84
Starlike conjugate	2.58	11.46	11.80	11.89
Background (anti-IgG/FITC)	2.54	1.77	2.54	2.54

* Free anti–Thy-1.2 mAb, classical anti–Thy-1.2-P-DOX (sample 1), and starlike anti–Thy–1.2-P-DOX (sample 6) at a dilution of 10 μg mAb/ml were incubated with mouse T-cell lymphoma EL4 or irrelevant control human colorectal carcinoma SW 620 cells (Control). Free anti-CD71/A mAb, classical anti-CD71/A-P-DOX (sample 2), starlike anti-CD17/A-P-DOX (sample 7), free anti-CD71/B mAb, classical anti-CD71/B-P-DOX (sample 3), and starlike anti-CD71/B-P-DOX (sample 8) at a dilution of 10 μg mAb/ml were incubated with human colorectal carcinoma SW 620. Binding of mAbs was detected by secondary FITC-labeled swine anti–mouse IgG. The secondary antibody alone was used as the background.

Inhibition of Cell Proliferation

Cytotoxic activity of the samples (samples 1–4 and 6–8) was determined by their antiproliferative capacity. Cell proliferation was investigated as [^{3}H]thymidine incorporation into the cell DNA, and inhibition was expressed as IC_{50}, i.e., the concentration of DOX equivalent necessary to inhibit the cell growth by 50%. The inhibition of cell proliferation was 10-fold higher if the EL4 cell line was exposed to the anti–Thy-1.2 targeted starlike conjugate (IC_{50} = 1.14 μM DOX) compared to the classic one (IC_{50} = 12.73 μM DOX) (Table III). Classic conjugate was twice as effective as the nontargeted conjugate PK1 (IC_{50} = 24.59 μM DOX). If SW 620 cells were incubated with the starlike conjugate targeted by CD71/B (IC_{50} = 0.25 μM DOX), the inhibition of proliferation was also 10-fold higher than with classic conjugates targeted by an identical antibody (IC_{50} = 2.76 μM DOX) (Table IV). The anti-CD71/B–targeted conjugates were more effective than conjugates targeted by anti-CD71/A, and starlike conjugates were always more potent than classic conjugates targeted with the same antibody.

Blood Clearance

We have compared the persistence of anti–Thy-1.2-targeted classic and starlike conjugates (samples 1 and 6) in blood circulation. Blood was taken 10 min, 0.5, 3, 6, 12, 24, and 48 h after intravenous application of the conjugates and/or free DOX, and the level of the drug was quantified by HPLC assay. Free DOX was detectable in the blood only in a low quantity 10 min (6%) and 30 min (1.5%) after the administration (Table V). In the sample taken 3 h after the administration of the drug, its level in blood was below the detection limit (<0.5%, i.e., <500 ng). All polymeric conjugates circulated in the bloodstream longer and in a higher quantity than free doxorubicin. The starlike conjugate remained in the blood circulation at a relatively high level, as 19% of injected dose was still left after 48 h. On the other hand, only 3.8% of classic conjugate and 2.9% of PK1 were detected at the same time interval.

Body Distribution

The anti–Thy-1.2 mAbs of the classic and starlike conjugate (samples 1 and 6) and the tyrosine tail of nontargeted PK1 (sample 5) were radiolabeled with ^{125}I and injected i.v. as a 1-μCi bolus into Balb/c mice. Distribution of radioiodinated samples into blood, liver, spleen, and kidney was analyzed at 0.5, 3, and 24 h after the injection. The blood level of radiolabeled samples was comparable to the amount measured by HPLC assay (blood clearance). Both methods, the quantification of DOX by HPLC and the measurement of radiolabeled antibodies, provided similar results. After 30 min, more than 50% of classic or starlike conjugate and less than 50% of PK1 was detected in the blood circulation (Table VI). After 3 h, the level of antibody-targeted conjugates was two times higher than the level of PK1. After 24 h, only starlike conjugate remained in a high concentration (26% of injected dose) in the blood. Both targeted conjugates were detected in higher amounts in liver and spleen. The level of PK1 in the kidney was higher than targeted conjugates at all time intervals tested.

Table III. Inhibition of Proliferation of T-Cell Lymphoma EL4 Detected by [^{3}H]Thymidine*

No.	Sample	IC_{50} ± SD (μM DOX)
1	Classical anti–Thy-1.2-P-DOX	12.73 ± 0.68
6	Starlike anti–Thy-1.2-P-DOX	1.14 ± 0.11
4	PK1	24.59 ± 1.33
	Doxorubicin	0.04 ± 0.00

* IC_{50} values were evaluated as the concentration of doxorubicin that after 48 h of *in vitro* cultivation, inhibits [^{3}H]thymidine incorporation into the DNA of EL4 cells to 50% of the control cell level. Experiments were repeated twice with similar results.

Table IV. Inhibition of Proliferation of Colorectal Carcinoma SW 620 Detected by [^{3}H]Thymidine*

No.	Sample	IC_{50} ± SD (μM DOX)
2	Classical anti–CD71/A-P-DOX	10.49 ± 0.39
7	Starlike anti–CD71/A-P-DOX	3.32 ± 0.11
3	Classical anti–CD71/B-P-DOX	2.76 ± 0.17
8	Starlike anti–CD71/B-P-DOX	0.25 ± 0.01
4	PK1	5.58 ± 0.05
	Doxorubicin	0.02 ± 0.00

* IC_{50} values were evaluated as the concentration of doxorubicin that, after 72 h of *in vitro* cultivation, inhibits [^{3}H]thymidine incorporation into the DNA of SW 620 cells to 50% of the control cell level. Experiments were repeated twice with similar results.

In Vivo Anticancer Activity

All mice with transplanted cancer were treated intraperitoneally every other day with five doses containing either 25 mg DOX/kg in the form of polymeric conjugate or 12.5 mg/kg of free doxorubicin. For T-cell lymphoma EL4, we have used a protective regimen; i.e., the treatment started 1 day after the injection of cancer cells. In contrast to the low anticancer activity of free DOX and a modest antitumor effect of the nontargeted PK1, it was possible to demonstrate a significant chemotherapeutic activity of both anti–Thy-1.2-P-DOX conjugates (Fig. 1). A slightly better result was obtained with the starlike conjugate because EL4 tumor never started to grow. In both cases we obtained long-term survivors (100%) at 60 days. Human colorectal carcinoma SW 620 was implanted into immunodeficient nu/nu mice. The therapeutic treatment started after the establishment of the tumor on day 3. The experimental group included mice with an average tumor size of 5 × 5 mm. The application of free DOX was without any significant effect on the tumor size (Fig. 2). Both anti-CD71–targeted starlike conjugates were more effective than classic conjugates. However, anti-CD71/A– or anti-CD71/B–targeted classic conjugates caused at least 50% retardation of tumor growth.

DISCUSSION

In this study we describe the biologic evaluation of two different antibody-targeted drug delivery systems. Both are based on the same HPMA copolymeric carrier containing doxorubicin and targeted with monoclonal antibody. The differences are in the methods of synthesis, attachment of targeting antibodies, and resultant physicochemical characteris-

Table V. Blood Clearance (HPLC Assay)*

No.	Sample	Percentage of injected dose versus time (h)						
		0.17	0.5	3	6	12	24	48
1	Classical anti–Thy-1.2-P-DOX	nd	64.0	52.5	40.0	23.0	9.2	3.8
6	Starlike anti–Thy-1.2-P-DOX	nd	65.0	51.5	nd	39.0	24.6	19.0
4	PK1	nd	44.5	30.0	14.9	9.7	5.8	2.9
	Doxorubicin	6.0	1.5	<0.5	<0.5	<0.5	<0.5	nd

* Free doxorubicin, classical, starlike, or PK1 conjugate, containing 0.1 mg DOX were injected intravenously into the Balb/c mice. Blood was taken at 10 min, 0.5, 3, 6, 12, 24, and 48 h after application of the samples. The amount of total DOX was determined by HPLC (see Materials and Methods). Five mice were used for each experimental group. SD was <5% in all cases; nd, not determined.

tics (8,9). Compared to the classic conjugates, the synthesis of the starlike conjugate represents a more precisely controlled chemistry. In this system, one antibody molecule is situated in the center of the conjugate, and cross-linking of polymer chains is prevented. Conjugation of the antibodies to HPMA copolymer-DOX molecules can involve the antibody-binding site and hence the antibody-binding potential. In classic conjugates, we have protected the antigen-binding site by dimethylmalein anhydride, but we did not see significant differences in the cytotoxicity of conjugates with protected and unprotected antibodies (9). Among other methods used to prevent inactivation of the antibody-binding site of the immunoglobulin molecule belongs an oriented binding of oxidized antibody to a polymer containing hydrazide groups (8,14–16) or binding of polymerized Fab-antibody fragments to a HPMA copolymeric carrier containing maleimide groups (14,17).

Generally, the effectiveness of the targeted conjugates depends on the character of antibodies (polyclonal or monoclonal), on their class (IgG or IgM) and subclass (IgG1, IgG2, or IgG3), specificity and affinity, on epitopes of the targeted receptor against which the antibodies are directed, and on the rate and efficiency of receptor-mediated endocytosis (18). In this paper we used three monoclonal antibodies conjugated to a HPMA copolymeric carrier in a classic or starlike system. Anti–Thy-1.2 mAbs (IgG3 isotype) in both classic and starlike conjugates showed lower MFI than original free mAb. If anti-CD71/A (IgG1 isotype) and/or anti-CD71/B (IgG2a isotype) mAbs were used as a targeting moiety, the binding of polymeric conjugates was almost comparable with that of original antibodies. This suggests that the binding ability of the resulting conjugate depends not only on the selected chemical procedure but also on the shape of the antibody molecule. Generally, the binding of classic conjugates to the targets was always superior to the binding of starlike conjugates.

Regardless of the antibody used, starlike conjugates were always more effective (up to 10 times) than classic conjugates. The cytotoxicity of both conjugates, classic and starlike, targeted with anti-CD71/B (IgG2a) was always considerably higher compared to conjugates targeted with anti-CD71/A (IgG1) mAb. Previously it was published that different efficacies of immunotoxins recognizing different epitopes on the surface CD2 molecule are related to the distance of the epitope from the cell membrane (19), immunotoxin containing antibodies directed against a distant epitope being less effective. In fact, we do not know what is more responsible for the difference in final cytotoxicity of the two starlike conjugates—the epitope of the TfR to which targeting antibodies are directed or the antibody isotype. A detailed analysis of epitope position is not available, but it was determined that neither antibody competes with the binding of transferrin to its receptor (Dr. V. Hořejší, personal communication).

Two different methods were used to determine the presence of administered conjugates in the peripheral blood of

Table VI. Body Distribution (^{125}I-Labeled Targeting Antibodies)*

	Percentage of injected dose vs. time PK1/classical anti-Thy-1.2-P-DOX/starlike anti-Thy-1.2-P-DOX (sample 4/1/6)		
	30 min	3 h	24 h
Blood	42.0/52.7/53.2	17.2/36.4/46.8	4.3/3.7/25.6
Liver	5.4/11.2/10.5	3.9/10.2/9.2	5.2/3.9/4.2
Spleen	0.3/1.3/nd	0.2/1.5/1.5	0.3/0.9/0.6
Kidney	5.6/1.6/nd	6.3/1.5/1.6	4.7/0.5/0.9

* The antibodies of the targeted classical and starlike conjugates, and the tyrosine tail of nontargeted conjugate, were radiolabeled with ^{125}I, and each sample was injected as an intravenous 1-μCi bolus into the Balb/c mice. Radioactivity in blood, liver, spleen, and kidney was analyzed as percentage of total injected dose after 0.5 3, and 24 h. Five mice were used for each experimental group. SD was <5% in all cases, nd, not determined.

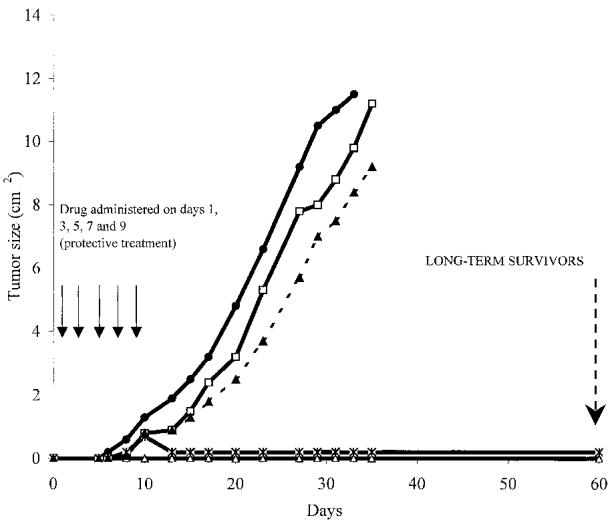

Fig. 1. Treatment of mouse T-cell lymphoma EL4 with saline (control; ●), free doxorubicin (□), PK1 (sample 4; ▲), classical anti–Thy-1.2-P-DOX (sample 1; ★), and starlike anti–Thy-1.2-P-DOX (sample 6; △). Five mice were used for each experimental group.

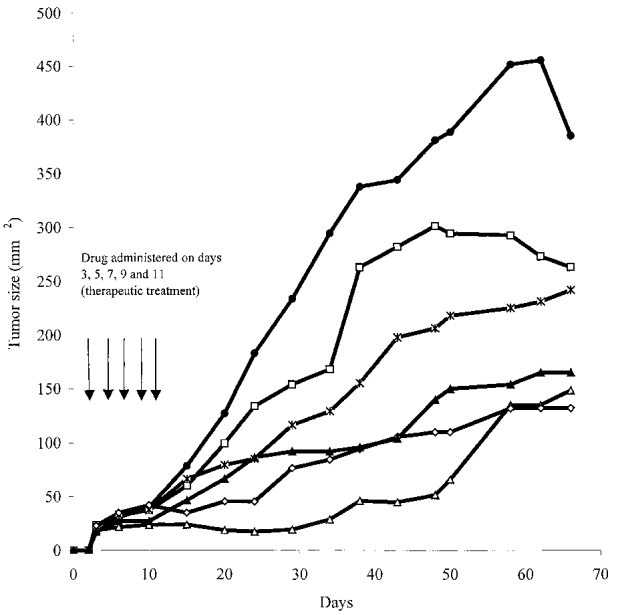

Fig. 2. Treatment of human colorectal carcinoma SW 620 with saline (control; ●), free doxorubicin (□), classical anti-CD71/A-P-DOX (sample 2; ▲), classical anti-CD71/B-P-DOX (sample 3; ★), starlike anti-CD71/A-P-DOX (sample 7; ◇), and starlike anti-CD71/B-P-DOX (sample 8; △). Eight mice were used for each experimental group.

mice; HPLC assay combined with extraction for quantification of free and polymer-bound DOX and radiolabeling to trace antibodies and/or HPMA carrier. Both methods yielded comparable results. In agreement with our previous data (20) we have confirmed that free DOX rapidly disappears from the bloodstream, whereas PK1 stays in the blood and in the other organs for a longer time in a higher concentration. The starlike conjugate is cleared from the blood much more slowly. Such a prolongation in blood circulation and hence a longer availability may explain the better antitumor activity in the human colorectal cancer system. Such higher efficiency of the starlike conjugate was not seen in mouse T-cell lymphoma, where the classic conjugate was also highly effective.

The most effective conjugate for the treatment of human colorectal carcinoma SW 620 was the starlike conjugate targeted by anti-CD71/B mAb that caused a significant retardation of tumor growth for a long time period, but a considerable antitumor effect was also seen with the anti-CD71/A–targeted starlike conjugate. Seymour *et al.* (21) were the first to use *in vivo* classic conjugate targeted to human colorectal carcinoma LS174T by B72.3 mAb. The inability of the conjugate to eradicate experimental cancer was explained as a blockage of the antigen-binding site of B72.3 mAb by soluble TAG72 antigen, which is released from the growing tumor. Flanagan *et al.* (22) used a classic conjugate for the treatment of mouse leukemia L1210. As a targeting moiety she used transferrin as a natural ligand of transferrin receptor. Surprisingly, such a targeted conjugate was less effective than nontargeted conjugate. Their explanation is a rapid exit of the conjugate from the peritoneal compartment, relatively broad cellular distribution of the transferrin receptor, or relation to the endosomal recycling of the transferrin receptor. The first antibody-targeted classic conjugate effective *in vivo* against experimental cancer model (mouse T-cell lymphoma EL4) was reported by Říhová *et al.* (4) and Ulbrich *et al.* (9). Classic conjugates targeted with anti-EL4 polyclonal antibody, antithymocyte globulin, or anti–Thy-1.2 mAb caused a significant retardation of tumor growth and an extension of the life span of treated mice. Recently, Kovář *et al.* (5) published data showing that classic conjugate targeted with anti–mouse CD71 mAb completely cured three of nine mice bearing B-cell lymphoma 38C13 and was considerably better than a conjugate targeted with transferrin. A starlike conjugate targeted with antiidiotype B1 mAb was able to completely cure five of nine mice with BCL1 leukemia after a single dose (6). We have demonstrated a comparable antitumor effect of classic and starlike conjugates targeted with anti–Thy-1.2 antibodies on mouse T-cell lymphoma and a superior effect of starlike conjugates targeted with anti-CD71 mAbs on human colorectal carcinoma. All targeted conjugates in either model were better than PK1 or free doxorubicin.

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