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Preclinical study

Albumin-based drug carriers: comparison between serum albumins of different species on pharmacokinetics and tumor uptake of the conjugate

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Albumin-based drug carrier systems have been developed in the field of chemotherapy to improve the passive tumor targeting properties of anti-cancer drugs. Usually, serum albumins of different species are used as carrier proteins, mostly of bovine (BSA), human (HSA) or rat (RSA) origin. The resulting albumin conjugates are often tested for anticancer activity in heterologous tumor models. No data is available whether the choice of the albumin species might influence the pharmacokinetics or the tumor uptake rates of the conjugates in vivo. Residualizingly ([^{111}In]DTPA) radiolabeled RSA, BSA or HSA were administered to Walker-256 carcinoma-bearing rats. No significant difference was found in the absolute or the weight-adjusted tumor uptake rates of the three albumin tracers. The tumors were the major catabolic sites accumulating 14–18% of the injected dose (ID). Low hepatic uptake rates were determined for all albumins (below 10% ID). Minor differences were found for hepatic uptake in favor of the autologous RSA (5.8% ID) versus HSA (6.9%) and BSA (8.0%). These differences might have occurred during the commercial preparation or the radiolabeling of the different batches. In addition, there are structural differences between the three albumins, which might have contributed, despite high sequence homologies above 70% for RSA, HSA and BSA. These minor differences in the distribution patterns of RSA, HSA or BSA might not decisively influence the results of drug targeting experiments in rats. For further studies with albumin conjugates, HSA was chosen as drug carrier in rodent animal models when considering later human use. In rats or nude mice multiple injections of various HSA–drug conjugates were well tolerated without signs of allergy or anaphylaxis. [© 1999 Lippincott Williams & Wilkins.]

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Introduction

Macromolecular drug carrier systems have been developed in the field of chemotherapy to improve pharmacokinetics and tumor targeting of anti-cancer drugs. For passive tumor targeting albumin-drug conjugates are frequently used. Due to impaired lymphatic drainage, macromolecules like albumin accumulate to a considerable extend in tumor tissues.[1,2] After endocytosis by tumor cells, albumin is lysosomally catabolized and the resulting amino acids are used for *de novo* tumor protein synthesis.[3] Covalently attached anti-cancer drugs are lysosomally released and might then exert anti-cancer activity.[4,5] Albumins of different species are used as drug carriers, mostly bovine (BSA), human (HSA) or rat (RSA) serum albumins. The resulting albumin conjugates were then tested for anti-cancer activity in heterologous rat, rabbit, mouse or nude mouse tumor models (Table 1).

We have recently studied the pharmacokinetics and the therapeutic effects of a methotrexate (MTX)-RSA conjugate in rat tumor models.[18,19,22,23] During preclinical development of these conjugates nude mice models with human xenograft tumors were used as well. The question arose whether MTX-RSA, MTX-HSA or even mouse albumin conjugates would be appropriate in xenograft models. We chose MTX-HSA in view of the human origin of the xenograft tumors and a future therapeutic use of these conjugates in man.[24] Surprisingly, there were

Table 1. Survey of albumin-based drug conjugates

Author	Species	Conjugated drug	Animal model
Chu *et al.*[6]	BSA	methotrexate	mouse
Chu *et al.*[7]	BSA	methotrexate	mouse
Soriano *et al.*[8]	BSA	methotrexate	mouse
Bostik *et al.*[9]	HSA	methotrexate	mouse
Bures *et al.*[10]	HSA	methotrexate	mouse
Yoon *et al.*[11]	rabbit-SA	methotrexate	rabbit
Tanaka *et al.*[12]	BSA	mitomycin C	mouse
Okhawa *et al.*[13]	BSA	doxorubicin	rat
Gabor *et al.*[14]	BSA	doxorubicin	rat
Pommerenke *et al.*[15]	BSA	doxorubicin	mouse
Takahashi *et al.*[16]	BSA	doxorubicin	rat
Dosio *et al.*[17]	HSA	taxol	mouse
Stehle *et al.*[18]	RSA	methotrexate	rat
Wunder *et al.*[19]	RSA	methotrexate	rat
Kratz *et al.*[20]	HSA	doxorubicin	mouse
Kratz *et al.*[21]	HSA	chlorambucil	mouse
Stehle *et al.*[22]	RSA	methotrexate	rat

Bovine (BSA), rabbit, human (HSA) and rat (RSA) albumin were used as drug carrier for a variety of cytostatic agents. The resulting albumin–drug conjugates were often studied in a heterogenous animal model.

no data available, whether the choice of BSA, RSA or HSA as a carrier might influence the pharmacokinetics or the tumor targeting properties of drug conjugates. To address this issue, we compared the tissue and tumor distribution of residualizingly radiolabeled RSA, BSA and HSA fractions in Walker-256 (W-256) carcinoma-bearing rats.

Material and Methods

Reagents

RSA (A4835), HSA (A8763) and BSA (A7638) were purchased from Sigma (Deisenhofen, Germany). The albumins were essentially globulin free. All reagents like *N*-bromo-succinimide, diethylenetriaminepentaacetic acid (DTPA), dimethylsulfoxide (DMSO) and Trypan blue were delivered by Aldrich (Steinheim, Germany). Indium-111 was obtained from Du Pont de Nemours (Bad Homburg, Germany). For separation of the compounds Centricon ultrafiltration units from Amicon (Witten, Germany) were used. All products for tumor cell culture were delivered by Gibco/BRL (Eggenstein, Germany): standard RPMI 1640 medium, fetal calf serum, penicillin-streptomycin solution (PenStrep), phosphate-buffered saline (Dulbecco's PBS) and Hank's balanced salt solution.

Preparation of the [^{111}In]DTPA–albumin conjugates

The coupling of DTPA to the albumin (RSA, HSA or BSA) was carried out using dicyclohexylcarbodiimide (DCC) and hydroxysuccinimide (HSI) for DTPA activation. DTPA was dissolved in DMSO (20 mg/ml). Then a 1.5 molar amount of DCC and a 5 molar amount of HSI were added. After 12 h the formation of the succinimidylester of DTPA (DTPA-SE) was completed. The activated DTPA-SE was added to the albumin solution (20%). After 30 min of gentle stirring of the mixture, the remaining DCC and dicyclohexylurea were removed from DTPA-albumin by filtration (Millipore; Stericup-GV, 0.22 μm Low Binding Duropore Membrane). The low molecular weight hydrophilic compounds were removed by an Amicon Ultrafiltration unit (30 kD[9]). $^{111}InCl_3$, dissolved in 0.1 M hydrochloric acid, was mixed with 10–20 μl of a 0.2 M sodium citrate solution to form indium citrate complexes. This mixture was added to 10–20 mg DTPA-albumin, dissolved in 0.17 M $NaHCO_3$. The labeling took place immediately. Low molecular weight compounds like non-bound indium or citrate were removed by centrifugation using a Centricon C30 ultrafiltration unit. Intermolecular cross-linking of the purified [^{111}In]DTPA-albumin tracer was ruled out by an analytical HPLC run. The labeling yield was about 97%, impurities were below 1% and the specific activities ranged from 37 to 100 MBq/mg protein.

Animal study

W-256 carcinoma. The W-256 carcinoma cells were obtained from the tumor bank of the German Cancer Research Center, Heidelberg. The tumor cells were cultivated using a standard RPMI 1640 medium enriched with 10% (v/v) of heat-inactivated fetal calf serum, L-glutamine and PenStrep. Viable cells were counted after Trypan blue uptake. Female SD rats received an intramuscular injection of 3×10^6 viable W-256 cells into their left hind leg, diluted in HBSS to a volume of 200 μl. The experiments were started when the tumors had reached an estimated weight of about 8–10 g or about 3–4% of the respective body weight of the rats.

Sequential scintigraphy and organ removal. Thirty female tumor-bearing SD rats, weighing 200–250 g, were kept under standard living conditions. The animal experiments had been approved

by the German Federal Government (Regierungspräsidium Karlsruhe AZ 100/1995 to GS and AW). All animals were placed in a prone position on a multihole collimator of a 10 inch gamma camera (Searle-Siemens; Pho-Gamma IV). For the on-line evaluation of the data a computer system specially adapted to the gamma camera was used (Gaede Medworker; Gaede, Freiburg, Germany). To study the distribution of the tracer substances in the animals, static images (5 min) were registrated after 1, 4, 8, 24, 48 and 72 h after tracer injection. The regions of interest (ROI) were marked, and the content of radioactivity in the thyroid gland, heart, liver, kidneys, urinary bladder and the tumor was evaluated. Throughout the experiments the rats were anesthetized by a mixture of halothane, N_2O and O_2 (1.5%/60%/38%). All tracer substances were administered by an i.v. injection into a lateral tail vein. After 1, 4, 8, 24, 48 and 72 h blood samples (20 μl per sample) were drawn after incising the tail tip. The equation 'blood volume=0.06 × body weight+0.77' was used to estimate the blood volume of the rats.[25] From these data the percentage of the injected radioactivity present in the blood at different times was calculated. The blood loss of the animals during the experiments was less than 1 ml or below 4% of the respective total blood volume. After final blood sampling the animals were sacrificed after 24 or 72 h. All organs and the tumor were removed after careful desanguination. The following organs were examined: heart, liver, kidneys, spleen, lungs, stomach, intestines, colon, the thyroid gland, tumor and the remaining body (muscles, bone, skin). The results were expressed as percent of radioactivity uptake per organ based on the initially injected amount of radioactivity. The specific activity of the organs was calculated from the percentage of the radioactivity uptake per organ divided by the body weight share in percent of the respective organs. A specific uptake rate of 2 for a tumor means that 4 times the amount of activity was found compared to a specific uptake rate of the carcass of 0.5. Thirty tumor-bearing rats were randomly allocated among three groups and received either RSA, HSA or BSA tracer. About 3.7 MBq [^{111}In]DTPA-RSA/HSA/BSA (100 μCi) was dissolved in 300 μl bicarbonate buffer (0.17 M, pH 8.4) prior to administration. About 100 μg of the tracer proteins was administered. Five rats in each group were sacrificed after 24 h and the remaining five rats after 72 h. For the descriptive analysis of the data, means ± SD were calculated. The *t*-test was used to evaluate the differences between the mean values.

Results

Thirty W-256-bearing rats received i.v. injections of residualizingly [^{111}In]DTPA-labeled albumins of different species (RSA, HSA and BSA). Groups with 10 rats for each albumin preparation were formed. Blood kinetics of these albumins were monitored and compared over 72 h after injection. Throughout the first 8 h after administration, the RSA, HSA and BSA tracers did not show differences in blood presence (Table 2). After 24 h a slight shift in favor of the homologous RSA tracer (7.6% of the injected dose) was observed compared to heterologous HSA (5.5%) or BSA (4.9%). The individual blood values of the 10 rats of the RSA group were all above the respective values measured for HSA or BSA. This difference increased until after 72 h, when 5% of the initially injected RSA tracer activity was found in blood compared to 2.8% of the HSA tracer and 2.4% of the BSA tracer (once again all individual values of the RSA group were higher than those of the HSA or BSA groups) (Figure 1).

After 24 and 72 h five rats of the respective groups were killed. Organs and tumors were removed, and evaluated for tracer accumulation. The major catabolic sites for RSA, HSA and BSA tracer were the W-256 tumors (Table 3). The tumor burden ranged from 3.8 to 4.9% of the body weight after 24 h and from 5.5 to 6.5% after 72 h. About 15–17% of the injected dose of the albumin tracers accumulated in the tumor tissue after 24 h and 13–14% after 72 h. Neither the total uptake rates nor the specific tumor uptake showed significant differences between the RSA, HSA or BSA tracer (Table 4). The liver was also identified as a catabolic site for albumins. Significant species differences were found. The liver accumulation in all rats of the HSA or BSA groups exceeded those of the RSA

Table 2. Blood concentrations of different albumin preparations of rat, human or bovine origin (RSA, HSA and BSA) over 72 h

Time (h)	[^{111}In]DTPA–RSA	[^{111}In]DPTA–HSA	[^{111}In]DTPA–BSA
1	67.5 (2.3)	69.6 (4.2)	68.0 (2.4)
4	49.8 (3.2)	48.8 (3.0)	48.8 (2.0)
8	37.3 (2.3)	35.6 (2.4)	33.8 (2.3)
24	16.4 (1.4)[a]	13.9 (1.0)[a]	12.5 (1.4)[a]
48	7.6 (0.6)[a]	5.5 (1.1)[a]	4.9 (0.4)[a]
72	5.0 (0.1)[a]	2.8 (0.6)[a]	2.4 (0.3)[a]

All albumins were residualizingly radiolabeled with [^{111}In]DTPA. W-256 carcinoma-bearing rats received the albumin tracers by an i.v. injection. The percentage of tracer present was calculated based on the initially administered amount [n=10 for the first 24 h, and n=5 for 48 and 72 h, mean (± SD), [a] *t*-test, $p<0.01$].

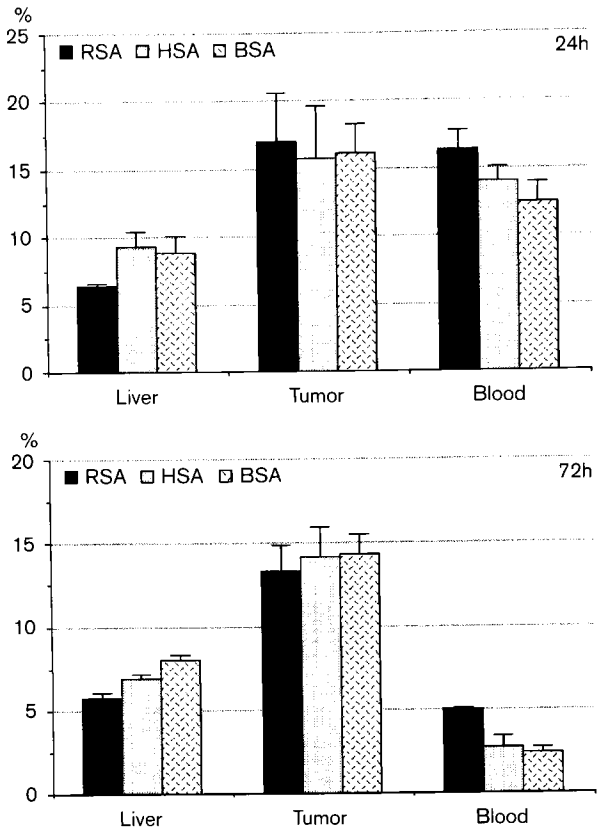

Figure 1. Organ and tumor uptake rates and circulating amount of three different radiolabeled albumin tracers (RSA, HSA and BSA) after 24 or 72 h. The percentages were calculated based on the initially administered amount of radioactivity (n=5 rats, mean).

group after 24 or 72 h (e.g. 9.3 or 8.8 versus 6.4%). The tracer uptake by the kidneys was uniform for all three albumin tracers ranging from about 4% of the injected dose after 24 to 6% after 72 h. The respective specific uptake rates were surpassing that of the tumor or the liver. The spleen accumulated less than 1% of the injected albumins. Significant differences were found after adjustment to organ weight. The specific uptake rates for RSA were 2.08, for HSA 2.56 and for BSA 3.18 after 72 h. After 24 h about 40% of the injected tracer was found in the carcass, about 20% confined to the skin and the remainder mostly to muscle tissue. The tracer loss due to excretion was below 25% after 24 h and about 40% after 72 h.

Discussion

For comparing the tumor targeting properties of different albumins, we selected a residualizing [^{111}In]DTPA protein label. After endocytosis and lysosomal digestion of the protein in target organs, the labels remain trapped at the catabolic sites. The catabolite responsible for intralysosomal accumulation of radioactivity after protein degradation was identified as [^{111}In]DTPA-ε-lysine, which was retained intracellularly with a half-life of several days.[26,27] For conjugating DTPA to the albumins we did not use the mixed anhydride procedure as inter- and intramolecular cross-linking, and tracer instability may occur with this technique. We chose a method based on the activation of DTPA by DCC and HSI for coupling DTPA to albumin. This technique permits a more selective conjugation and a molar loading rate of DTPA close to 1:1 without

Table 3. Organ and tumor uptake rates of different albumin preparations of rat, human or bovine origin (RSA, HSA and BSA) after 24 or 72 h

	[^{111}In]DTPA–RSA	[^{111}In]DTPA–HSA	[^{111}In]DTPA–BSA
After 24 h			
tumor	17.3 (4.6)	15.7 (3.8)	16.1 (2.2)
liver	6.4 (0.2)[a]	9.3 (1.0)[a]	8.8 (2.0)[a]
kidneys	4.3 (0.3)	4.3 (0.2)	4.3 (0.6)
spleen	0.8 (0.2)	0.9 (0.3)	0.9 (0.1)
lung	2.3 (0.5)	1.5 (0.1)	1.7 (0.2)
heart	0.4 (0.1)	0.4 (0.1)	0.3 (0.1)
intestines	5.4 (0.5)	5.1 (0.7)	4.8 (0.6)
carcass	38.2 (4.4)	39.3 (3.0)	40.7 (2.2)
loss	24.9	23.5	22.9
tumor weight (g)	11.3 (3.5)	8.9 (2.2)	9.6 (1.1)
tumor % bw	4.9 (1.5)	4.2 (1.0)	3.8 (0.3)
After 72 h			
tumor[a]	13.2 (1.5)	14.2 (1.8)	14.3 (2.2)
liver	5.8 (0.3)[a]	6.9 (0.3)[a]	8.0 (0.3)[a]
kidneys	6.1 (0.2)	6.2 (0.1)	6.0 (0.4)
spleen	0.9 (0.1)	0.9 (0.1)	1.0 (0.2)
lung	1.4 (0.5)	1.4 (0.1)	1.1 (0.2)
heart	0.2 (0.0)	0.2 (0.1)	0.2 (0.1)
intestines	6.1 (0.9)	4.0 (0.4)	4.9 (0.6)
carcass	25.2 (2.5)	24.9 (3.0)	25.7 (1.1)
loss	41.1	41.3	40.5
tumor weight (g)	13.8 (1.5)	12.5 (1.8)	12.6 (0.9)
tumor % bw	6.5 (1.5)	5.5 (1.0)	5.4 (0.5)

All albumins were residualizingly radiolabeled with [^{111}In]DPTA. W-256 carcinoma-bearing rats received i.v. injections of the albumin tracers. The percentage of tracer presence was calculated based on the initially administered amount. Five rats were sacrificed after 24 h and another five rats after 72 h. The tracer uptake rates of all major organs, the tumor, the carcass (including skin, bones and muscle) and the amount of missing tracer were evaluated. Tumor weight (g) and respective body weight share (% bw) of the tumors are also given [n=5, mean (±SD), [a]t-test, $p<0.01$].

Table 4. Specific uptake rates of different albumin preparations of rat, human or bovine origin (RSA, HSA and BSA) after 24 or 72 h

	[^{111}In]DTPA–RSA	[^{111}In]DTPA–HSA	[^{111}In]DTPA–BSA
After 24 h			
tumor	3.53 (0.25)	3.76 (0.34)	3.57 (0.43)
liver	1.58 (0.13)[a]	2.06 (0.21)[a]	2.12 (0.35)[a]
kidneys	5.03 (0.76)	4.58 (0.49)	4.97 (0.78)
spleen	1.99 (0.16)[a]	2.44 (0.37)[a]	2.77 (0.34)[a]
lung	3.99 (0.74)	2.95 (0.28)	3.39 (0.41)
heart	0.93 (0.16)	1.01 (0.12)	0.71 (0.16)
intestines	0.79 (0.09)	0.86 (0.18)	0.78 (0.12)
carcass	0.47 (0.05)	0.47 (0.03)	0.49 (0.03)
After 72 h			
tumor	2.06 (0.30)	2.49 (0.27)	2.65 (0.12)
liver	1.39 (0.11)	1.51 (0.13)	2.12 (0.18)[a]
kidneys	6.07 (0.13)	6.12 (0.51)	6.89 (0.21)
spleen	2.08 (0.24)	2.56 (0.44)	3.18 (0.54)[a]
lung	2.48 (1.08)	2.55 (0.37)	2.53 (0.31)
heart	0.51 (0.01)	0.53 (0.20)	0.61 (0.23)
intestines	1.05 (0.21)	0.71 (0.20)	0.83 (0.18)
carcass	0.32 (0.03)	0.31 (0.01)	0.30 (0.02)

All albumins were residualizingly radiolabeled with [^{111}In]DTPA. The percentage of tracer present was calculated based on the initially administered amount and adjusted to the respective weight. Five rats were sacrificed after 24 h and another five rats after 72 h. The tracer uptake rates of all major organs, the tumor, the carcass (including skin, bones and muscle) and the amount of missing tracer were evaluated [n=5, mean (±SD), [a]t-test, $p<0.01$].

protein cross-linking.[2] The tumor uptake rates, measured for residualizingly radiolabeled RSA, HSA or BSA in W-256 carcinoma, did not show any significant difference over 72 h. About 15% of the three injected albumin tracers accumulated in the W-256 carcinomas. All tumors were about the same size (12–14 g after 72 h). Neither the absolute tumor uptake rates nor the specific activity revealed any preferences for RSA, BSA or HSA in the rat. All tumors were the major catabolic sites for albumins in this study.

The liver was identified as another important catabolic site of albumin. The uptake rate was well below 10% of the injected dose during the experiment. Low uptake hepatic rates of albumin are also reasonable from a physiologic point of view. Up to 50% of the liver synthetic capacity is confined to albumin production by hepatocytes.[28] Major albumin catabolism by hepatocytes would generate a futile nitrogen and energy wasting cycle. High rates of albumin tracer accumulation by the liver are either due to tracer instability (e.g. accumulation of unchelated indium) or caused by denaturation of albumin during conjugation procedures. We have recently studied the impact of MTX loading rates on the integrity of albumin molecules in the same animal model. A loading rate of 5 mol MTX already doubled the hepatic uptake rate of the conjugate (12%) and an average loading rate of 10 mol MTX per albumin resulted in a hepatic uptake of more than 60% of the injected dose. Not the uptake by hepatocytes, but a macrophage scavenger receptor-mediated mechanism was identified as being responsible for liver accumulation of these polyanionic drug conjugates.[18]

Consequently, we consider low hepatic uptake rates of residualizingly labeled drug-albumin conjugates as an *in vivo* quality control to the integrity of the albumin molecule after the conjugation procedure. Interestingly, slight but statistically significant differences in the hepatic uptake rates between RSA (5.8%) and HSA (6.9%) or BSA (8.0%) were detected. This might also explain the lower blood concentrations observed in this study for HSA and BSA tracers after 24–72 h. These differences in uptake rates or blood concentrations were consistent in all individual rats from the respective groups. Several explanations for these findings might apply. Slight differences during the commercial preparation or the radiolabeling of the different batches might have occurred. In addition, although a high sequence homology exists between RSA, HSA (73% to RSA, 75.6% to BSA) and BSA (70% to RSA), there are differences between these albumins. At pH 7.5 a net negative charge of 12 was calculated for RSA, 15 for HSA and 17 for BSA. BSA differs from RSA and HSA due to an internal deletion of one amino acid at position 116. In addition, after biosynthesis albumins might undergo alterations *in vivo*. This is mostly caused by the formation of glucose adducts to albumins (about 1% of albumin) targeting these AGE-albumins to the hepatic macrophage system, as well. Another organ involved in the removal of altered proteins is the spleen. Although, the uptake rates for all albumin fractions ranged below 1% of the injected dose in the spleen, the weight adjustment of these samples consistently showed the lowest rates for RSA (1.99), intermediate for HSA (2.44) and highest for BSA (2.77). In our experience these slight differences in the hepatic or splenic distribution patterns of RSA, HSA or BSA did not decisively influence the results of drug targeting experiments in rats. In rats or nude mice multiple injections of HSA-drug conjugates were well tolerated without any signs for allergy. For further preclinical studies with albumin conjugates in rodents we chose HSA as drug carrier in view of later human use.

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