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Pharmacokinetics of methotrexate–albumin conjugates in tumor-bearing rats

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Linking chemotherapeutic drugs to a macromolecular carrier system may enhance tumor targeting, reduce toxicity and overcome drug resistance mechanisms. As an elementary model to evaluate the pharmacological properties of macromolecular drug carrier systems we chose rat serum albumin (RSA) for carrier and methotrexate (MTX) as antineoplastic drug. The conjugation procedure yielded conjugates with an approximate 1:1 molar loading rate (MTX(1)–RSA). In the first part of the study a residualizing [^{111}In]DTPA protein label was used for mapping *in vivo* the catabolic sites of the native carrier protein and of the MTX(1)–RSA drug conjugate in Walker 256 carcinosarcoma bearing rats. The tumor accumulation was about 14% of the injected dose for the RSA and MTX(1)–RSA tracers after 24 h. Tracer entrapment by organs with an active mononuclear phagocyte system was low (liver below 7% and spleen below 1.5% of the injected dose after 24 h). The 1:1 conjugation of MTX to RSA did not decisively alter the pharmacokinetic properties nor the tumor or tissue distribution of the native carrier protein RSA. In the second part of the study the different properties of the MTX(1)–RSA conjugate were compared with MTX *in vivo*. About 2 mg MTX/kg body weight either of the drug conjugate or of the original drug were injected after being additionally spiked with radiolabeled tracers. Plasma concentrations were simultaneously determined by immunological and radioactive means. After 24 h about 12% MTX(1)–RSA was found in circulation compared to 0.03% MTX. Favorable tumor accumulation rates of about 14% were achieved for MTX(1)–RSA versus 0.04% for MTX. About 45-fold more of the injected dose of [^{3}H]MTX accumulated in the liver as compared to the tumor (1.5 versus 0.03%) after 24 h. Conjugation of MTX to RSA reversed this ratio in favor of the tumor to 1:1.4 (13.6 versus 9.6%). In conclusion, the potential therapeutic benefit of the MTX(1)–RSA conjugate lies in its very long tumor exposure time and its improved tumor accumulation rate compared to conventional MTX. In addition the conjugation to albumin might enhance the therapeutic effects over those achieved by long-term continuous infusion of MTX, as MTX(1)–RSA enters the cells by a different uptake mechanism. This might also help to circumvent MTX resistance mechanisms, such as a reduction in folate receptor numbers or impaired MTX polyglutamylation.

Key words: Drug carriers, methotrexate, pharmacokinetics, pharmacology, 256 carcinosarcoma Walker, residualizing labels, scintigraphy.

Introduction

After i.v. administration, low molecular weight lipophilic drugs are predominately trapped by the liver, while hydrophilic drugs are rapidly cleared from circulation by the kidneys. In both cases tumor exposure time for conventional chemotherapeutic drugs is decisively reduced. In addition, the high interstitial pressure found in solid tumors does not favor accumulation of low molecular weight drugs in the tumor, instead the administration of low molecular weight drugs results in preferential distribution to normal tissues.[1] The concept to improve the unfavorable pharmacokinetic properties of low molecular weight drugs ('toxophore') by using a drug carrier system ('heptophore') was originally put forward by Paul Ehrlich about 90 years ago. Unfortunately, a variety of obstacles stands between the drug carrier complex present in circulation and the ultimate target area of the drug inside tumor cells. Barriers to surmount are, for example, the endothelial cell lining, the basal lamina, the cells of the mononuclear phagocyte system, hepatobiliary removal or excretion by the kidneys, and membrane systems of the target cells.[2–5]

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A simple and lucid way to achieve tumor accumulation of a drug is to use the most abundant serum protein in animals and man, albumin, as a drug carrier.[6] Albumin can readily leave the vascular system by transcytosis. The native albumin molecule is not doomed for untimely degradation by specific receptor-mediated uptake in the mononuclear phagocyte system or by hepatocytes as no sugar side chains have been found on the molecule. A long biological half-life of albumin, amounting to 2.2 days in the rat and to about 19 days in man, favors tumor accumulation. Proliferating cells are able to incorporate albumin by fluid phase endocytosis and to degrade the carrier in their lysosomal compartment, intracellularly liberating the conjugated drug. Native albumin is neither immunogenic nor excreted by the kidneys. It is nontoxic, biodegradable and easily available. Albumins resist denaturation by chemical agents or heat more than other serum proteins, allowing efficient virus inactivation and offering promising conditions for the conjugation of drugs. Daily administration of more than 40 g albumin in man (about 10% of the albumin pool) is feasible, allowing adequate dosage of drug conjugates. An excellent up to date review on the properties of albumin is available.[7] We reported that tumors are a major catabolic site of albumin in rats bearing Walker 256 carcinosarcomas, ovarian 342 tumors and Novikoff 186 hepatomas.[8-10] There is evidence that plasma proteins, which are dominated by albumin, play an important role in the nitrogen and energy metabolism of tumors *in vivo*.[11] Based on these observations we re-evaluated the conception to employ serum albumin as a carrier for covalently linked chemotherapeutic drugs like methotrexate (MTX).[6] Methotrexate-rat serum albumin (MTX(1)-RSA) conjugates were prepared with loading rates ranging from 1 to 20 mol MTX per 1 mol albumin. Loading ratios in excess of 3 mol of MTX per 1 mol albumin lead to a reduced tumor uptake. Only loading rates close to 1:1 resulted in optimal tumor targeting of the conjugates.[6]

In the first part of this study we will show that MTX(1)-RSA enjoyed the same pharmacokinetic properties, and tissue and tumor distribution as native RSA. In the second part the altered pharmacokinetics of the MTX(1)-RSA conjugates will be compared to original MTX.

Material and methods

Reagents

RSA and MTX were purchased from Sigma (Deisenhofen, Germany). Methotrexate sodium salt [3′,5′,7-^3H(N)]MTX was obtained from Moravek Biochemicals (Brea, CA). For solubilizing the tissues and the blood samples containing [^{3}H]MTX, we used Beckman Tissue Solubilizer (BTS-450). READY-SOLV NA was used as scintillation cocktail from Beckman Instruments (Irvine, CA). For the immunologic determination of methotrexate the EMIT® MTX Assay (Syva Company, San Jose, CA) was used. ^{111}In was bought from Du Pont de Nemours (Bad Homburg, Germany). Centricon ultrafiltration units from Amicon (Witten, Germany) were used for separation of the compounds. All products for tumor cell culture were delivered by Gibco/BRL (Eggenstein, Germany): standard RPMI 1640 medium, fetal calf serum (FCS), penicillin-streptomycin solution (PenStrep), phosphate buffered saline (Dulbecco's PBS), trypsin-EDTA and Hank's balanced salt solution (HBSS).

Preparation of the MTX–RSA conjugates and radiolabeling of the conjugates

MTX-RSA conjugates were prepared according to a recently published procedure.[6] Dicyclohexylcarbodiimide (DCC) and *N*-hydroxy-succinimide (HSI) were used for activating and linking MTX to RSA. To achieve a loading rate of approximately 1 mol MTX per 1 mol RSA, 15 mg MTX was added to 1.5 g RSA. The binding rate of MTX to RSA was calculated after a photometric determination of the amount of unbound MTX in the filtrate. For the determination of the distribution patterns of MTX(1)-RSA in tumor bearing rats, the conjugates were tagged with a residualizing [^{111}In]DTPA label to RSA. The coupling of DTPA to RSA or MTX(1)-RSA was carried out using DCC and HSI for DTPA activation, as previously described.[10] Unbound reagents were separated by washing with 0.17 M sodium bicarbonate solution in a Centricon ultrafiltration C30 unit. For radiolabeling 185 MBq $^{111}InCl_3$ (5 mCi) was mixed with 5 µl of a 0.2 M sodium citrate solution, and then added to 10 mg DTPA-RSA or DTPA-MTX(1)-RSA dissolved in 1 ml 0.17 M sodium bicarbonate. Unbound tracer was separated by a Centricon ultrafiltration C30 unit. The labeling yield was about 97%. An analytical HPLC control run of the purified [^{111}In]DTPA-RSA or [^{111}In]DTPA-MTX(1)-RSA tracers showed impurities below 1%. The loading rate of the DTPA label was also adjusted to a molar ratio of approximately 1:1 with RSA. The conjugation technique did not lead to the formation of multimers of albumin. The HPLC profiles of radiolabeled [^{111}In]DTPA-RSA and of [^{111}In]DTPA-MTX(1)-RSA matched the profile of RSA that had

been obtained before the chemical modification by MTX or DTPA conjugation.

Animal studies

Female Sprague Dawley (SD) rats, weighing 200-250 g, were obtained from Zentrale Versuchstieranstalt (Hannover, Germany). The rats were kept under standard living conditions in the Central Animal Laboratories of the German Cancer Research Center, Heidelberg. The animal experiments had been approved by the German Federal Government (Regierungspräsidium Karlsruhe AZ 72/1994 and 100/1995 to GS). As a tumor model the Walker 256 carcinosarcoma was chosen. Walker 256 cells were obtained from the tumor cell bank of the German Cancer Research Center, Heidelberg. Detailed information on the cell culture procedures have recently been published.[10]

Pharmacokinetic experiments of [^{111}In]DTPA–RSA and [^{111}In]DTPA–MTX(1)–RSA in tumor bearing rats

Twenty female SD rats received an intramuscular injection of about 3×10^6 viable Walker 256 carcinosarcoma cells into their left hind leg. The experiments were started when the tumors had reached an estimated weight of about 5-8 g, or about 2-4% of the respective body weight of the rats. After randomization all rats received either i.v. injections of [^{111}In]DTPA-RSA (100 μCi = 3.7 MBq; 100 μg) or of [^{111}In]DTPA-MTX(1)-RSA (100 μCi = 3.7 MBq; 100 μg of the conjugate), dissolved in 300 μl bicarbonate buffer (0.17 M, pH 8.4). The animals were placed in the prone position on a high energy multihole collimator (420 keV) of a 10 inch γ-camera (Searle, Pho-Gamma IV). A computer system specially adapted to the γ-camera was used for the on-line evaluation of the data (Gaede Medworker; Gaede, Freiburg, Germany). To study the distribution of the tracer substance in the animals, static images (5 min) were registrated 5 min, and 1, 4, 8, 24, 48 and 72 h after tracer injection. The regions of interest (ROI) were marked, and the content of radioactivity in heart, liver, kidneys, urinary bladder, the tumor and the carcass was evaluated. The percentage of activity present in each ROI was calculated from the counts of that area in relation to the whole body count rate based on the initial scintigram performed 5 min after tracer injection. Throughout the experiments the rats were anesthetized by a mixture of halothane, N_2O and O_2 (1.5%/60%/38%). All substances were administered by an i.v. injection into a lateral tail vein. At 1, 4, 8, 24, 48 and 72 h after tracer administration triplicate blood samples (20 μl per sample) were drawn after incising the tail tip. A reference curve was prepared using triplicate serial dilutions of the tracer substance. After each blood sampling procedure the initial standard tracer dilution was recounted to adjust for the radioactive decay of ^{111}In. The equation 'blood volume (ml) = 0.06 × body weight (g) + 0.77' was used to estimate the blood volume of the rats.[12] From these data the percentage of the injected radioactivity present in the blood at different times was calculated. The blood loss of the animals was less than 1 ml or below 5% of the respective total blood volume. After the final scintigraphy and blood sampling five animals were sacrificed either after 24 or 72 h post-injection, and the organs and the tumor were removed. During 72 h the body weight share of the tumor had increased to about 6.5% due to tumor growth and the rats did not yet show signs of cachexia. After determination of the weight, the radioactivity of the samples was measured in a γ-counter. The results were expressed as percent of radioactivity uptake per organ initially injected to the animal. The specific uptake of radioactivity of the organs was calculated from the percentage of the radioactivity per organ divided by the body weight share in percent of the respective organs. For example a specific uptake rate of 2 for a tumor means that four times the amount of activity was found compared to a specific uptake of the carcass of 0.5.

Pharmacokinetic experiments of MTX(1)–RSA and conventional MTX in tumor bearing rats

Thirty rats bearing tumors of about 5 g were randomly divided into two groups. The 15 rats of the first group received an i.v. injection of the MTX(1)-RSA conjugate (2 mg/kg body weight based on the amount of MTX conjugated to RSA). This solution was spiked with 100 μg of [^{111}In]DTPA-MTX(1)-RSA (3.7 MBq, 100 μCi) for later determination of the radioactivity. The remaining 15 rats received 2 mg/kg body weight of conventional MTX, containing 0.37 MBq [^{3}H]MTX (10 μCi). The mixture of radioactive and of 'cold' material was chosen to allow for simultaneous determination of blood pharmacokinetics by radioactive and immunological means. The same doses of MTX had shown anti-tumor effects in the carrier-bound and original form.[13] After 5 min, and 1, 4, 8 and 24 h, three rats of the MTX(1)-RSA group were sacrificed. After 5, 15

and 30 min and 1 and 24 h, three rats of the MTX group were killed. Blood, tumor and major organs were removed. For both groups the content of radioactivity (%) in plasma based on the initially administered amount was determined, as described above. In addition the amount of MTX or MTX(1)-RSA present in plasma was measured immunologically by the Syva EMIT MTX Assay. For the determination of conventional MTX standard dilutions were used (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 μmol/l MTX) as provided from Syva. The sheep antibody reactive to MTX of the EMIT MTX Assay showed cross-reactivity with MTX(1)-RSA conjugates. A new standard curve for MTX(1)-RSA was established using serial dilutions of samples with known MTX content (0.5, 0.8, 1.1, 1.4, 1.7 and 2.0 μmol/l MTX bound to RSA). Plasma concentrations of MTX and MTX(1)-RSA were determined and used for area under curve (AUC) calculations. Absolute amounts (percent of injected dose) of MTX and MTX(1)-RSA present in plasma and in whole blood were calculated, after taking the hematocrit into account, and demonstrating that neither conventional MTX nor MTX(1)-RSA conjugates displayed a binding affinity to blood cells. For determination of the tumor uptake rates, both legs of the rat were exarticulated. The tumor uptake of [^{111}In]DTPA-MTX(1)-RSA was calculated after measuring the difference in radioactivity between the tumor bearing and the tumor-free leg. For the determination of [^{3}H]MTX both legs were completely solubilized, using a Beckman Tissue Solubilizer BTS-450. Aliquots were counted in scintillation vials after addition of a LS cocktail. The tumor uptake rates of [^{3}H]MTX (percent initially injected dose) were estimated based on standard dilutions from the initially injected amount of radioactivity and by a calculation of the radioactive counts in tumor bearing and the tumor-free hind leg.

Statistics

For the descriptive analysis of the data mean values and standard deviation were calculated. Pharmacokinetic parameters like the AUC were determined using the computer program TOPFIT 2.0 (Thomae Optimized Pharmacokinetic Fitting Program for the PC).[14] The AUC was calculated employing the logarithmic trapezoidal rule. The exponential extrapolation of the first two data points to the time of administration was calculated. For determination of the total AUC (0 h-∞) the terminal elimination constant λ_z was used from the last point of the curve (pp. 2-11 to 2-62 in Heinzel *et al.*[14]).

Results

Pharmacokinetics and organ and tumor distribution of radiolabeled RSA and radiolabeled MTX(1)–RSA

Ten tumor bearing rats received an i.v. injection of [^{111}In]DTPA-RSA. Another 10 rats received [^{111}In]MTX(1)-RSA. Blood sampling and sequential scintigraphy was carried out for all 20 rats over a 24 h period. Five rats of each group were sacrificed after 24 h, the remaining five rats after 72 h. Radioactivity was determined in all samples. Blood pharmacokinetics are shown in Table 1. Over 72 h both radiolabeled compounds were present in blood at approximately the same percentage. After 24 h 15.7% of the initially injected RSA tracer and 13.4% of the MTX(1)-RSA tracer were detected, declining further to 6% after 48 h and to 4.5 or 3.2% after 72 h. The blood concentrations of the MTX(1)-RSA conjugate were slightly lower. After 72 h all major organs and the tumor were removed. In terms of absolute uptake rates, organs like the heart, lung, thymus and thyroid gland were negligible for both tracer substances. Although a considerable amount of tracer activity was found in the cutaneous tissues both specific activities calculated on body weight basis did not show high tracer accumulation rates significantly exceeding the specific tracer uptake of the remaining carcass (Table 2). The tumor area dominated tracer accumulation in all animals in terms of the absolute uptake rates (approximately 14%, based on the initially injected amount of tracer). About 25% of the total activity (at 72 h) still present in the rats was confined to the tumor area. The absolute uptake rates of the liver and of both kidneys ranged between 6 and 8% of the initially injected dose. The specific uptake rates of the

Table 1. Pharmacokinetics of radiolabeled RSA and of radiolabeled MTX(1)–RSA in blood

Time	RSA		MTX(1)–RSA	
(h)	Mean	SD	Mean	SD
1	65.7	5.5	56.8	3.6
4	48.5	5.8	41.0	3.0
8	36.3	3.9	30.1	1.9
24	15.7	1.7	13.4	0.9
48	6.9	0.5	6.1	0.9
72	4.5	0.1	3.2	0.4

Percentages of tracer presence in circulation are shown, based on the initially injected amount of [^{111}In]DTPA–RSA or [^{111}In]DTPA–MTX(1)–RSA in rats bearing Walker 256 carcinosarcomas over a period of 72 h (3.7 MBq tracer was administered; tumors sharing approximately 5–7% of the body weight; $n = 10$ for each group).

tumor region (2.2) exceeded the background rates (0.3) by about seven times and was higher than that of the liver (1.5). Although the kidneys are known to play only a minor role in albumin catabolism, high specific uptake rates after 72 h were found. These uptake rates reflect the role of the kidneys in recycling peptide fragments. The role of the spleen was negligible in the absolute amount of tracer uptake (rates from 0.6 to 0.9% of the injected amounts). The specific activity ranged from 1.5 to 1.9, being slightly higher than that of the liver tissue. The data presented in this first section show that the MTX(1)-RSA conjugate behaved *in vivo* like native RSA. Considerable tumor accumulation was detected. The uptake rates for both tracers by the liver and the spleen, which possess a highly active mononuclear phagocyte system, were low in absolute and in relative terms for RSA and MTX(1)-RSA tracers.

Pharmacokinetics, and organ and tumor distribution of MTX(1)–RSA compared to conventional MTX

For this experiment 30 tumor bearing rats were randomly distributed among two groups. The first group received an i.v. injection of 2 mg MTX/kg of MTX(1)-RSA, containing a share of 3.7 MBq [^{111}In]DTPA-MTX(1)-RSA (100 μCi). The second group received 2 mg/kg of conventional MTX spiked with 0.37 MBq [^{3}H]MTX (10 μCi). Three animals of each group were sacrificed at a time, the organs, the tumor and the blood were removed, and MTX was determined immunologically in plasma by an enzyme immunoassay (EMIT MTX Assay) or by radioactivity.

Table 2. A comparison of tissue and tumor uptake rates (%) of the radiolabeled native carrier protein ([^{111}In]-DTPA–RSA) and of its MTX derivative ([^{111}In]DTPA–MTX(1)–RSA) in rats bearing a Walker 256 carcinosarcoma

	RSA		MTX(1)–RSA	
	Mean	SD	Mean	SD
Tumor				
% upt	14.22	2.10	14.84	2.01
% bwt	6.52	0.60	6.63	1.04
upt/bwt	2.19	0.31	2.25	0.22
Kidneys				
% upt	6.76	0.24	7.76	0.53
% bwt	0.98	0.06	1.07	0.07
upt/bwt	6.90	0.19	7.32	0.86
Liver				
% upt	6.38	0.39	6.17	0.55
% bwt	4.15	0.32	4.62	0.15
upt/bwt	1.54	0.12	1.34	0.12
Carcass				
% upt	26.96	3.76	23.01	3.62
% bwt	81.20	0.80	80.83	1.32
upt/bwt	0.33	0.05	0.28	0.04

The organs were removed after 72 h (% upt = tracer uptake in percent, based on the initially administered amount; % bwt = share of the body weight in percent; upt/bwt = specific tracer uptake per percent of the body weight; mean, SD, $n = 5$).

Table 3. Plasma concentrations (μmol/l) of MTX after i.v. injection of MTX(1)–RSA conjugates or of conventional MTX over a period of 24 h

Time (h)	MTX(1)–RSA (μmol MTX/l)		Conventional MTX (μmol MTX/l)	
	Mean	SD	Mean	SD
0.08	85.85	3.90	12.32	0.84
0.25			3.32	0.41
0.50			1.80	0.17
1.00	64.91	3.13	0.44	0.17
4.00	46.85	2.41		
8.00	37.38	1.55		
24.00	17.87	0.97	0.01	

All rats received an injection of 2 mg MTX/kg body weight either of the free drug or of MTX conjugated to RSA. The plasma concentrations were measured immunologically in triplicates by the EMIT MTX Assay. At each time three rats were sacrificed.

Already after 5 min significant differences in the concentrations were obvious (86 mol/l MTX(1)-RSA versus 12 μmol/l conventional MTX as determined by the EMIT test). After 1 h the respective concentrations were 65 μmol/l MTX(1)-RSA versus 0.44 μmol/l for MTX, and 18 to less than 0.01 μmol/l after 24 h (Table 3). AUC calculations were performed for MTX(1)-RSA conjugates and for conventional MTX to compare the relative plasma availability of the conjugates in relation to conventional MTX (Table 4). The area calculations for different periods of time showed a decisive increase in the plasma MTX(1)-RSA compared to that of conventional MTX ranging from 94- to 127-fold. In addition, the percentage of MTX(1)-RSA and MTX present in blood was calculated from the immunologically measured concentration values. The completely different pharmacokinetic behavior of MTX(1)-RSA and of conventional MTX required the use of logarithmic scaling for illustration (Figure 1). After 1 h a magnitude of 3 less conventional MTX was found in blood compared to MTX(1)-RSA. After 24 h about 14% of the MTX(1)-RSA was present in circulation compared to 0.01% of MTX tracer. The amounts of MTX or MTX(1)-RSA in blood, as measured immunologically, were mostly in agreement with the data based on the presence of [^{3}H]MTX or of [^{111}In]DTPA-MTX(1)-RSA tracers (Table 5 and Figure 2). Five minutes after injection only about 5% of the initially administered amount of [^{3}H]MTX was still present in

Table 4. AUC calculations after i.v. injection of MTX(1)–RSA conjugates or conventional MTX over a period of 24 h

Time	MTX(1)–RSA		Conventional MTX		Factor
	AUC (μmol h/l)	AUC (%)	AUC (μmol h/l)	AUC (%)	
0 to 5 min	6.95	0.60	1.36	14.96	
5 min to 24 h	847.45	73.32	7.70	84.57	110.0
0 min to 24 h	854.40	73.92	9.06	99.53	94.2
24 h to ∞	301.39	26.08	0.04	0.47	
0 min to ∞	1155.79	100.00	9.11	100.00	126.8

Both groups received injections equal to 2 mg MTX/kg body weight either of MTX(1)–RSA or of conventional MTX. The plasma concentrations (μmol/l) of MTX(1)–RSA and of MTX were determined using the EMIT MTX Assay. The AUC was calculated employing the logarithmic trapezoidal rule. The exponential extrapolation of the first two data points to the time of administration was calculated. For determination of the total AUC (0 h to ∞) the terminal elimination constant λ_z was used from the last point of the curve (TOPFIT 2.0, Thomae Optimized Pharmacokinetic Fitting Program for the PC).

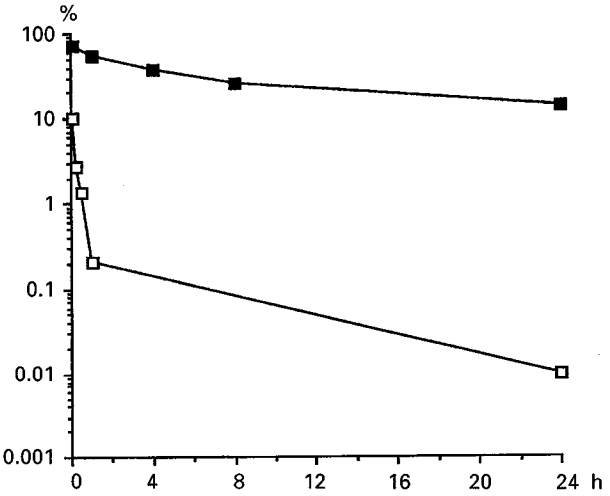

Figure 1. Pharmacokinetics of MTX(1)–RSA (■) and of conventional MTX (□) in blood. The percentages of the initially injected amount of MTX are given based on immunological determination (EMIT MTX Assay) over 24 h. Three rats bearing a Walker 256 carcinosarcoma had been killed at a time.

circulation. In contrast, almost 70% of [^{111}In]DTPA-MTX(1)-RSA was detected in the blood pool. After 24 h the difference between MTX(1)-RSA and MTX in the circulation was 400-fold as calculated by radioactivity.

These differences in blood pharmacokinetics were also reflected by the tumor uptake rates (Table 6). After 1 h about 4% of the injected dose of the [^{111}In]DTPA-MTX(1)-RSA conjugate was detected in

Table 5. Comparison of the radioactivity present in blood after injection of [^{111}In]DTPA–MTX(1)–RSA or after conventional [^{3}H]MTX in rats bearing a Walker 256 carcinosarcoma over 24 h

Time	[^{111}In]MTX(1)–RSA (%)		MTX(1)–RSA (EMIT; %)	
(h)	Mean	SD	Mean	SD
0.08	68.67	4.12	73.07	7.79
1.00	52.13	2.27	54.83	4.05
4.00	33.80	1.31	37.87	2.29
8.00	22.37	0.40	25.33	1.89
24.00	11.60	1.47	14.23	0.67
	[^{3}H]MTX (%)		MTX (EMIT; %)	
	Mean	SD	Mean	SD
0.08	4.65	0.81	9.89	0.71
0.25	1.03	0.20	2.70	0.40
0.50	1.07	0.34	1.33	0.12
1.00	0.05	0.02	0.21	0.18
24.00	0.03	0.04	0.01	0.01

The percentages are calculated either on the basis of the initially injected amount of radioactivity or the injected amount of MTX/MTX(1)–RSA (2 mg/kg body weight MTX) as measured by the EMIT MTX Assay.

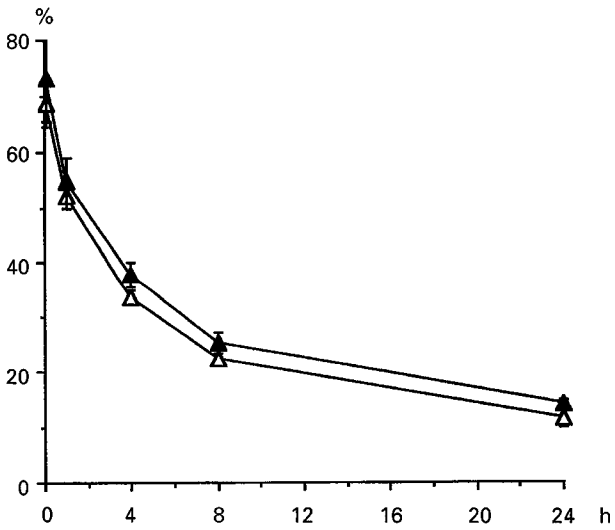

Figure 2. Comparative pharmacokinetics of MTX(1)–RSA in blood. The percentages of the initially injected amount of MTX(1)–RSA are given based on immunological determination (EMIT MTX Assay; ▲) and measured by radioactivity (^{111}In; △) over 24 h. Three rats bearing a Walker 256 carcinosarcoma had been killed at a time.

the tumor area. For conventional [^{3}H]MTX tumor accumulation rates of only 0.1% of the injected dose were measured (40 times less than for MTX(1)-RSA). After 24 h the [^{3}H]MTX uptake rates of the three rats were 0.03% (tumor 2.1 g wet weight), 0.02% (tumor

2.0 g) and 0.08% (tumor 2.9 g) of the initially injected dose of approximately 0.4 mg (2 mg MTX/kg body weight). From these data it can be estimated that approximately 40, 57 and 110 ng MTX/g tumor was trapped in tumor tissue, provided that the [^{3}H]MTX tracer had remained stable *in vivo*. An uptake of about 12.5 μg MTX/g tumor was estimated for MTX(1)-RSA, provided that the radioindium label would reflect the MTX presence, which it did in blood (Table 5).

Apart from renal excretion a considerable amount of conventional MTX is metabolized by the liver. After 5 min about 24% of the injected amount of [^{3}H]MTX was hepatically confined (Table 7). At that time about 8% of the injected amount of MTX(1)-RSA was found in the liver. After attaining an early peak of about 24% [^{3}H]MTX disappeared rapidly from the liver thereafter (5.1% after 30 min, 2% after 1 h). After 24 h 1.8% of the injected amount of the [^{3}H]MTX radioactivity was still trapped by the liver compared with 9.6% for [^{111}In]DTPA-MTX(1)-RSA. The conjugation of MTX to RSA caused a considerable shift in distribution rates between the tumor and the liver. After 24 h about 45-fold more [^{3}H]MTX-derived radioactivity was located in the liver compared to the tumor. Conjugation to albumin reversed this ratio in favor of the tumor.

Table 6. Tumor uptake rates (percent initially injected dose) of [^{111}In]DTPA–MTX(1)–RSA or [^{3}H]MTX in rats bearing a Walker 256 carcinosarcoma over 24 h

Time	[^{111}In]MTX(1)–RSA		[^{3}H]MTX	
(h)	Mean	SD	Mean	SD
0.08	1.27	0.18		
1.00	4.15	0.97	0.11	0.03
4.00	9.21	1.58		
8.00	9.39	2.29		
24.00	13.57	1.96	0.04	0.03

Table 7. Liver uptake rates (percent of the initially injected dose) of [^{111}In]DTPA–MTX(1)–RSA or [^{3}H]MTX in rats bearing a Walker 256 carcinosarcoma over a 24 h period (at each time three rats were sacrificed and the liver removed for determination of radioactivity)

Time	[^{111}In]DTPA–MTX(1)–RSA		[^{3}H]MTX	
(h)	Mean	SD	Mean	SD
0.08	8.12	0.46	23.71	1.66
0.25			11.31	1.82
0.50			5.13	0.55
1.00	9.54	1.53	2.03	0.44
4.00	9.24	0.48		
8.00	12.67	1.09		
24.00	9.58	0.51	1.81	0.56

About 1.4-fold more radioactivity derived from [^{111}In]DTPA-MTX(1)-RSA tracer was found in tumor (weight approximately 6% of total body weight) compared to the liver (weight approximately 4.5% of total body weight)

Discussion

Linking chemotherapeutic drugs to a macromolecular carrier system may enhance tumor targeting, improve solubility of lipophilic chemotherapeutic drugs, reduce toxicity and overcome drug resistance mechanisms.[5] As an elementary model to evaluate the pharmacological properties of macromolecular drug carrier systems we chose RSA for carrier, MTX as antineoplastic drug and a 1:1 loading rate. A residualizing radioactive protein label was used for mapping *in vivo* the catabolic sites of the native carrier and of the drug conjugate. Residualizing radiolabels remain lysosomally trapped at the sites of protein degradation with *in vivo* half-lives of several days.[15-18] We have recently evaluated the properties of a [^{131}I]tyramine-deoxisorbitol and an [^{111}In]DTPA residualizing radiolabel attached to native albumin.[10] Both labels showed similar tumor accumulation rates and comparable body retention rates over 72 h. [^{111}In]DTPA-albumin was preferred for this study not only due to its advantage for metabolic studies (better γ detection), but also in view of conducting future patient studies.

Basic evaluations of drug carrier systems should include an experiment comparing the tissue distribution of the original carrier protein and of the later drug carrier conjugate. In our experience SDS-PAGE electrophoresis, gel permeation chromatography or HPLC analysis might not suffice to claim that the chemical linking of a drug to its carrier protein did not effect the integrity of the conjugate. In the first part of this study we compared the blood circulation rates, and the tissue and the tumor uptake of residualizingly labeled [^{111}In]DTPA-RSA and [^{111}In]DTPA-MTX(1)-RSA. DTPA and MTX had been linked to RSA by using the free ε-amino groups of lysine residues present on the RSA molecule. MTX(1)-RSA was prepared first and then radiolabeled with [^{111}In]DTPA. The presented data show that there are on an average about three lysine residues available for attaching drugs or labels to albumin without damaging substantial amounts of the carrier protein. Radiolabeled RSA and MTX(1)-RSA displayed similar circulation rates with about 15% of the injected dose present after 24 h and about 4% after 72 h. After 72 h the uptake rates by the Walker 256 tumors (14%), the kidneys and the liver were comparable for both tracers, as well. The kidneys

took up about 7% of the injected dose and showed the highest specific activity (about 7) of all tissues studied. Catabolites, such as [^{111}In]DTPA-lysine which constitutes the major lysosomally trapped compound, will slowly leak from degradation sites and will subsequently be trapped in the kidneys, as recent experimental evidence suggested.[19,20] Analysis of urine and feces demonstrated that the main excretory product of an ^{111}In-labeled antibody and its Fab fragment was [^{111}In]DTPA-ε-lysine.[17] Although high specific uptake rates by the kidneys were found for RSA and MTX(1)-RSA alike, a subacute toxicity study of MTX-HSA carried out in mice did not reveal any nephrotoxicity after a cumulative dose of up to 20 mg MTX/kg body weight bound to albumin over an 8 week period (personal communication, HH Fiebig, Tumor Biology Clinic, Freiburg). In an ongoing phase I clinical trial 30 and more repeated injections of MTX-HSA (50 mg MTX/m^2) were carried out in some patients over more than 12 months and were tolerated without any signs of renal impairment.[21]

The liver and the spleen are major sites of the mononuclear phagocyte system formed primarily by hepatic Kupffer cells and fixed splenic macrophages. One of its functions is to clear circulating colloids, bacteria, viruses and foreign or denatured proteins. Fragments of potential antigens are recycled to the macrophage cell surface by MHC class II transport proteins and are potentially immunogenic. For achieving optimal therapeutic efficacy it is essential for a drug conjugate not to be trapped by the mononuclear phagocyte system. Antigenicity caused by the conjugate or damage to the mononuclear phagocyte system might result from entrapment.[22] Recently we have demonstrated that by increasing the molar load of MTX to the carrier protein from 1 up to 20, the mononuclear phagocyte system was preferentially targeted at the expense of the tumor tissue.[6] MTX-RSA conjugates bearing on average 10 MTX molecules were removed from circulation by members of the scavenger receptor family on macrophages as identified by competition experiments with the receptor ligand maleylated bovine serum albumin.[6] The rapidly increasing tracer accumulation rates in the liver and the spleen after conjugation of 5, 10 or 20 mol MTX per 1 mol RSA are shown in Table 8. In contrast, these high specific uptake rates by liver and spleen were not observed for the MTX(1)-RSA or RSA tracer used in this study. Only about 6% of the injected doses of RSA and MTX(1)-RSA tracers were found in the rat livers and less than 1.5% in the spleens. The specific uptake rates were below that of the tumor tissue (1.5 versus 2.2).

Conventional MTX disappeared rapidly from circulation after i.v. administration.[23,24] After 30 min only about 1% of the injected dose of MTX remained present in circulation compared to more than 50% of the injected dose of MTX(1)-RSA. All plasma concentrations had been determined independently by using either radioactivity or an enzyme immunoassay. About 0.01-0.03% of MTX was found in the circulation after 24 h, whereas 11.6-14.3% of MTX(1)-RSA was detected. Compared to conventional MTX, an increase in the relative plasma presence for MTX(1)-RSA was achieved by factors ranging from 94 to 127, as determined by an enzyme immune assay method (EMIT MTX Assay) and AUC evaluation. The data measured from the same samples by radioactivity or immunologically were in good agreement over a 24 h period, confirming that most of the circulating MTX remained bound to the carrier. Due to the rapid *in vivo* elimination rates of the small amounts of MTX, being released from the conjugate after intracellular degradation, unbound MTX might not play a decisive role in the pharmacokinetics of the conjugate.

Conventional MTX shares the fate of other low molecular weight anti-cancer drugs, that are rapidly removed from blood by either the kidneys, the liver or the fatty tissue, resulting in a low overall uptake rate in solid tumors due to the very short exposure time. Although MTX has been studied by many scientists since the early 1940s and a detailed picture of its antineoplastic action *in vitro* has been drawn, data on the absolute uptake rates of conventional MTX obtained in solid tumor tissue are scarce.[25-30] We determined the tumor uptake rates of MTX in percent of the initially administered amount for rats bearing Walker 256 carcinosarcomas using conventional [^{3}H]MTX. Walker 256 carcinosarcoma shows an extremely high tumor doubling rate and displays high metabolic activity. The uptake of MTX tracer into these tumors was small (0.11% of the injected dose after 1 h, and 0.04% after 24 h). Therefore, we did not attempt to check the [^{3}H]MTX tracer by HPLC after

Table 8. Comparison of specific tracer uptake rates of radiolabeled native [^{111}In]DTPA–RSA and of [^{111}In]-DTPA–MTX(x)–RSA conjugates (x=1, 5, 10 or 20 mol MTX per 1 mol RSA) by organs with a highly active monocyte macrophage system [mean ± SD, n=5 for RSA and MTX(1)–RSA, and n=3 for MTX(5, 10, 20)–RSA]

	Liver	Spleen
[^{111}In]DTPA–RSA	1.75 ± 0.15	1.99 ± 0.06
[^{111}In]DTPA–MTX(1)–RSA	1.64 ± 0.16	2.20 ± 0.60
[^{111}In]DTPA–MTX(5)–RSA	3.68 ± 0.28	3.91 ± 0.36
[^{111}In]DTPA–MTX(10)–RSA	7.73 ± 0.88	4.55 ± 0.39
[^{111}In]DTPA–MTX(20)–RSA	12.55 ± 0.67	5.20 ± 0.87

tissue solubilization. The 3′ and 5′ positions of ^{3}H at the phenyl part and the 7 position at the pteridine ring in the MTX tracer molecule make rapid exchange of tritium with hydrogen unlikely. From this data it was estimated that approximately 40–110 ng MTX/g tumor was trapped after 24 h. These estimated low uptake rates from three tumor bearing animals is supported by data from Kipp *et al.* In rats bearing a R-1 rhabdomyosarcoma about 120 ng MTX/g tumor was detected by HPLC, 24 h after administration of three times 10 mg MTX/kg.[30] MTX was predominately excreted by the kidneys with 1.8% of the injected tracer radioactivity left in the liver after 24 h. About 45-fold more of the remaining MTX tracer radioactivity was found in the liver compared to tumor tissue. The conjugation to albumin shifted this ratio in favor of the target tissue. The tumor accumulated about 1.5 times more of the conjugate tracer as compared to the liver.

In conclusion, the conjugation of MTX to RSA at an approximate 1:1 molar ratio did not decisively alter the pharmacokinetic properties of the native drug carrier. A preferential uptake of these drug conjugates by organs with active mononuclear phagocyte systems was not observed. Signs for immunogenicity of these conjugates were not noticed. Favorable tumor accumulation rates compared to healthy non-target tissues were achieved by the prolonged presence of the MTX(1)-RSA conjugate in circulation. Long plasma circulation rates were also observed after the administration of MTX-HSA in the phase I patient trial. Half-lives of about 15–18 days *in vivo* were determined by the EMIT MTX test, comparable to the known half-life of 19 days of native human serum albumin.[21,31]

The potential therapeutic benefit of MTX(1)-RSA conjugates lies in its very long tumor exposure time and its improved tumor accumulation rates compared to conventional MTX. In addition conjugation to albumin might enhance the therapeutic effects of MTX over those achieved by long-term continuous infusion, as MTX(1)-RSA enters the cells by a different uptake mechanism. This might also help to circumvent MTX resistance mechanisms, such as a reduction in folate receptor numbers or impaired MTX polyglutamylation.

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