Anti-Cancer Drugs 2002, **13,** pp. 615–623

Report

Efficacy and tolerability of an aminopterin–albumin conjugate in tumor-bearing rats

Paul Kremer,[1] Gernot Hartung,[2] Ulrike Bauder-Wüst,[3] Hans-Hermann Schrenk,[3] Andreas Wunder,[3] Stefan Heckl,[4] Uwe Zillmann[5] and Hannsjörg Sinn[3]

[1]Neurosurgical Department, Kopfklinikum, University of Heidelberg, 69120 Heidelberg, Germany. [2]Department of Hematology and Oncology, University of Rostock, 18056 Rostock, Germany. [3]Department of Radiochemistry and Radiopharmacology, [4]Department of Oncological Diagnostics and Therapy, and [5]Central Animal Laboratories, German Cancer Research Center, 69120 Heidelberg, Germany.

The antifolate aminopterin (AMPT) was developed before methotrexate (MTX), but was not clinically established or generally used due its increased toxicity compared to MTX. Recently, we reported on the increased metabolism of albumin conjugates such as methotrexate–albumin (MTX–SA) in malignant tumors and the feasibility of using albumin as a carrier for drug targeting. Consequently, AMPT was covalently bound to serum albumin (AMPT–SA) at a 1:1 molar ratio. Biodistribution, tolerability and efficacy of this novel conjugate were studied in Walker-256 (W-256) carcinoma-bearing rats. As compared to native albumin, the same biodistribution and plasma clearance were found for AMPT–SA, which achieved 20.1% tumor uptake (estimated uptake per g tumor 6.4%) within 24 h after i.v. administration in rats. In a randomized study, AMPT–SA, repeatedly i.v. injected, was compared with low-molecular-weight AMPT. Depending on the molar concentration, the maximum tolerated dose (MTD) of AMPT covalently bound to SA was twice that of unbound AMPT (three repeated injections of 1.0 mg AMPT–SA/kg body weight versus three repeated injections of 0.5 mg AMPT/kg body weight; $p=0.0006$). Efficacy was studied at the level of the MTD and MTD/2, and demonstrated that AMPT–SA was significantly more active. At the MTD/2 in W-256 carinoma-bearing rats, AMPT–SA achieved a 100% volume reduction and an optimal volume reduction during treatment/control (T/C) of 8.3% compared to a 53% volume reduction of AMPT and a T/C of 16.5% ($p=0.032$). Tumor relapses were reduced and occurred later in the AMPT–SA group (two tumor recurrences for AMPT–SA versus seven for AMPT; $p=0.05$). In this comparative study, the AMPT–SA conjugate showed high antitumor activity *in vivo* and a favorable toxicity compared to low-molecular-weight AMPT. These effects are attributed to the albumin carrier which seems to be an effective tool for selective tumor drug targeting. [© 2002 Lippincott Williams & Wilkins.]

Correspondence to P Kremer, Neurosurgical Department, Kopfklinikum Heidelberg, Im Neuenheimer Feld 400, 69120 Heidelberg, Germany.
Tel: (+49) 6221566300; Fax: (+49) 6221565534;
E-mail: paul.kremer@med.uni-heidelberg.de

Key words: Albumin, aminopterin, antifolate, drug targeting.

Introduction

In the 1940s the antifolate aminopterin (AMPT) was the first antimetabolite to be introduced for the treatment of children with acute leukemia. Its transient success was quite remarkable[1,2] and was attributed to the development of modern chemotherapy. AMPT then was replaced by a similar antifolate, methotrexate (MTX), in the 1950s because murine studies demonstrated that MTX had a similar antitumor effect, but was less toxic.[3] MTX is still one of the most widely used chemotherapeutic agents for acute lymphoblastic leukemia in children, osteogenic sarcoma, choriocarcinoma, lymphoma, head and neck tumors, and other solid tumors. It also is used to treat inflammatory diseases such as rheumatoid arthritis and psoriasis. In comparison to MTX, however, AMPT shows a 20-fold increase in the V_{max}/K_m quotient for the enzyme folylpolyglutamate synthetase,[4] and a greater uptake and polyglutamation in lymphoblasts and myeloblasts.[5] Recently, AMPT was re-evaluated in a phase I trial in 20 patients with malignancies which were refractory to other treatments and showed promising activity in various tumors.[6]

In malignant tumors, the albumin turnover is increased. Like glucose and free amino acids, circulating albumin provides amino acids and energy for tumor nutrition and growth. Proliferating tumor cells consume substantial amounts of albumin[7–11] by endocytosis and after its lysosomal break down they

use the released amino acids for *de novo* synthesis of proteins.[12] In previous studies, albumin was also chosen for drug targeting. In most cases multiple drug molecules were loaded on the protein in order to increase the therapeutic benefit.[13–19] However, this overloading of albumin altered or damaged the quaternary configuration of the protein. As demonstrated by an *in vivo* study using albumin conjugates loaded with 5, 10, 20 or more drug molecules, the altered albumin was removed rapidly from the circulation and trapped by the monocyte–phagocyte system. Only albumin molecules with a loading ratio of close to 1:1 exhibit optimal tumor targeting, because these conjugates show an *in vivo* behavior like that of native albumin.[20] Recently, we reported on the 1:1 conjugation of MTX to albumin (MTX–SA), demonstrating a 60% tumor cure rate in Walker-256 (W-256) carcinoma-bearing rats without toxic side effects.[21,22] MTX–SA also proved to be highly effective in xenograft studies.[23] Meanwhile, MTX–SA has entered clinical phase I/II development.[24] To overcome the problem of the known toxicity of AMPT while maintaining its therapeutic potential, we sought to develop a suitable albumin-based drug-targeting strategy for this highly active antimetabolite, and performed the first animal study in W-256 carinoma-bearing rats with AMPT coupled to albumin at a 1:1 molar ratio to determine its toxicity and anti-cancer activity.

Material and methods

Labeling and loading techniques

AMPT activation. Aminopterin hydrate (AMPT; molecular weight 440.42 Da;. Aldrich, Steinheim, Germany) was dissolved in dimethylsulfoxide (DMSO) with a concentration of 10 mg/ml. To the clear yellow solution a 1.5-fold molar quantity of dicyclohexyl-carbodiimide (DCC) and a 10-fold molar quantity of hydroxysuccinimide (HSI) were added. After 14–15 h at room temperature, the reaction to the succinimidyl-ester (AMPT–HSIE) was finished, as seen by the presence of crystallized dicyclohexylurea (DCHU).

Covalent coupling of the activated AMPT to albumin. The clear yellow AMPT–HSIE solution in DMSO was added under slow stirring to a solution of human serum albumin (SA, molecular weight 68 kDa; Pharma Dessau, Dessau, Germany; 50 mg SA/ml 0.17 M $NaHCO_3$ at pH 8.5). at a 1.5:1 molar ratio. After 15 min the primarily clear solution clouded, depending on the formation of colloidal aggregates of non-reacted DCC and DCHU still dissolved in DMSO. After 30 min of reaction time, the colloidal aggregates were separated via a sterile filter (0.22 μm; Millipore, Molsheim, France), whereas DMSO and non-covalently bound AMPT were separated via ultrafiltration by means of a pressurized stirred cell equipped with a membrane filter (YM 30; Millipore). HPLC-SEC was used for purity control.

HPLC–SEC conditions:

Precolumn:	Phenomenex GFC-4000, 4 mm L × 3 mm ID
Column:	Zorbax GF 250,
Eluent 1:	0.25 M Li acetate, 0.05 M Li citrate pH 7.4 (Ampuwa)
Eluent 1:	95% methanol, 5% water (HPLC grade/Ampuwa)
Flow:	0–15 min eluent 1 (100%); 1.0 ml/min
Gradient:	15–30 min eluent 2 (from 0 to 100%); 1.3 ml/min
	30–45 min eluent 2 (100%)
	45–50 min eluent 2 (from 100 to 0%)
	50–60 min eluent 1 (100%); 1.0 ml/min
Pressure:	about 65 bar

The detection was done at 370 nm using a UV-vis detector (Merck, Darmstadt, Germany).

Labeling of AMPT–SA with ^{111}In. Diethylene-triamine-penta-acetic acid (DTPA) was dissolved under heating in DMSO to a concentration of 20 mg/ml. After cooling to room temperature, a 1.2-fold molar quantity of DCC and a 10-fold molar quantity of HSI were added. Within 24 h at room temperature the activation to the succinimidylester was finished. The clear, colorless DMSO solution was slowly added in a 2-fold molar surplus to the protein solution (10 mg AMPT–SA/ml 0.17 M $NaHCO_3$, pH 8.5). After 15 min, white colloidal aggregates had formed, representing unreacted DCC and DCHU. The colloidal aggregates were separated via a sterile filter (0.22 μm; Millipore), whereas DMSO and free DTPA were separated via ultrafiltration in a C30 microconcentration unit (Millipore). DTPA-activated AMPT was labeled with $^{111}InCl_3$ by adding the calculated quantity of radioactivity as a ^{111}In–citrate complex to 10 mg protein. Directly after adding the ^{111}In–citrate complex, unbound ^{111}In was separated by ultrafiltration using a Millipore C30 unit. Using this technique 98% of the ^{111}In activity was bound to DTPA–AMPT–SA as residualizing label.[25–27]

W-256 carcinoma

W-256 was chosen as a model for a highly proliferating and antifolate-sensitive tumor.[28] The cells were obtained from the tumor bank of the German Cancer Research Center (Heidelberg) and stored at −196°C. After thawing, the W-256 cells were cultivated using a standard RPMI medium enriched with 10% (v/v) heat-inactivated fetal calf serum and 1% L-glutamine. The culture medium was changed every 48 h. The final concentration was adjusted to 10^6 cells/ml with PBS. For seeding, intramuscular inoculation of 5×10^6 W-256 viable cells in a volume of 200 µl RPMI was applied at the left hind leg.

Animal studies and data evaluation

All animal experiments were approved by the German Federal Government (Regierungspräsidium Karlsruhe 35-0185.81/124/98). Female, specific pathogen-free (SPF) Sprague-Dawley rats ($n=139$) weighing 180–210 g were used for this study. The animals were kept under standardized SPF conditions in a microisolator. After tumor seeding in the right hind leg, the tumor radii were measured with calipers and the tumor volumes were calculated using the formula (length × width^2)/2, where length (a) is the largest side and width (b) the smallest side perpendicular to the length ($a \times b^2$)/2.[29]

Data were evaluated using specifically designed software by plotting the relative tumor volume against time. Relative tumor volumes were calculated for each individual tumor by dividing the tumor volume on day x by the tumor volume on day 0 at the time of randomization. Growth curves were analyzed in terms of maximal tumor inhibition [treated/control (T/C) = (mean tumor volume of treated rat/mean tumor volume of control rat) × 100]. Tumor volume doubling time was noted at 48 h, which correlated well with previous studies.[30] Statistical data analysis was performed using Fisher's Exact test. Tumor volumes of each treatment group were compared to those of the control group.

Pharmacokinetics and biodistribution

Pharmacokinetics, biodistribution and tumor uptake of [^{111}In]DTPA–SA and [^{111}In]DTPA–AMPT–SA were studied after i.v. tracer administration (3.7 MBq) in four animals. Plasma clearance was determined by taking blood samples of 20 µl at 5 min, and 1, 4, 8, 24, 48, 72 and 96 h after i.v. injection. The blood samples were drawn by clipping the tail tip. Plasma sample count rates were measured in a γ-counter (Berthold, Wildbad, Germany).

Biodistribution and tumor uptake were studied non-invasively in three W-256 carcinoma-bearing rats using sequential scintigraphy which was performed 5 min, and 1, 4, 24 and 48 h after tracer administration (7.4 MBq, [^{111}In]DTPA–AMPT–SA). For sequential scintigraphy, the animals were anesthetized using a mixture of halothane, N_2O, and O_2 (1.5, 60 and 38%) and placed in a prone position on a multihole collimator (420 keV) of a 10-in γ-camera (Pho-Gamma IV; Searle-Siemens, Erlangen, Germany). For online evaluation of the data, a computer system was connected to the γ-camera (Gaede Medworker; Gaede, Freiburg, Germany). The whole body counts, and the counts from the regions of interest (ROIs) over the thyroid gland, liver, heart, kidneys, urinary bladder and the tumors were collected to determine the time-dependent biodistribution and tumor uptake.

Determination of maximal tolerated dose (MTD) and drug efficacy

No data on the MTD for repeated injections of AMPT and AMPT–SA in SD rats were available. Therefore, based on the data of previously conducted *in vivo* experiments with MTX–SA in tumor-bearing rats with an albumin half-life of 2.5 days[21] or tumor-bearing nude mice,[23] a schedule of three or four repeated i.v. drug administrations was selected. Because of the increased toxicity of AMPT compared to equimolar doses of MTX,[2,5] a reduced starting dose of 0.5 mg AMPT/kg or 0.5 mg AMPT–SA/kg was chosen. Repeated i.v. injections on day 1, 3, 7 and 10 were performed until signs of toxicity such as weight loss, mucositis, diarrhea and shaggy fur were noted. During treatment the body weight and behavior as well as tumor volumes were monitored daily. The MTD for AMPT and AMPT–SA was established in groups of 10 animals per substance. The dose was escalated to repeated injections of 1.0 mg/kg body weight according to the same administration schedule in cases of no toxicity. MTD in tumor-bearing rats receiving repeated i.v. administrations was defined as the dose at which 20% of animals died (LD_{20}) and/or 20% body weight loss occurred. Gross necropsies were conducted in all animals.

For evaluation of antitumor activity, the SD rats bearing W-256 carcinomas were treated at the MTD and MTD/2 either with AMPT or AMPT–SA. Groups of 15 animals were used, which were randomly divided to receive repeated AMPT or AMPT–SA injections. The control group ($n=12$) received saline injections according to the same administration schedule. Treatment was started after the tumor volumes had grown 1500 mm^3 or more, which was usually reached 3 days after tumor inoculation. The body weights, side effects and tumor volumes were monitored daily. The studies were terminated when tumor volumes reached a critical size (3 cm in diameter or more) or, in cases of long-term tumor remission, when regrowth occurred, or at week 4 after the last administration of treatment.

Results

Analytic control of the albumin conjugates

Before ultrafiltration for purification the chemical yield was estimated by the original 1:1 molar mixture which contained a 80% yield of the activated AMPT to the protein. Using HPLC-SEC the albumin dimer fraction of AMPT–SA was eluted with a retention time 8.17 min, whereas the monomer albumin fraction of AMPT–SA was eluted at 9.06 min.The retention time of unbound AMPT was 15.78 min (Figure 1).

Pharmacokinetics and biodistribution

The plasma clearances of [^{111}In]DTPA–AMPT–SA were identical to those of [^{111}In]DTPA–SA, with a plasma half-life of 2.5 days (Figure 2). Sequential scintigraphy performed 5 min, and 1, 4, 24 and 48 h after administration of [^{111}In]DTPA–AMPT–SA demonstrated a normal distribution of the conjugate. Sequential scintigraphy also showed a 8.4% uptake of [^{111}In]DTPA–AMPT–SA within the tumor 1 h after administration which increased to 20.1% 2 days after i.v. administration. The mean tumor volume at this time was 3.13 cm^3. Therefore, the uptake of the conjugate could be estimated at 6.4%/g tumor 48 h after i.v. administration (all data of sequential scintigraphy are presented in Table 1 and Figure 3).

Tolerability of AMPT–SA versus AMPT

Tolerability of AMPT–SA and low-molecular-weight AMPT was studied in a total of 60 animals (10 animals per drug and dosage), which in a first set of experiments received repeated i.v. injections of 0.5 mg AMPT or 0.5 mg AMPT–SA/kg body weight until the onset of toxicity. After the third injection, two thirds of the animals treated with 3×0.5 mg AMPT/kg body weight demonstrated side effects such as mucositis, diarrhea and shaggy fur, whereas the animals who had received three equimolar injections of AMPT–SA showed no side effects. By continuing treatment with a fourth injection of 0.5 mg AMPT/kg toxicity increased. All animals developed shaggy fur, severe mucositis, diarrhea and remarkable weight loss, and three of them died (Table 2). In contrast, none of the animals that received four injections of 0.5 mg/kg AMPT–SA had toxic reactions. This difference in tolerability was highly statistically significant

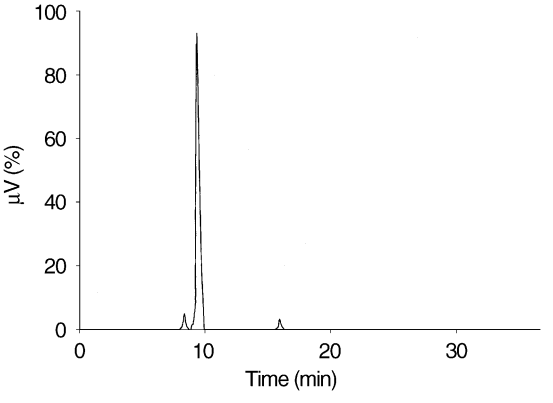

Figure 1. HPLC analysis of AMPT–SA by three-channel recording: dimer fraction <9 min, albumin-conjugate fraction 9–10 min and low-molecular-weight fraction for AMPT 15 min.

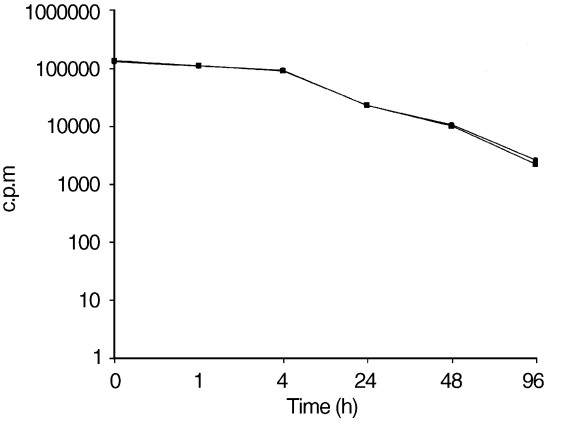

Figure 2. Plasma clearance of [^{111}In]DTPA–SA (squares) and [^{111}In]DTPA–AMPT–SA (circles) (3.7 MBq). Values are given in c.p.m. A total of 0.1 mg albumin was injected per rat.

Table 1. Sequential scintigraphy after i.v. administered [^{111}In]DTPA–AMPT–SA (7.4 MBq) in W-256 carcinoma-bearing rats

	Time				
	5 min	1 h	4 h	24 h	48 h
Whole body activity	103635 (100%)	101751 (100%)	96050 (100%)	72274 (100%)	48361 (100%)
Heart	8868 (8.6%)	10345 (10.2%)	7037 (7.3%)	3983 (5.5%)	2325 (4.8%)
Liver	20212 (19.5%)	21509 (21.1%)	13434 (14.0%)	8737 (12.1%)	5273 (10.9%)
Tumor	4930 (4.8%)	8535 (8.4%)	18143 (18.8%)	14279 (19.7%)	9720 (20.1%)

The ROIs of the heart, liver and the tumor are measured as c.p.m., and are related to the whole body activity over time.

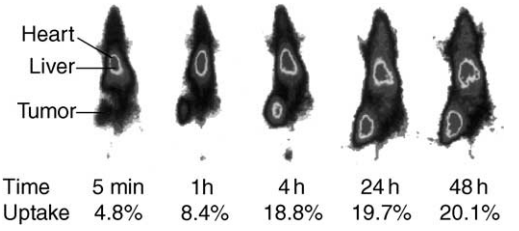

Figure 3. Images of sequential scintigraphy over time after i.v. administered [^{111}In]DTPA–AMPT–SA (7.4 MBq) in W-256 carcinoma-bearing rats.

($p=0.0006$). Over a period of 4 days, the surviving animals recovered completely. In this study, the MTD for AMPT was fixed at three repeated i.v. injections of 0.5 mg AMPT/kg body weight on days 1, 3 and 7. Since no toxic reaction was seen after four repeated injections of 0.5 mg AMPT–SA/kg body weight, the dose was escalated to repeated i.v. injections of 1.0 mg AMPT–SA/kg body weight. Side effects were observed in 70% of the animals after three repeated injections of 1.0 mg AMPT–SA. Toxicity also increased after the fourth injection, which was lethal in four rats. Thus, the MTD for AMPT–SA was defined for three repeated i.v. injections at 1.0 mg/kg body weight, which is twice the molar dose level of unbound AMPT.

Efficacy of AMPT–SA versus AMPT

Maximal antitumor effects are expected at a level close to the MTD, particularly for drugs that interact directly with DNA or DNA synthesis, such as antimetabolites for which AMPT is representative. Repeated i.v. injections at the level of the MTD and MTD/2 on days 1, 3 and 7 for both substances, unbound AMPT and AMPT–SA, were administered to 60 randomly allocated W-256 carcinoma-bearing rats. Using this administration schedule, no severe side effects were noted. Because of the high proliferating activity of the W-256 carcinomas, the untreated control groups demonstrated rapid tumor progression within 10 days after tumor seeding; thus, 11 of 12 control animals had to be sacrificed early on in accordance with the German Animal Welfare Regulations.

Table 2. Toxicity of AMPT and AMPT–SA in W-256 carcinoma-bearing SD rats

	Lethality (until 7 days after last administration)		Side effects (mucositis, diarrhea, shaggy fur) (%)	Body weight		Evaluation
	Total	% Total		BWL (%) (maximal loss)	BW (%) 7 days after last administration	
AMPT[a] (3 × 0.25 mg/kg)	0/10	0	30	1.3 ± 5.8	105.5 ± 56.7	MTD/2
AMPT[a] (3 × 0.5 mg/kg)	0/10	0	66	7.3 ± 7.6	103.5 ± 5.3	MTD
AMPT[b] (4 × 0.5 mg/kg	3/10	30	100	22 ± 13.1	95.1 ± 8.1	toxic
AMPT–SA[b] (4 × 0.5 mg/kg	0/10	0	20	0.3 ± 6.2	108.4 ± 5.9	MTD/2
AMPT–SA[a] (3 × 1.0 mg/kg	0/10	0	70	6.9 ± 3.8	102.7 ± 6.9	MTD
AMPT–SA[b] (4 × 1.0 mg/kg	4/10	40	100	17.2 ± 12.2	93.4 ± 8.5	toxic

[a]Drug administrations on day 1, 3 and 7.
[b]Drug administrations on day 1, 3, 7 and 10.

Table 3. Activity of AMPT–SA and AMPT at MTD and MTD/2 in W-256 carcinoma-bearing SD rats

	MTD level	Optimal T/C (days) (%)	Complete tumor remission (%) (7 days after last administration)	*p* value: AMPT–SA versus AMPT	Tumor relapse (%) (days after last administration)	*p* value: AMPT–SA versus AMPT
AMPT (3 × 0.25 mg/kg)	MTD/2	16.5 (7)	53.3	NA	46.6 (2–6)	NA
AMPT–SA (3 × 0.5 mg/kg	MTD/2	8.3 (7)	100	0.0032	13.3 (10/24)	0.05
AMPT (3 × 0.5 mg/kg	MTD	8.2 (7)	100	0.0032	26.6 (14)	NS
AMPT–SA (3 × 1.0 mg/kg	MTD	8.0 (7)	100	0.0032	6.6 (22)	NS

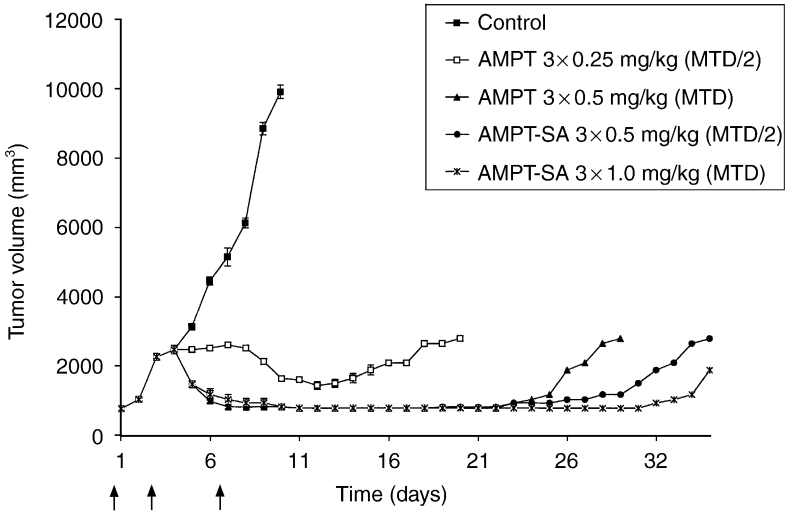

Figure 4. Activity of AMPT–SA compared to unbound AMPT in W-256 carcinoma-bearing rats. Drug treatment is indicated by arrows. Mean tumor volumes are shown. Tumor recurred for AMPT at the MTD in four animals beginning from day 14 versus in two animals at day 10 and 24 for AMPT–SA at the MTD/2. Only one animal had tumor relapse on day 22 for AMPT–SA at the MTD group.

AMPT–SA showed high antitumor activity at the MTD and MTD/2, resulting in long-term tumor remissions (Table 3 and Figure 4). Only in the MTD group did unbound AMPT demonstrate substantial antitumor activity. Three repeated equimolar injections of 0.5 mg/kg body weight for AMPT (MTD) achieved activity similar to that seen for AMPT–SA at the MTD/2, with complete remissions 7 days after the last administration as demonstrated by the same T/C values of 8.2 for AMPT (MTD) and 8.3 for AMPT–SA (MTD/2). At MTD/2, significantly more tumor relapses were seen with AMPT (46.6%) than with the corresponding MTD/2 group of AMPT–SA (13.6%). The differences in T/C values ($p \leqslant 0.0032$) and tumor relapses ($p \leqslant 0.05$) were highly significant in favor of AMPT–SA (Table 3 and Figure 4). In addition, tumor remissions induced by AMPT–SA were sustained for a longer period than in the AMPT-treated animals (Table 3 and Figure 4).

Discussion

Throughout the last three decades attempts have been launched to exploit albumin as a carrier system for cytostatic drugs. MTX, in particular, was considered to be promising for covalent linkage to albumin.[13,14,16,17,31] Initially, the discrepant molecular weights of MTX and SA was the focus of all efforts to improve the therapeutic efficacy of these conjugates by increasing the molar load from originally about 10 molecules MTX conjugated per albumin molecule up to MTX-albumin conjugates carrying

56 mol MTX/mol albumin.[17,31] Data from radiopharmacokinetic *in vivo* studies in W-256 carcinoma-bearing rats suggested that albumin conjugates with an increasing molar excess of MTX (more than 3 mol) were targeting predominantly the liver macrophage system rather than tumor tissue.[12] Later on, MTX–albumin conjugates with low loading rates close to a molar ratio of 1:1 (MTX–SA) showed improved tumor targeting properties and high antitumor activity.[12,21,23] Meanwhile, MTX–SA has entered clinical phase I/II trials.[24] Similarly, the covalent binding of 5-aminofluorescein to SA (AFLc–SA) in a 1:1 ratio demonstrated the enhanced tumor-targeting properties of albumin in brain tumors and has entered phase I/II trials for intraoperative laser-induced fluorescence detection in malignant glioma surgery.[32]

Recently, AMPT was re-evaluated in a phase I trial in 20 patients with refractory malignancies. It showed promising activity, achieving complete response in one patient with endometrial carcinoma and stable disease in seven patients.[6] AMPT might be an even more active antifolate than MTX for conjugation to albumin due to its 20-fold higher V_{max}/K_m quotient for the enzyme folylpolyglutamate synthetase.[4] Therefore, this study was conducted to investigate the biodistribution, uptake, tolerability and efficacy of 1:1 molar loaded AMPT–SA in W-265 carcinoma-bearing rats. As expected, AMPT–SA showed similar properties with respect to biodistribution, plasma clearance, and tumor uptake as compared to native albumin, MTX–SA or AFLc–SA.[21,32] Therefore, it is very likely that AMPT–SA will show increased antitumor activity in cancers expressing enhanced permeability and retention effects,[33–35] as has also been reported for MTX-HSA recently.[23]

In the study reported here, we established the MTD for unbound AMPT given i.v. on days 1, 3 and 7 at 0.5 mg AMPT/kg body weight (max. BWL=7.3%; BW 7 days after last injection=103.5%). Continuing treatment with a fourth administration on day 10 caused severe AMPT-induced toxicity in the treated animals, as manifested by mucositis, diarrhea and a maximal weight loss of 22%. Compared to equimolar doses of AMPT, the conjugate AMPT–SA was less toxic and demonstrated an increased MTD level, which was established for three repeated i.v. injections at 1.0 mg AMPT–SA/kg body weight on days 1, 3 and 7 (max BWL=6.9%; BW 7 days after last injection=102.7%). This remarkable difference in the MTD levels for AMPT and AMPT–SA is associated with the different total body distribution of AMPT and AMPT–SA. Even for the majority of low-molecular-weight chemotherapeutic drugs, severe systemic toxicity was seen after repeated i.v. injections. However, despite its distinctly prolonged circulating time, the conjugate AMPT–SA did not reach physiologically proliferating tissue by the same route as unbound AMPT; thus, AMPT–SA did not show severe side effects at equimolar dose levels.

At the MTD and MTD/2, AMPT–SA demonstrated a high antitumor activity, resulting in long-term tumor remissions. In contrast, significantly more tumor relapses were seen in the MTD/2 group for AMPT than in the corresponding MTD/2 group for AMPT–SA. The differences in T/C values ($p \leqslant 0.0032$) and tumor relapses ($p \leqslant 0.05$) were highly significant. At three repeated equimolar injections of 0.5 mg AMPT/kg body weight (MTD) and 3×0.5 mg AMPT–SA/kg body weight (MTD/2), both were capable of producing complete remissions. However, a lower and later tumor recurrence rate was observed for AMPT–SA.

Similar to the results observed for MTX–SA in animal studies, AMPT–SA also has superior effects and less toxicity than the unbound drug. Therefore, AMPT–SA might be a promising candidate for further preclinical testing in human xenograft-bearing nude mice and possibly for clinical testing. It may be assumed that AMPT–SA exhibits its cytostatic effects in a similar way as has recently been described for MTX–SA. The albumin conjugate is internalized into the cell via endocytosis; thereafter, the drug is cleaved from the lysosomally degraded albumin and liberated into the cell.[36] Due to its powerful inhibition of the enzyme folylpolyglutamate synthetase, it might be an even more active cytostatic drug than MTX–SA.

Acknowledgments

This study was supported by the Tumorzentrum Heidelberg/Mannheim. We thank Mr Eskerski and Mr Dähmel for preparing the animal studies, and Ms Sherryl Sundell for substantial help in editing.

References

1. Farber S, Diamond LK, Mercer RD, Sylvester RF, Wolff JA. Temporary remissions in acute leukemia in children produced by folic acid antagonist 4-aminopteroylglutamic acid (aminopterin). *N Engl J Med* 1948; **238**: 787–93.
2. Bertino JR. Ode to methotrexate. *J Clin Oncol* 1993; **11**: 5–14.

3. Goldin A, Venditti JM, Humphreys SR, Don D, Mantel N, Greenhouse SW. A quantitative comparison of the antileukemic effectiveness of two folic acid antagonists in mice. *J Natl Cancer Inst* 1955; **15**: 1657–64.
4. Garrow TA, Shane B. Purification and general properties of human folylpolyglutamate synthetase. In: Ayling JE, Nair MG, Baugh CM, eds. *Chemistry and biology of pteridines and folates*. New York: Plenum 1993: 659–62.
5. Smith A, Hum M, Winick N, *et al.* A case for the use of aminopterin in treatment of patients with leukemia based upon metabolic studies of blasts *in vitro*. *Clin Cancer Res* 1996; **2**: 1–5.
6. Ratliff AF, Wilson JW, Hum M, *et al.* Phase I and pharmacokinetic trial of aminopterin in patients with refractory malignancies. *J Clin Oncol* 1998; **16**: 1458–64.
7. Babson AL, Winnick T. Protein transfer in tumor-bearing rats. *Cancer Res* 1954; **14**: 606–11.
8. Jewell WR, Krishnan EC, Schloerb PR. Apparent cellular ingress of albumin in Walker 256 tumor and rat muscle. *Cancer Res* 1975; **35**: 405–8.
9. Pittman RC, Carew TE, Glass CK, Green SR, Talor CAJ, Attie AD. Radioiodinated, intracellularly trapped ligand for determining the sites of plasma protein degradation. *in vivo*. *J Biochem* 1983; **212**: 791–800.
10. Andersson C, Iresjo BM, Lundholm K. Identification of tissue sites for increased albumin degradation in carcinoma-bearing mice. *J Surg Res* 1991; **50**: 156–62.
11. Wunder A, Stehle G, Sinn H, *et al.* Enhanced albumin uptake by rats tumors. *Int J Oncol* 1997; **11**: 497–507.
12. Stehle G, Sinn H, Wunder A, *et al.* Plasma protein (albumin) catabolism by the tumor itself—implications for tumor metabolism and the genesis of cachexia. *Crit Rev Oncol Hematol* 1997; **26**: 77–100.
13. Jacobs SA, D'Urso-Scott M, Bertino JR. Some biochemical and pharmacologic properties of amethopterin–albumin. *Ann NY Acad Sci* 1971; **186**: 284–6.
14. Chu BC, Fan CC, Howell SB. Activity of free and carrier-bound methotrexate against transport-deficient and high dihydrofolate dehydrogenase-containing methotrexate-resistant L1210 cells. *J Nat Cancer Inst* 1981; **66**: 121–4.
15. Soriano L, Rivera-Fillat MP, Grau-Oliete MR. Evolution of acute lymphoblastic leukemia in mice treated with carrier-bound methotrexate and levamisole. *Chemotherapy* 1987; **33**: 123–8.
16. Halbert GW, Florence AT, Stuart JF. Characterization of *in-vitro* drug release and biological activity of methotrexate–bovine serum albumin conjugates. *J Pharm Pharmacol* 1987; **39**: 871–6.
17. Bures L, Bostik J, Motycka K, Spundova M, Rehak L. The use of protein as a carrier of methotrexate for experimental cancer chemotherapy. III. Human serum albumin–methotrexate derivative, its preparation and basic testing. *Neoplasma* 1988; **35**: 329–42.
18. Bostik J, Bures L, Spundova M. The use of protein as a carrier of methotrexate for experimental cancer chemotherapy. IV. Therapy of murine melanoma B16 by human serum albumin–methotrexate derivative. *Neoplasma* 1988; **35**: 343–9.
19. Kim CK, Hwang SJ, Lee MG. The organ targetability of small and large albumin microspheres containing free and HSA conjugated methotrexate. *Int J Pharm* 1993; **89**: 91–102.
20. Stehle G, Sinn H, Wunder A, *et al.* The loading rate determines tumor targeting of methotrexate–albumin conjugates in rats. *Anti-Cancer Drugs* 1997; **8**: 677–85.
21. Stehle G, Wunder A, Sinn H, *et al.* Pharmacokinetics of methotrexate–albumin conjugates in tumor bearing rats. *Anti-Cancer Drugs* 1997; **8**: 835–44.
22. Wunder A, Stehle G, Schrenk HH, *et al.* Antitumor activity of methotrexate–albumin conjugates in rats bearing a Walker-256 carcinoma. *Int J Cancer* 1998; **76**: 884–90.
23. Burger AM, Hartung G, Stehle G, Sinn H, Fiebig HH. Pre-clinical evaluation of a methotrexate–albumin conjugate (MTX–HSA) in human tumor xenografts *in vivo*. *Int J Cancer* 2001; **92**: 718–24.
24. Hartung G, Stehle G, Sinn H, *et al.* Phase I trial of a methotrexate-albumin (MTX-HSA) conjugate in a weekly intravenous bolus regimen in cancer patients. *Clin Cancer Res* 1999; **5**: 753–9.
25. Jain RK. Delivery of novel therapeutic agents in tumors: physiological barriers and strategies. *J Natl Cancer Inst* 1989; **81**: 570–6.
26. Sinn H, Schrenk HH, Friedrich EA, Schilling U, Maier-Borst W. Design of compounds having an enhanced tumour uptake, using serum albumin as a carrier. Part I. *Nucl Med Biol* 1990; **17**: 819–27.
27. Schilling U, Friedrich EA, Sinn H, Schrenk HH, Clorius JH, Maier-Borst W. Design of compounds having enhanced tumour uptake, using serum albumin as a carrier. Part II. *In vivo* studies. *Nucl Med Biol* 1992; **19**: 685–95.
28. Smith GM. A comparison of the effects of cytotoxic agents on the Walker 256 tumour growing in the rat and at the hamster cheek pouch. *Br J Cancer* 1969; **23**: 88–94.
29. Geran RI, Greenberg NH, MacDonald MM, *et al.* Protocols for screening chemical agents and natural products against tumor and other biological systems. *Cancer Chemother Rep* 1972; **3**: 1–103.
30. Fogt F, Wan J, O'Hara C, *et al.* Flow cytometric measurement of cell cycle kinetics in rat Walker-256 carcinoma following *in vivo* pulse labelling with bromodeoxyuridine. *Cytometry* 1991; **12**: 33–41.
31. Magnenat G, Schindler R, Isliker H. Transport d'agents cytostatiques par les protéines plasmatiques: III. Activité antitumorale *in vitro* de conjugués cytostatique–azoprotéines. *Eur J Cancer* 1969; **5**: 33–40.
32. Kremer P, Wunder A, Sinn H, *et al.* Laser-induced fluorescence detection of malignant gliomas using fluorescein-labeled serum albumin: experimental and preliminary clinical results. *Neurol Res* 2000; **22**: 481–9.
33. Loadman PM, Bibby M, Double JA, *et al.* Pharmacokinetics of PK1 and doxorubicin in experimental colon tumor models with

differing responses to PK1. *Clin Cancer Res* 1999; **5**: 3682–8.
34. Muggia FM. Doxorubicin–polymer conjugates: further demonstration of the concept of enhanced permeability and retention. *Clin Cancer Res* 1999; **5**: 7–8.
35. Vasey PA, Kaye SB, Morrsion R, *et al.* Phase I clinical and pharmacokinetic study of PK1 (HPMA co-polymer doxorubicin): first member of a new class of chemotherapeutic agents—drug–polymer conjugates,. *Clin Cancer Res* 1999; **5**: 83–94.
36. Weigand M, Hartung G, Roboz J, *et al.* Mode of action of methotrexate-albumin in a human T-cell leukemia line and activity against an MTX-resistant clone. *Anti-Cancer Drug Des* 2002; **7**: 1–8.

(Received 4 December 2001; revised version received 26 March 2002)