Antitumor Properties and Toxicity of Dextran-methotrexate Conjugates are Dependent on the Molecular Weight of the Carrier

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Abstract. *Methotrexate (MTX) is widely utilized in the clinical treatment of many forms of cancer. However, the drug has a short plasma half-life and causes toxic effects on normal proliferating cells. Conjugation with carriers is a possible way to alter these disadvantageous pharmacokinetics. Our aim was to synthesize dextran-MTX (D-MTX) conjugates, using carriers with molecular weights (Mw) ranging from 10 kDa to 500 kDa. Their in vitro and in vivo properties were compared with free MTX. The in vitro studies revealed that D-MTX conjugates had 4- to 10-fold lower antiproliferative effects against neoplastic cell lines compared to free MTX. There was a negative relationship between the Mw of the carrier and the antiproliferative effect of the respective conjugate. The data obtained in a mouse leukemia P388 in vivo model suggested that a lower in vitro antiproliferative effect of the conjugates does not result in diminished antileukemic activity in vivo. The toxicity of the conjugates was greater in comparison with the parent drug and tended to rise with increasing Mw. However, no superiority over free MTX in terms of an antileukemic effect was demonstrated. In particular, the D-MTX conjugate based on the dextran with Mw 10 kDa showed a comparable antileukemic effect with an even lower toxicity than that of free MTX. The data suggest that at least the toxicity of conjugates is dependent on the Mw of the carrier. This fact should be taken into account when designing new anticancer polymer-drug compounds.*

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Methotrexate (MTX) is one of the oldest and most widely utilized chemotherapy drugs, not only in anticancer therapy, but also in the treatment of autoimmune diseases and for the prevention of graft-*versus*-host disease after hematopoietic transplantation (1, 2). Its metabolism and mechanism of action is well understood due to extensive studies conducted over the past decades. MTX primarily acts as an inhibitor of the enzyme dihydrofolate reductase, which results in hindering the synthesis of DNA precursors and, therefore, inhibition of cell proliferation. The drug also competes with reduced folates for transport and polyglutamation (2, 3). Despite its unequivocal advantages, there are also some disadvantageous issues regarding the clinical use of MTX. The drug has a very short plasma half-life, comprising its efficacy in anticancer chemotherapy. Moreover, it causes non-specific overall toxic effects on normal proliferating cells when applied in high doses. The problem of drug resistance is another point of concern, especially in patients receiving long-term therapy with MTX (2).

There have been numerous attempts to solve the problems addressed above. Conjugation with different carriers is a strategy frequently applied to alter the pharmacokinetic behavior of anticancer agents. This process often results in prolongation of the effect, alteration of the toxicity profile and a reduction in the immunogenecity of parental drugs or proteins (4). Two possible effects of conjugation on the antitumor properties of chemotherapy drugs have been outlined to date. First, coupling these agents with different carriers was shown to increase their plasma half-life, since macromolecules with high molecular weights (Mw) have lower clearance rates (5). The second effect is mediated by passive targeting of drug-carrier conjugates, due to increased permeability of the tumor vasculature and retention of macromolecules in the tumor site (6). The possibility of coupling MTX with macromolecules has also been extensively

investigated. Among other authors, we have recently reported studies on the coupling of MTX to different carriers: fibrinogen (7, 8), albumin (9), branched polypeptide (10), polyethylene glycol (PEG) (11) and dextrans (12, 13). There is also an encouraging report about a clinical trial of albumin-MTX conjugate in cancer patients (14).

Dextrans are glucose polymers, which have been used in clinical medicine for several decades as plasma volume expanders and antithrombolytic agents. More recently, dextrans have been utilized as carriers for the delivery of drugs and proteins, mainly to increase the longevity of therapeutic agents in the circulation and reduce their immunogenicity. Dextrans are soluble in water and contain a large number of hydroxyl groups which can be easily conjugated to drugs and proteins by either direct attachment or through a linker (4). Another advantage of these macromolecules is that they are neutral in their native form (5). Comprehensive reviews, focusing on studies with dextran conjugates, have been published recently (4, 5). While the results of these investigations were often encouraging, they have not resulted in marketing of the conjugates.

Several reports on conjugates of MTX with dextrans have appeared to date. They mainly concentrated on dextrans with Mw 40 and 70 kDa. Manabe *et al.* coupled 40 kDa dextran to MTX and anti-HLA IgG1 antibody. They reported that the conjugate had a significantly higher cytotoxic effect *in vitro* on the HLA-bearing cells (15). Shih *et al.* used a similar strategy, when they coupled 40 kDa dextran with MTX and anti-carcinoembryonic antigen monoclonal antibody. The conjugate showed greater inhibition of tumor growth in a human tumor xenograft model *in vivo* (16, 17). Onishi *et al.* developed a conjugate of MTX with dextran 70 kDa, modified by decylenediamine, and reported that the inhibitory effect of the conjugate on dihydrofolate reductase was approximately 20 times less than of the free MTX (18, 19).

However, to our knowledge, investigations into the effect of the Mw of dextran macromolecules on the biological properties of MTX-conjugates are conspicuously lacking. This seems to be a particularly gross oversight, as the Mw of dextran carriers is known to significantly affect the physico-chemical properties of their conjugates *in vivo* (4, 5). Thus, the aim of our study was the synthesis of several dextran-MTX (D-MTX) conjugates, to investigate their *in vitro* cytotoxicity against human and murine neoplastic cell lines and to compare them with free MTX. The well-established P388 mouse leukemia model was also used to characterize the antitumor and toxic profile of the conjugates *in vivo*.

Materials and Methods

Conjugation of MTX with dextrans. Five D-MTX conjugates were synthesized using dextran with different Mw as the carriers (T_{10}, T_{40}, T_{70}, T_{110} and T_{500}, respectively). All dextrans were obtained from Pharmacia (Fine Chemicals AB, Uppsala, Sweden). MTX was obtained from Lachema (Brno, Czech Republic). The conjugates were coded as T10-MTX (Mw 10 kDa), T40-MTX (Mw 40 kDa), T70-MTX (Mw 70 kDa), T110-MTX (Mw 110 kDa) and T500-MTX (Mw 500 kDa), according to the molecular weight of the carrier.

The first stage of synthesis requires MTX to be present in the form of a free acid. To obtain this form, the pH of MTX was adjusted using 1M hydrochloric acid to pH 3.0-4.0. The yellow precipitate obtained during this reaction was lyophilized and dissolved in dry dimethylformamide (POCH, Gliwice, Poland). Residues of the acid form of MTX dissolved in dimethylformamide and undissolved inorganic salts were removed by centrifugation. In the second stage, MTX anhydride was prepared by reaction of the acid form of MTX with N,N'-dicyclohexylcarbodimide (Fluka, Busch, Switzerland), at the molar ratio 1:0.9. The reaction was carried out in dimethylformamide at 4˚C for 48 h in the dark. Finally, the mixture was centrifuged to remove dicyclohexylurea.

In the third stage, the actual conjugating stage, pure MTX anhydride was added very slowly under the surface of the stirred dextran solution in 0.05 M Na_2CO_3. The reaction was allowed to proceed at pH 10.3 for 5 min. The pH of the reaction mixture was subsequently adjusted to 7.0 by addition of 2 M KH_2PO_4 (POCH, Gliwice, Poland). The conjugates were dialyzed for 48 h against 0.1 M $NaHCO_3$ (POCH), to remove free MTX. The concentration of MTX (mg/ml) in the conjugate was calculated by dividing the absorbance in 0.1 M sodium bicarbonate at 302 nm by 22.7 ($\varepsilon_{302\ nm}$, 1 mM, 1 N NaOH). The concentration of dextran in the conjugates was calculated using the phenol-acetone reagent, as described elsewhere (20). The absorbance of the reaction mixture was measured after 60 min and 37˚C at 568 nm. The conjugates were purified by gel filtration on Sephadex G-25 (Fine Chemicals AB, Uppsala, Sweden).The stability of the prepared conjugates was assessed under different conditions and the compounds were stored at –20˚C after lyophilization. Free MTX was used as the reference compound.

Cell lines. A549 (human non-small cell lung carcinoma), SW707 (human colon adenocarcinoma) and P388 (murine leukemia) were obtained from the American Type Culture Collection (Rockville, MD, USA) and were maintained in culture or frozen in the Cell Culture Collection of the Institute of Immunology and Experimental Therapy, Wroclaw, Poland. Twenty-four h before addition of the tested compounds, the cells were plated in 96-well plates (Sarstedt, Germany) at a density of $0.5x10^4$ cells per well and were cultured in the mixture of RPMI 1640 and Opti-MEM (1:1). The medium was supplemented with 2 mM glutamine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 100 mg/ml streptomycin (Polfa, Tarchomin, Poland), 100 U/ml penicillin (Polfa) and 5% fetal bovine serum (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). The cells were cultured at 37˚C in a humid atmosphere saturated with 5% CO_2. For *in vivo* experiments, passages of P388 leukemia cells in DBA/2 mice were carried out according to the NIH/NCI standard screening protocols *in vivo* (21, 22).

Antiproliferative assays in vitro. The *in vitro* cytotoxic effect of all agents was examined after 72-h exposure of the cultured cells to varying concentrations of the tested compounds, using the SRB assay as described by Skehan *et al.* (23). Briefly, the cells were attached to the bottom of plastic wells by fixing them with cold 50% (80% in the case of the P388 cell line) trichloroacetic acid (TCA, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) on the top of the culture medium in each well. The plates were incubated at 4˚C for 1 h and

then washed 5 times with tap water. The background optical density was measured in the wells filled with culture medium, without the cells. The cellular material fixed with TCA was stained with 0.4% sulforhodamine B (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) dissolved in 1% acetic acid (POCH) for 30 min. Unbound dye was removed by rinsing (4x) with 1% acetic acid. The protein-bound dye was extracted with 10 mM unbuffered TRIS base (POCH) for determination of optical density (at 540 nm) in a computer-interfaced, 96-well microtiter plate reader Multiskan RC photometer (Labsystems, Helsinki, Finland).

The results were presented in terms of IC_{50} values. The IC_{50} is the concentration of the tested agent which inhibits the proliferation of 50% of the cancer cell population. Average IC_{50} values for each compound were calculated using data from 3 independent experiments.

Mice: Male and female (C57Bl/6 x DBA/2)F1 ($B_6D_2F_1$) mice, aged 16-24 weeks and weighing 22-29 g were used. Each separate experiment was performed on animals of the same gender. The mice were supplied by the Animal Breeding Centre of the Medical Academy, Wroclaw, Poland, and were maintained in standard laboratory conditions. Experiments were performed according to the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing and Education issued by the New York Academy of Sciences' Ad Hoc Committee on Animal Research and were approved by the First Local Ethical Committee for the use of Laboratory Animals, Wroclaw, Poland.

The mice were injected with 10^6 leukemia (P388) cells *i.p.* (day 1) and, 24 h later (day 0), each mouse was injected once *i.p.* with the appropriate agent. All doses were based on the molarity of the MTX in the conjugates. Mice body weight and survival data were collected on a daily basis for the duration of the experiment. In the first experiment, the animals were randomly divided into 7 groups of 7-8 mice. The animals in the control and the MTX groups were administered 0.9% saline solution and 40 mg/kg of free MTX, respectively. Another 5 groups were administered 40 mg/kg of T10-MTX, T40-MTX, T70-MTX, T110-MTX or T500-MTX conjugates, respectively. In the second experiment, the animals were randomly divided into 4 groups of 5-6 mice. The animals in the control and MTX groups were administered 0.9% saline solution or 160 mg/kg of free MTX, respectively. Another 2 groups were administered 160 mg/kg of T10-MTX or T40-MTX conjugates, respectively.

Data handling: The antitumor effect was evaluated as the increase in life span (ILS) of treated mice over the control, calculated by the following formula: (MSTT/MSTC) x 100 – 100, where MSTT is the median survival time of treated animals, and MSTC is the median survival time of untreated control mice. The overall toxicity in the experimental groups was assessed using a number of treated mice, which died earlier than the day of the first death registered in the control group.

Statistical evaluation. The analysis of *in vitro* data was performed by 2-way ANOVA with *post-hoc* Tukey comparison after natural logarithmic data transformation to meet parametric assumptions. The cell line and Mw of the carrier were used as independent factors. Cuzick's nonparametric test for trend was used to explore the relationship between the Mw of the carrier and the IC_{50} values (24). The survival data in the experimental *in vivo* groups were compared using the Cox's F test with Bonferroni correction for multiple comparisons ($p_{adjusted} = p_{counted}$ x N, where N = number of pairwise comparisons). *P* values less than 0.05 were considered significant.

Results

Characterization of synthesized conjugates. All conjugates had approximately the same level of substitution (0.0011-0.0019 per mol of glucose in the *in vitro* and first *in vivo* experiment and 0.016-0.018 in the second *in vivo* experiment). The reaction and purification steps were monitored by high performance liquid chromatography to confirm high purity and reasonable yield. Monitoring of the elution profile at 302 nm revealed one fraction of the drug (Figure 1).

In vitro antiproliferative activity of D-MTX conjugates against human and mouse cancer cell lines. The results of the *in vitro* experiments are summarized in Table I. There were differences in the cell line sensitivity to the free MTX and all conjugates, as can be seen from the IC_{50} values. The P388 cell line was the most sensitive to cytostatic agents and had an approximately 10-fold lower IC_{50} in comparison with the A549 and SW707 cell lines ($p<0.001$). The sensitivity of SW707 was slightly higher than that of A549, though still statistically significant ($p<0.05$, Figure 2).

The studies *in vitro* revealed that all D-MTX conjugates had approximately 4- to 10-fold higher IC_{50} values in comparison with free MTX, therefore demonstrating a lower antiproliferative effect. This difference was statistically significant for all compounds tested ($p<0.001$). Moreover, the conjugate with the lowest Mw of the carrier (T10-MTX) also revealed the lowest IC_{50} among all conjugates. The differences *versus* T40-MTX ($p<0.05$), T70-MTX ($p<0.05$), T110-MTX ($p<0.01$) and T500-MTX ($p<0.001$) were all statistically significant. However, differences between the IC_{50} values of the rest of the conjugates with higher Mw carriers (T40-MTX, T70-MTX, T110-MTX and T500-MTX) were not statistically significant (Table I).

Preliminary data plotting revealed that the IC_{50} values tend to rise consistently with the Mw of the carrier used for conjugation (Figure 2). Cuzick's test was applied to formally test this hypothesis. Indeed, further analysis showed that there was a statistically significant trend of increasing IC_{50} values with the Mw of the dextran carrier in the A549 ($p<0.05$), SW707 ($p<0.01$) and P388 ($p<0.05$) cell lines.

In vivo toxicity and antileukemic effect of D-MTX conjugates. Whether the decreased antiproliferative effect of conjugates *in vitro* would result in diminished antileukemic activity *in vivo* was also investigated. $B_6D_2F_1$ mice bearing P388 leukemia were injected *i.p.* once with MTX or one of the D-MTX conjugates at a dose of 40 mg/kg. The results of the experiment are summarized in Table II.

The groups treated with T10-MTX and T40-MTX conjugates had the highest ILS among all experimental groups. These two groups also showed significantly prolonged survival rates in comparison with the control group ($p<0.01$

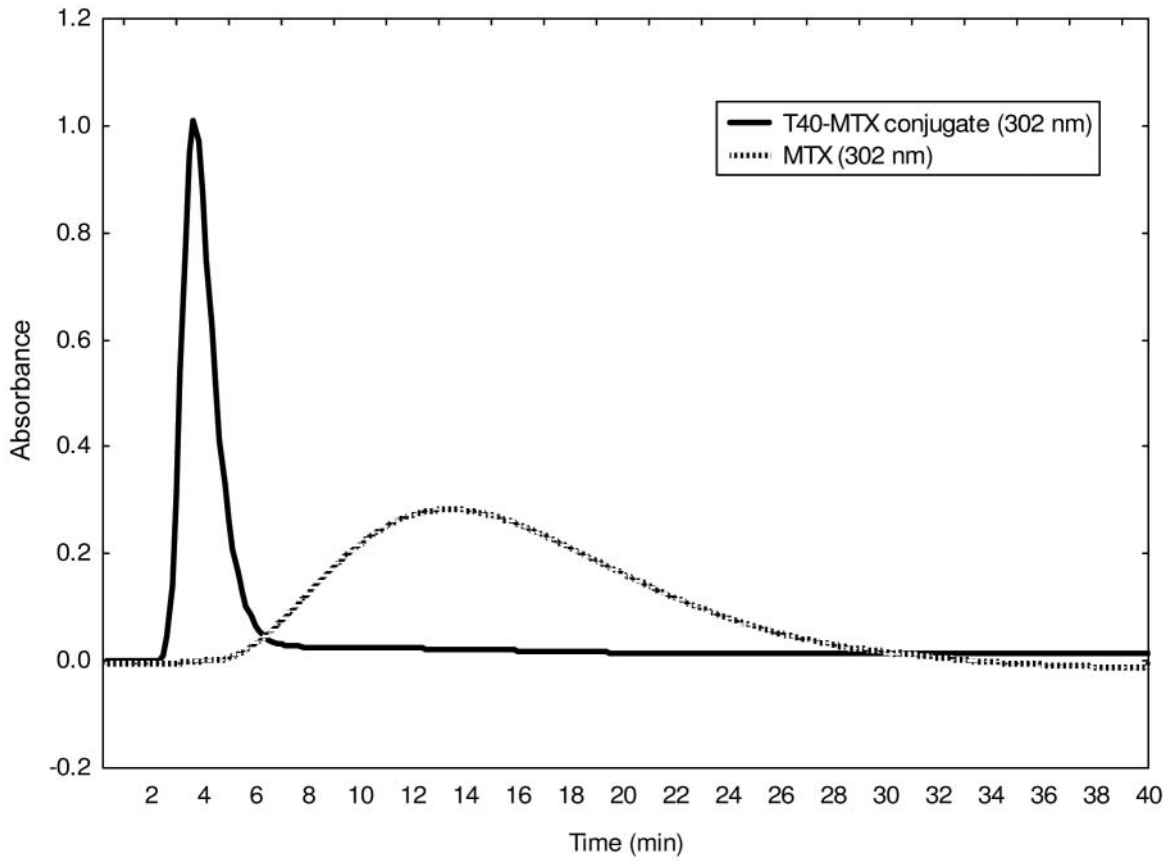

Figure 1. *Gel filtration of T40-MTX conjugate and MTX on Sephadex-G25 (0.4 x 18cm). Eluent: 0.05 M phosphate buffer, pH 7.0, flow 0.53 ml min^{-1}. Chromatograms of the other compounds were similar and are therefore not presented.*

and $p<0.05$, respectively). The median survival time in the groups treated with T10-MTX and T40-MTX conjugates suggests that they tend to have slightly higher antileukemic activity in comparison with the free drug. However, the advantages of the T10-MTX- and T40-MTX-treated groups over free MTX were not statistically significant in this model.

Some mice died before the mice in the control group (Figure 3), demonstrating that the conjugates were generally more toxic than free MTX. The data on body weight changes confirmed this observation (Figure 4). However, in the group treated with the T10-MTX conjugate, no lethal toxicity was observed. This group also experienced the least negative effect on body weight of the experimental mice. Moreover, in the group treated with the T40-MTX conjugate, only one mouse died before the control group and this group still had a higher ILS in comparison with the control and free MTX-treated groups.

Antileukemic properties and toxicity of T10-MTX and T40-MTX conjugates in the high-dose chemotherapy model. Previous experiments have shown that the two T10-MTX and T40-MTX conjugates induced the highest ILS among the experimental groups. However, whether the conjugates are

Table I. *Antiproliferative activity in vitro of different D-MTX conjugates in comparison with free MTX.*

Compound	IC_{50} (SD) μg/ml		
	Cell line		
	A549	SW707	P388
MTX[a]	0.054±0.001	0.033±0,004	0.0046±0.0003
T10-MTX[b]	0.304±0.105	0.202±0.054	0.0199±0.0077
T40-MTX	0.388±0.031	0.333±0.053	0.0314±0.0093
T70-MTX	0.436±0.063	0.343±0.079	0.0277±0.0137
T110-MTX	0.427±0.049	0.341±0.013	0.0321±0.0046
T500-MTX	0.473±0.067	0.462±0.077	0.0366±0.0052

[a]all conjugates revealed weaker *in vitro* antiproliferative activity than MTX alone ($p<0.001$).
[b]this compound revealed the highest *in vitro* antiproliferative activity among all conjugates. T10-MTX *vs*: T40-MTX ($p<0.05$), T70-MTX ($p<0.05$), T110-MTX ($p<0.01$) and T500-MTX ($p<0.001$).

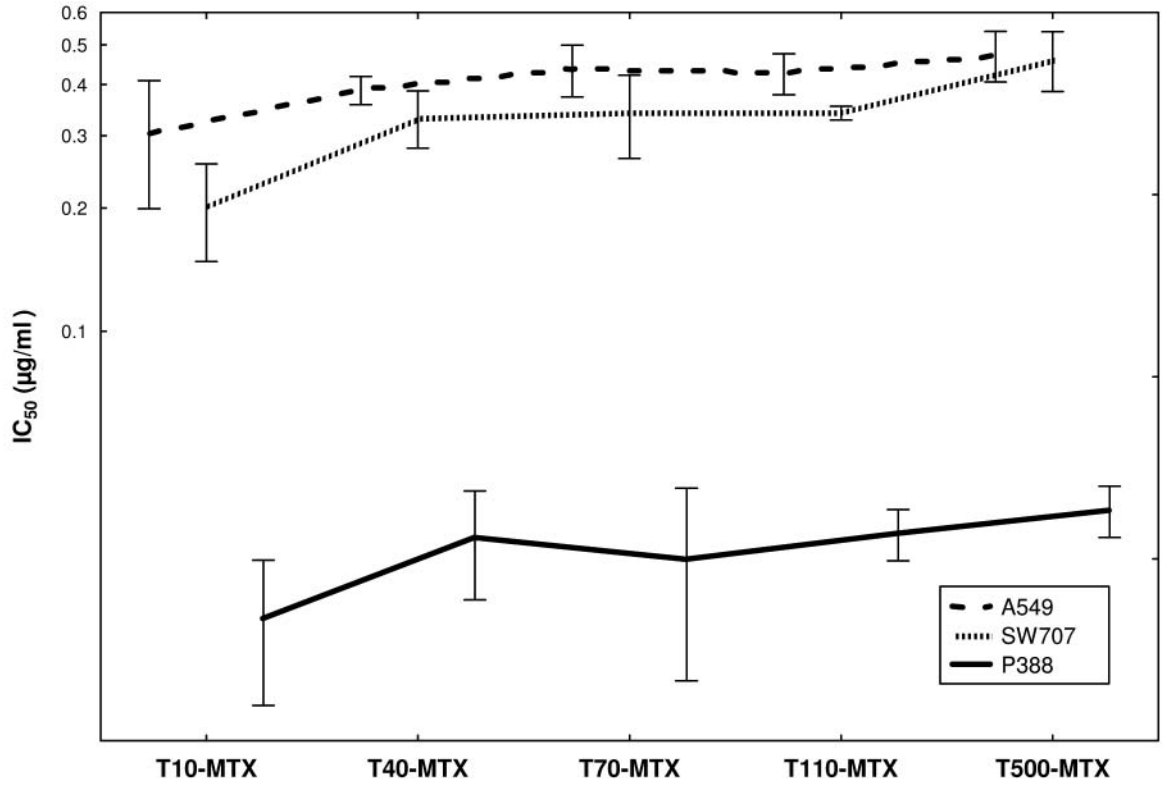

Figure 2. *Relationship between molecular weight of the carrier used for conjugation and IC_{50} values (SD) in 3 cancer cell lines. Cuzick's test demonstrated that there was a statistically significant trend for increasing IC_{50} values with the molecular weight of the dextran in the A549 ($p<0.05$), SW707 ($p<0.01$) and P388 ($p<0.05$) cell lines.*

preferable over free MTX was not entirely clear in the model used. Therefore, the two most promising conjugates, T10-MTX and T40-MTX, were tested in the model with high-dose chemotherapy, on the hypothesis that the difference in these compounds would, thus, be more pronounced in comparison with the parent drug. We were also interested in the potential toxic effect of such a therapy. $B_6D_2F_1$ mice carrying P388 leukemia cells were *i.p.* injected once with MTX or either of the two D-MTX conjugates at a dose of 160 mg/kg. The results of this experiment are summarized in the Table III.

The data presented therein indicate that the T40-MTX conjugate was already lethally toxic at a dose level of 160 mg/kg, since all mice in the group treated with this compound died before the control group. The change in average body weight confirmed this observation (Figure 5). On the contrary, the toxic effect of the T10-MTX conjugate was much lower than that of free MTX and was of shorter duration (Figure 5). In addition, T10-MTX still had an ILS value comparable to that of the free MTX-treated group and differences in survival times between these two groups were not statistically significant. Such distinct biological properties of T10-MTX and T40-MTX compounds clearly suggest that at least the toxicity of the conjugates is strictly dependent on the Mw of the carrier.

Table II. *Survival data of leukemia-bearing mice treated with free MTX or different D-MTX conjugates, expressed as an increase in life span (ILS %).*

Group	N[a]	ILS %	Median[b]	L[c] (%)
Control	7	0	11.0	
MTX, 40 mg/kg	8	45	16.0	0 (0)
T10-MTX conjugate, 40 mg/kg	8	55	17.0	0 (0)
T40-MTX conjugate, 40 mg/kg	8	59	17.5	1 (12.5)
T70-MTX conjugate, 40 mg/kg	8	–36	7.0	5 (62.5)
T110-MTX conjugate, 40 mg/kg	8	–45	6.0	8 (100.0)
T500-MTX conjugate, 40 mg/kg	8	–36	7.0	5 (62.5)

[a]N, number of mice in group
[b]Median survival time (days)
[c]Number of mice which died before the control group

Discussion

Conjugates of MTX with dextrans were reported to have better antitumor properties in comparison with the free drug (15-17). Dextrans have been frequently used as carriers, not only for antitumor drugs, but also for proteins and imaging agents (5). There are number of commercially available dextrans with different Mw. As reviewed elsewhere (4), the

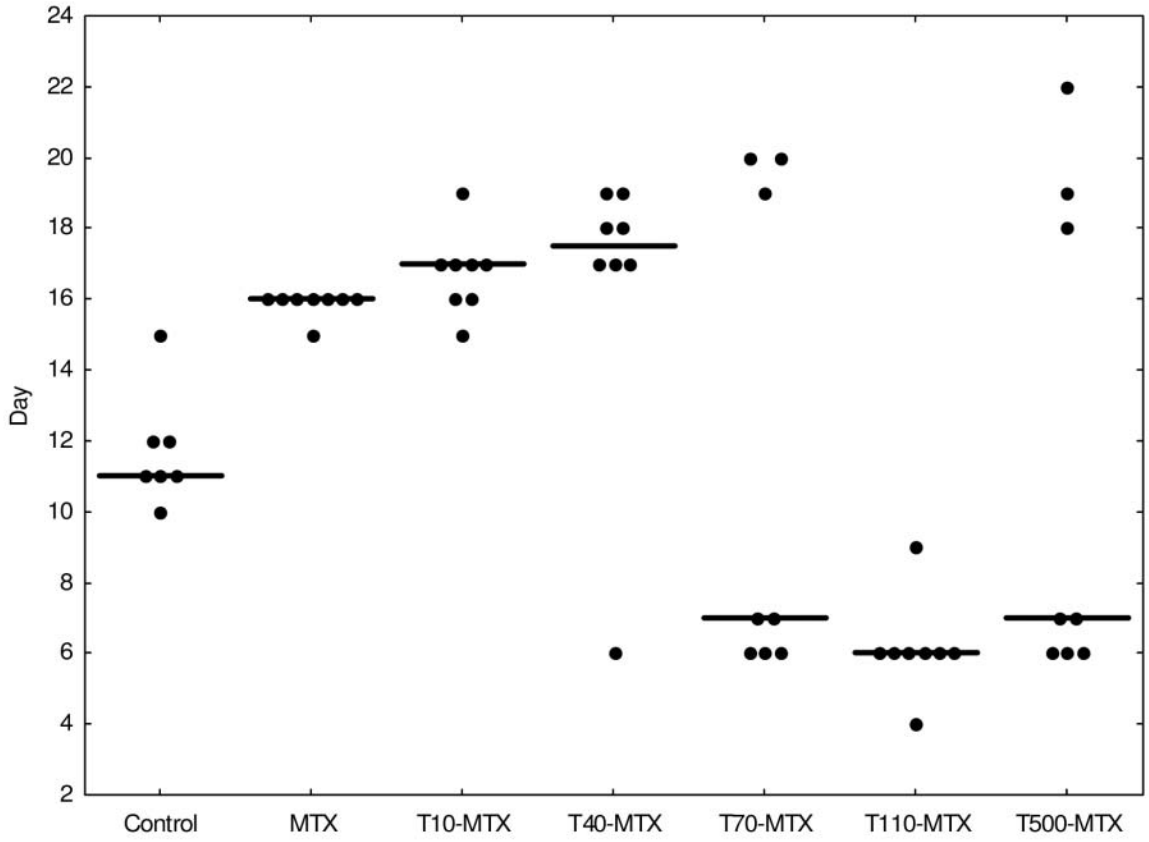

Figure 3. *Median and individual survival times for treated and control mice.*

pharmacokinetics, renal clearance and metabolism of dextrans are strongly dependent on their Mw. It is also believed that the kinetics of drugs attached to polymers are substantially affected by the pharmacokinetics of the carrier macromolecules. However, to our knowledge, there is a lack of studies investigating the influence of the dextran's Mw on the antitumor properties and toxicity of their conjugates with antitumor drugs. Our hypothesis was that MTX coupled with dextrans would result in conjugates with different biological behaviors, depending on the Mw.

All the tested compounds revealed lower *in vitro* cytotoxicity in comparison with free MTX. This appears to be general for all conjugates of macromolecule carriers with MTX, since lower *in vitro* cytotoxicities as compared to free MTX were also previously reported for PEG-MTX (11), fibrinogen-MTX (7), albumin-MTX (9) and dextran-peptide-MTX (12) conjugates. The mechanism of the *in vitro* cytotoxic effects of the conjugates is not entirely clear. However, it can be speculated that their diminished *in vitro* cytotoxicity might be explained by difficult cellular uptake of D-MTX compounds, due to the larger molecular sizes.

Further analysis has also shown that IC_{50} values rise consistently with increasing Mw of the dextran carrier used. These results indicate that the *in vitro* cytotoxic effect is dependent on the Mw of the carrier macromolecule and that it decreases from light to heavier dextrans (Figure 2). The conjugate of MTX with the T_{10} dextran (T10-MTX), the lightest one, had the lowest statistically significant IC_{50} among all the conjugates tested. These data are consistent with the recently published report by Riebeseel *et al.* (11). In their studies on different PEG-MTX conjugates, these authors showed that the *in vitro* cytotoxicity of the conjugates lessened with increasing carrier size. Our data further support the hypothesis that the differences in the *in vitro* cytotoxicities of the conjugates are due to different rates of cellular uptake, which, in turn, depend on the Mw of the compound. We also speculate that the rate of cellular uptake is the primary factor distinguishing different conjugates, because it was also shown that the size of the carrier macromolecule did not have a significant influence on the inhibition of the target enzyme (11). This fact suggests that the intracellular activity of the conjugates is comparable, regardless of the carrier Mw. However, additional studies are needed to investigate the exact mechanism of conjugate activity *in vitro*.

The sensitivities of the 3 cell lines were not identical. The murine leukemia P388 line was the most sensitive to free

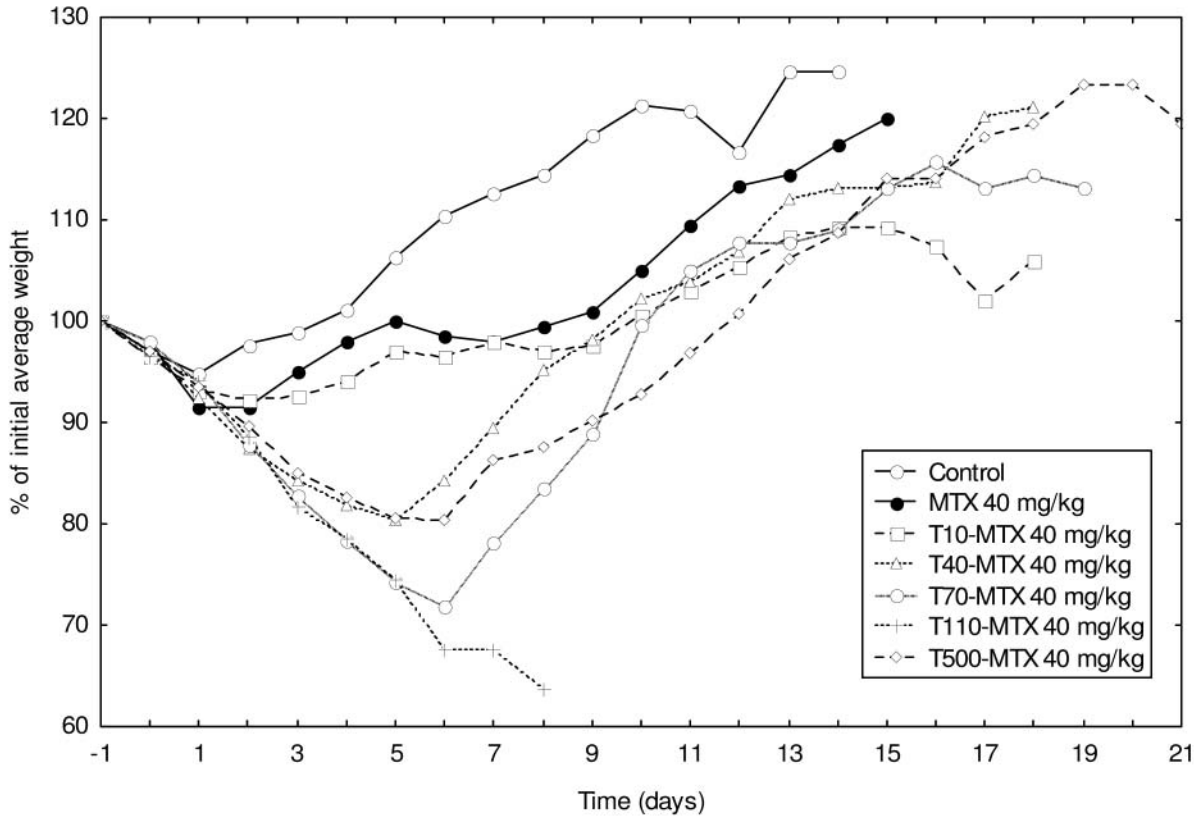

Figure 4. *Dynamics of body weight in leukemia-bearing mice treated with either MTX or one D-MTX conjugate.*

MTX and the human lung cancer A549 cell line was the least. Notably, this order of cell line sensitivity was also preserved in the conjugates (Table I). Instead of generally higher IC_{50} values for conjugates in comparison with free MTX, the P388 cell line was, again, the most sensitive to their cytotoxic effect.

In vivo comparative experiments were then performed to investigate whether the decreased *in vitro* cytotoxicity of the conjugates would result in diminished antileukemic activity *in vivo*. The well-established murine leukemia P388 model was used for this purpose. We were also interested in determining the overall toxicity of the conjugates. The survival data revealed that the conjugates with higher Mw were significantly more toxic than free MTX. Between 62.5% and 100% of the mice died due to the toxic effects of the conjugates (Table II) in the groups treated with the T70-MTX, T110-MTX and T500-MTX conjugates. The overall toxicity of the T40-MTX conjugate was also significant, since one mouse died before those of the control group and the average weight of the mice treated with this conjugate decreased in the first week after drug injection (Figure 4). However, all mice which survived this initial toxic effect showed significantly longer survival in comparison with the control group and slightly higher, though not significant, median survival time in comparison with the free MTX-treated group. As regards T10-MTX, this conjugate had the lowest toxicity as can be seen from the average weight dynamics (Figure 4) and overall survival compared with the free MTX-treated group (Table II, Figure 3). The data clearly showed that the toxicity of the conjugates was dependent on the Mw of the dextran carrier and was higher in the case of compounds with Mw ≥40 kDa. The mice, treated with either T70-MTX or T500-MTX conjugates and surviving the first week, tended to live even longer than mice in the free MTX-, T10-MTX- and

Table III. *Survival data of leukemia-bearing mice treated with free MTX or either the T10-MTX or T40-MTX conjugate, expressed as an increase in life span (ILS %).*

Group	N[a]	ILS %	Median[b]	L[c] (%)
Control	6	0	11.0	
MTX, 160 mg/kg	6	68	18.5	0 (0)
T10-MTX conjugate, 160 mg/kg	6	55	17.0	0 (0)
T40-MTX conjugate, 160 mg/kg	5	–46	6.0	5 (100)

[a]N, number of mice in group
[b]Median survival time (days)
[c]Number of mice which died before those of the control group

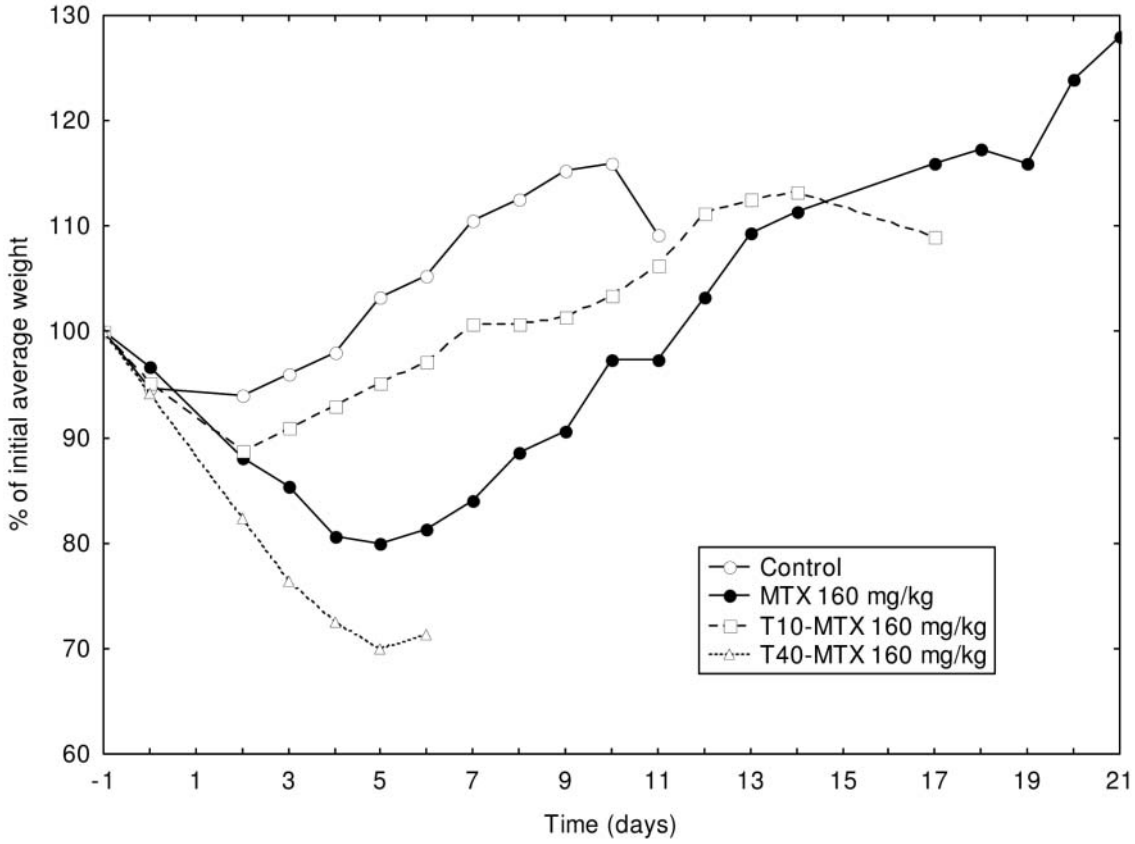

Figure 5. *Dynamics of body weight in leukemia-bearing mice treated with free MTX or either the T10-MTX or T40-MTX conjugate.*

T40-MTX-treated groups. However, due to the profound toxic effects of the conjugates with high Mw causing high mortality in these groups, the overall survival was significantly worse in comparison to the free MTX-treated group (Figure 3). Therefore, we could not clearly establish the dependency of the antileukemic effect on the Mw of the carrier macromolecule in this model. Our data support the opinion of Seymour *et al.*, that higher Mw polymer-drug conjugates could be potentially more efficacious, but also more toxic due to their lower rate of whole body excretion (6). It seems that, when designing new polymer-drug compounds, a compromise between their antitumor effects and overall toxicity must be made.

Two conjugates, T10-MTX and T40-MTX, were chosen for further investigation due to their low toxicity in comparison to the others. Their effects were investigated in the model using high-dose chemotherapy. Our hypothesis was that the differences in these compounds would be more pronounced in comparison with the parent drug when higher chemotherapy doses were used. The dose of 160 mg/kg was based on the molarity of MTX in the conjugates. The results of this experiment provided further evidence that the properties of D-MTX compounds are strictly dependent on the Mw of the carrier. The group treated with the T10-MTX conjugate had an ILS comparable to that of the group treated with free MTX and the difference in overall survival was not statistically significant between these two groups. Notably, the toxicity of the T10-MTX conjugate was less and lasted a shorter time than that of either free MTX or T40-MTX, as is clearly seen from the average weight changes (Figure 5). However, the T40-MTX conjugate was already lethally toxic at the dose used, since all the mice in group treated by the compound died during the first week after drug injection (Table III). We explain this dramatic difference in the toxicity of these two conjugates by the differences in their pharmacokinetics. It was shown that dextrans with Mw <40 kDa are excreted unrestrictedly, while those with Mw ≥40 kDa are not able to pass unchanged through the pores of the glomerular capillary walls, due to their large molecular size (5). Thus, the increased toxicity of the D-MTX conjugates with Mw ≥40 kDa could be explained by the prolonged circulation and, therefore, increased systemic toxic effect on the normal proliferating cells of the body.

The data from our *in vivo* experiments suggest that the lower *in vitro* cytotoxic effects of the conjugates did not result in diminished antileukemic activity *in vivo*. This discrepancy between *in vitro* and *in vivo* results is in accordance with our and other's previous studies on fibrinogen-MTX and

PEG-MTX conjugates (7,11). We agree with the opinion stated by Wosikowski *et al.*, that the advantage of carrier macromolecule conjugates with MTX over free MTX cannot be demonstrated fully in *in vitro* studies (9). Moreover, Riebeseel *et al.* have shown that the antitumor activities of PEG-MTX conjugates rose with the increase of the carrier's Mw (11). However, due to the toxicity of our D-MTX conjugates with higher Mw, we were not able to demonstrate their superiority over free MTX with regard to an antileukemic effect, though a trend could be seen in mice surviving the initial toxicity after drug administration (Figure 3). We are currently conducting studies in the multiple low-dose administration models, to verify whether it is possible to minimize the toxic and maximize the antitumor effects of these conjugates.

In conclusion, the data presented suggest that the biological properties of D-MTX conjugates are dependent on the Mw of the carrier macromolecules. This fact should be taken in account when designing new experimental anticancer polymer-drug compounds.

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