In Vitro and in Vivo Antitumor Activity of Methotrexate Conjugated to Human Serum Albumin in Human Cancer Cells

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ABSTRACT

To avoid systemic toxicity of the cytotoxic drug methotrexate (MTX) and to improve tumor selectivity, MTX was bound to human serum albumin (HSA) as a drug carrier. To understand more about the mechanism of action of MTX conjugated to HSA (MTX-HSA), the uptake of MTX-HSA into the cell was determined as well as the effect of MTX-HSA on thymidylate synthase (TS), cell cycle distribution, and cell proliferation. Different uptake kinetics were observed for [3H]MTX and [3H]MTX-HSA. However, similar uptake kinetics were measured for 125I-HSA and 125I-MTX-HSA (2.1 and 1.8 pmol/107 cells/h when cells were treated with 10 μM 125I-HSA and 125I-MTX-HSA, respectively), suggesting that MTX-HSA enters the cells by albumin-mediated endocytosis. We observed no effect of MTX-HSA on TS when folate receptor-expressing KB cells were treated for 4 h (IC50 >50 μM). However, 24 h after incubation, MTX-HSA inhibited TS with an IC50 of 6.9 μM. In addition, we found that MTX-HSA had a delayed effect on the cell cycle compared with MTX and that this effect could be inhibited with the lysosomal inhibitor methylamine, suggesting that MTX-HSA activity is dependent on lysosomal processes. The proliferation of different wild-type and MTX-resistant tumor cell lines was inhibited at IC50 concentrations between 2 and 78 μM, respectively. MTX-HSA accumulates in vivo in the tumor tissue. Local concentrations of 18–29 μM were measured, which are effective antiproliferative concentrations as determined in vitro. We also investigated the antitumor activity of MTX-HSA in vivo in different human tumor xenografts grown s.c. in nude mice. Fourteen tumors from eight different tissues were tested. Nine of 14 tumors (64%) showed a clear response with tumor inhibition, stasis, or regression; 5 of 14 (36%) gave a moderate response with tumor growth delay or no response. In conclusion, MTX-HSA is effectively taken up by the cells via albumin receptor- or folate receptor-mediated endocytosis and time-dependently released as an active compound into the cytosol to exert an inhibiting effect on TS and to induce cell cycle alterations. In vivo, effective concentrations of MTX-HSA were reached in tumor tissue to exhibit antitumor activity.

INTRODUCTION

MTX continues to play an important role in the treatment of a variety of malignancies and is well understood with respect to its mechanism of action at the molecular level. However, the clinical application of this drug is limited by its toxic dose-related side effects. The lack of selectivity of the low molecular weight antitumor drug MTX is closely related to its pharmacokinetic properties, i.e., short half-life in the bloodstream and rapid diffusion throughout the body resulting in an essentially uniform tissue distribution. An approach to alter the pharmacokinetic behavior and overcome the toxicity of MTX in normal tissue is to couple MTX to HSA. HSA has a biological half-life of 19 days, in contrast to the half-life of 2–3 h of MTX in humans (1, 2). In addition, HSA was found to accumulate in tumor tissue because of the absence of a functioning lymphatic network [enhanced permeability and retention (3, 4)]. Furthermore, tumor cells possess elaborate mechanisms to internalize albumin, which serves as a nutrient for proliferating tumors, making HSA a suitable protein as potential drug delivery system (5–7). The main advantages of HSA as a macromolecular carrier for MTX can be summarized as follows: it has no toxicity or immunogenicity (in humans); it is biodegradable; the chemistry of HSA allows easy conjugation with MTX; it accumulates selectively in tumor tissue; it is stable in circulation with a long half-life; there is easy access to all tumor locations via the blood circulation; it has high chemical stability (up to 60°C; pH 1–10; 8 M urea); and it is convenient to administer.

The considerable discrepancy in the molecular weight of MTX (M, 454) and albumin (M, 66,500) tempted researchers to load multiple drug molecules on one carrier molecule (8–12). The optimal therapeutic efficacy of MTX protein conjugates was thought to be achieved by increasing the number of molecules of...
MTX attached to the carrier. However, it was recently shown that only loading rates of close to 1 molecule of MTX per molecule of albumin offer optional conditions for targeting MTX-albumin conjugates in tumor tissue (13). The accumulation of MTX conjugates in tumor tissue declined dramatically with an increasing molecular load of MTX linked to albumin. Furthermore, highly loaded MTX-albumin conjugates behaved as denatured albumin and were therefore rapidly removed from the circulation by the monocytic macrophage system of the liver.

With an approximate loading ratio of 1 molecule of MTX per molecule HSA, MTX-HSA exhibits a favorable toxicity profile in cancer patients. No MTX-HSA-specific antibodies were observed in the serum samples of patients (data not shown). In a clinical Phase I trial (14), tumor regressions were observed in 3 of 17 patients treated with MTX-HSA. Phase II clinical trials are currently ongoing.

The promising clinical results obtained with MTX-HSA led us to characterize the mechanism of action of MTX-HSA in more detail. The uptake of MTX-HSA into the cell was determined as well as the effect of MTX-HSA on TS, cell cycle distribution, and cell proliferation. In addition, we investigated the antitumor activity of MTX-HSA in vivo in different human tumor xenografts grown s.c. in nude mice and determined the accumulation of MTX-HSA in tumor tissue.

MATERIAL AND METHODS

MTX-HSA. MTX-HSA was synthesized by IDT (Desau, Germany) by coupling MTX (Heumann Pharma, Nuremberg, Germany) to HSA derived from blood donors (Octapharm, Vienna, Austria). Synthesis of MTX-HSA was described elsewhere (13). Concentrations are all based on the molarity of MTX in the conjugate. The molarity and the coupling degree of the MTX-HSA batches were determined by means of liquid chromatography-electrospray ionization-mass spectrometry. Up to four regions that correspond to the uncoupled HSA, HSA conjugates in tumor tissue (13). The accumulation of MTX conjugates remained in the [3H]MTX-HSA preparation. Therefore, a control experiment was performed with 0.05 μM [3H]MTX (specific activity, 4.54 mCi/mmol), 2 μM [3H]MTX (specific activity, 500 mCi/mmol; Biotrend, Cologne, Germany), and 9.88 μM [125I]HSA-MTX (specific activity, 0.384 Ci/mmol; Biotrend), or 9.88 μM [125I]HSA-MTX (specific activity, 0.440 Ci/mmol; Biotrend) were added. [3H]MTX-HSA was synthesized from [3H]MTX (Biotrend) and HSA by the same method as MTX-HSA (13). [3H]MTX-HSA, [3H]MTX, [125I]HSA-MTX, and [125I]HSA uptake studies were performed at 37°C. Negative controls were done each time at 4°C on ice to quanitate the amount of nonspecific binding. The [3H]MTX-HSA preparation was purified by Sephadex G25 columns, as described, to remove the unconjugated [3H]MTX. However, ≤0.07% [3H]MTX remained in the [3H]MTX-HSA preparation. Therefore, a control experiment was performed with 0.05 μM [3H]MTX (specific activity, 4.54 mCi/mmol; Biotrend, Cologne, Germany). BXF 1299 was provided by Prof. H. H. Fiebig (Oncotest, Freiburg, Germany).

Uptake Studies. DU-145 cells were seeded at a density of 5 × 10⁶ cells/10 ml RPMI 1640 + 10% FCS (Pan Systems, Aidenbach, Germany) in 58-cm² culture dishes. Culture medium was removed 2 days later, and 3–3.2 ml of HBSS buffer [107 mM NaCl, 20 mM HEPES, 26.2 mM NaHCO₃, 5.3 mM KCl, 1.9 mM CaCl₂, 1 mM MgCl₂, and 7 mM glucose (pH 7.4)] containing a final concentration of 50 μM [3H]MTX-HSA (specific activity, 4.54 mCi/mmol), 2 μM [3H]MTX (specific activity, 500 mCi/mmol; Biotrend, Cologne, Germany), 9.88 μM [125I]HSA-MTX (specific activity, 0.384 Ci/mmol; Biotrend), or 9.88 μM [125I]HSA-MTX (specific activity, 0.440 Ci/mmol; Biotrend) were added. [3H]MTX-HSA was synthesized from [3H]MTX (Biotrend) and HSA by the same method as MTX-HSA (13). [3H]MTX-HSA, [3H]MTX, [125I]HSA-MTX, and [125I]HSA uptake studies were performed at 37°C. Negative controls were done each time at 4°C on ice to quanitate the amount of nonspecific binding. The [3H]MTX-HSA preparation was purified by Sephadex G25 columns, as described, to remove the unconjugated [3H]MTX. However, ≤0.07% [3H]MTX remained in the [3H]MTX-HSA preparation. Therefore, a control experiment was performed with 0.05 μM [3H]MTX (specific activity, 4.54 mCi/mmol; Biotrend, Cologne, Germany). BXF 1299 was provided by Prof. H. H. Fiebig (Oncotest, Freiburg, Germany).
activity, 4.54 mCi/mmol), which represents 0.1% of un conjugated [3H]MTX in the [3H]MTX-HSA uptake studies.

After incubation, the medium was removed, and the monolayers were washed twice with 5 ml of ice-cold HBSS + HSA (2 mg/ml). The cells were detached with a cell scraper, sus pended in 5 ml of ice-cold HBSS + HSA (2 mg/ml), and transferred to 15-ml centrifuge tubes. The dishes were rinsed with 5 ml of ice-cold HBSS + HSA (2 mg/ml). The cells were centrifuged at 4°C for 10 min at 290 × g. The cell pellets were resuspended in 0.5 ml of double-distilled water, and the radioactivity was counted with liquid scintillation. An extra dish was used for cell number determination on the day of experiment.

**TS in Situ Inhibition Assay.** Inhibition of TS was determined in intact cells by measuring the antifolate-induced inhibition of TS-catalyzed conversion of [3H]dUMP to dTMP, during which reaction the release of [3H]H2O in the medium is impaired, as originally described by Yalowich and Kalman (21), using the modifications described previously (22, 23). Monolayer cells were plated at 4 × 105 cells in 6-well plates. Twenty four h later, exposure to MTX or MTX-HSA was initiated (five to seven drug concentrations covering 3 logs). Blanks were incubated without drugs and cells. Three conditions were tested: (a) a short (4-h) incubation period; (b) a 4-h drug incubation, after which the drugs were washed away, and the cells were suspended in drug-free medium for another 20 h; and (c) long term incubation (24 h). One h before the end of the incubation, 2⁻⁷[³⁵S]deoxyctydine (final concentration, 1 μM; specific activity, 2.5 Ci/mmol) was added. We used 2⁻⁷[³⁵S]deoxyctydine instead of 2⁻⁷[³⁵S]deoxyctydine because phosphorylation of deoxyctydine is dependent on the activity of thymidine kinase, which is usually lower in nondividing cells, is cell cycle de pendent, and can be down-regulated when DNA synthesis is inhibited. The initial activating enzyme deoxyctydine kinase is independent of the cell cycle, and 2⁻⁷[³⁵S]deoxyctydine gave similar relative inhibition patterns, but with a higher signal (23).

At the end of this incubation, 150 μl of the supernatant were collected and transferred to a 3-ml capped Falcon tube. The cells were put on ice, and 150 μl of 35% ice-cold trichloroacetic acid were added together with 750 μl of 10% activated charcoal solution (10 g of washed charcoal, 0.5 g of dextran and 2.5 g of BSA in 100 ml of water). After vortexing, the cells were left on ice for 30 min and then centrifuged at 800 × g for 30 min at 4°C. A 450-μl sample of the supernatant was then transferred to a scintillation vial and counted for radioactivity. After subtraction of the mean blank counts, the data were evaluated by calculating the TS IC50 values: the concentration of drug needed to inhibit 50% of the activity compared with the control TS.

**Cell Cycle Distribution.** CCRF-CEM cells were seeded in RPMI 1640 enriched with 5% FCS (Pan Systems) at a density of 2.5 × 10⁴ cells/ml in 12-well plates, and test compounds were added immediately after seeding. The plates were returned to the incubator for the indicated time periods, and then 0.5-ml aliquots were removed from each well and stained with 0.5 ml of sulforhodamine/4¢,6-diamidino-2-phenylindole ready-to-use solution from Partec (Münster, Germany). Samples were ana lyzed with a particle analyzing system (PAS; Partec), and 20,000 cells were investigated with regard to cell cycle dis tribution using WinList software (Verity Software House, Topsham, ME).

**Growth Inhibition Studies.** Suspension cells (leukemia cell lines) were plated in 1-ml aliquots at a density of 1.25 × 10⁵ cells/ml in individual wells of a 24-well culture plate. Drugs were added at the time of cell plating. Growth inhibition was assessed after 72 h of incubation via trypan blue exclusion (19). Monolayer cells (KB/MDCK cells) were plated in 1-ml aliquots at a density of 2 × 10⁵ cells/well in a 24-well culture plate. Drugs were added 24 h after plating the cells. Growth inhibition was assessed after 72 h of incubation. Cells were detached by trypsinization, and cell counts and viability were determined by trypan blue exclusion (20). Survival studies with DU-145 cells were performed in 96-well plates. A total of 2000 cells were plated in each well and treated 24 h later. After 6 days in culture, the cells were fixed in 10% trichloroacetic acid and stained with 0.4% sulforhodamine B in 1% acetic acid. The bound dye was solubilized in 200 μl of 10 mM unbuffered Tris solution, and the absorbance was determined at a wavelength of 515 nm in an ELISA microplate reader (Bio-Tek EL340; Deelux, Gödenstorf, Germany) in triplicate. Untreated control wells were assigned a value of 100%, and the IC50 was defined as the dose required to inhibit the absorbance measured at 515 nm to 50% of the control value.

**Tissue Distribution Study.** Albino female rats (n = 12) of the Sprague Dawley strain were inoculated in the midline of the thigh muscle with Walker-256 carcinoma cells (5 × 10⁶), and tumor growth was monitored over a period of 7 days. After 7 days, the tumors were measured, and six rats were selected for the study. A group of four rats received single i.v. doses (13.2 μmol/kg MTX-HSA corresponding to 6 mg/kg MTX) of [14C]MTX-HSA (specific activity, 11.5 mCi/mol; American Radiolabeled Chemicals, St. Louis, MO). These animals were then sacrificed at 1, 3, 8, and 48 h after administration of the dose. The animals were subjected to autoradiography. Sections were made at three or four different levels of the body to include tumor and liver where possible. Freeze-dried sections were powdered with talc and placed against imaging plates, which were stored in a lead imaging box for an exposure period of 7 days. Distribution of radioactivity was determined using a Fuji BAS 1500 bioimage analyzer (Raytek Instruments) and associated Tina and Seescan software. Concentrations were reported in nmol [14C]MTX-HSA/g tissue. The specific radioactivity of the dosed test substance was used for the calculation of concentration. The molar concentrations were estimated by assuming that 1 g of tissue is equivalent to 1 ml.

**Human Tumor Xenografts.** The tumors were induced in NMRI nu/nu mice by s.c. implantation of tumor fragments or s.c. inoculation of tumor cells. Treatment started at tumor sizes of 20–50 mm². Between six and eight mice were used per dose group. MTX-HSA doses up to the maximum tolerated dose and equivalent MTX doses were administered i.v. three times in weekly intervals. The maximum tolerated dose was defined as the dose at which <10% death and/or <20% body weight loss occurred. The doses used were as follows: 22 μmol/kg MTX HSA corresponding to 10 mg/kg MTX; 33 μmol/kg MTX-HSA corresponding to 15 mg/kg MTX; 44 μmol/kg MTX-HSA corresponding to 20 mg/kg MTX; 110 μmol/kg MTX corresponding to 50 mg/kg MTX; and 220 μmol/kg MTX corresponding to 100 mg/kg MTX. In some studies, this general design was slightly changed. Changes in tumor size and side effects were
recorded. The tumor volume was calculated using the formula \( a \times b^2 / 2 \) (a = larger diameter of the tumor, b = smaller diameter of the tumor). The antitumor activity was calculated by comparing the tumor volume of the treated group (T) on the treatment day as depicted in the Table 3 with the control group (C) or with the initial tumor volume \( (T_0) \), resulting in \( T/C \) values (percentages) and \( T/T_0 \) values (percentages). The control group was treated with vehicle alone (0.9% NaCl). The defined scores are explained in “Results.” The experiment was finished when tumor regrowth or severe side effects were observed.

RESULTS

Accumulation of MTX, HSA, and MTX-HSA in Human Prostate Cancer Cells. The uptake of \([ ^3H ]\)MTX and \([ ^3H ]\)MTX-HSA was measured in the human prostate cancer cell line DU-145. As shown in Fig. 1A, \([ ^3H ]\)MTX uptake is very rapid and reaches a plateau after 20 min. The cells accumulate 6 pmol \([ ^3H ]\)MTX/10^7 cells when treated with 2 \( \mu \)M \([ ^3H ]\)MTX for 60 min. After treatment of the cells with 50 \( \mu \)M \([ ^3H ]\)MTX-HSA for 60 min, accumulation of 6 pmol \([ ^3H ]\)MTX-HSA/10^7 cells was also measured. However, the accumulation continues to increase and reaches 15 pmol \([ ^3H ]\)MTX-HSA/10^7 cells after 3 h of incubation. Although the MTX-HSA solution was purified by Sephadex columns and used immediately before the experiments started, \( \approx 0.07\% \) free \([ ^3H ]\)MTX is still present in the purified solution. During the time of the experiment, the amount free MTX did not increase. This means that an incubation with 50 \( \mu \)M \([ ^3H ]\)MTX-HSA exposes cells additionally to at least 0.035 \( \mu \)M \([ ^3H ]\)MTX. Therefore, 0.05 \( \mu \)M \([ ^3H ]\)MTX itself was also investigated in the experiment. The accumulation in the cells when incubated with 0.05 \( \mu \)M \([ ^3H ]\)MTX reached 0.2 pmol/10^7 cells after 10 min and 1 pmol/10^7 cells after 3 h, which was considerably lower than the observed 15 pmol/10^7 cells after 3 h of incubation with 50 \( \mu \)M \([ ^3H ]\)MTX-HSA, indicating that the effect of the unconjugated \([ ^3H ]\)MTX is negligible.

These results demonstrate that higher concentrations of MTX-HSA compared with MTX are necessary to obtain similar intracellular concentrations and that distinct mechanisms are involved in the uptake of MTX and MTX-HSA. It has been hypothesized that intracellular MTX-HSA accumulation occurs through HSA-mediated endocytosis (7). If this is true, the uptake kinetics of MTX-HSA and HSA should be comparable. We therefore determined the accumulation of \([ ^{125}I ]\)HSA-MTX and \([ ^{125}I ]\)HSA in DU-145 cells treated with 10 \( \mu \)M \([ ^{125}I ]\)HSA-MTX or \([ ^{125}I ]\)HSA. Very similar accumulation kinetics were observed in \([ ^{125}I ]\)HSA-MTX and \([ ^{125}I ]\)HSA. After 30 min of incubation, 1.2 pmol \([ ^{125}I ]\)HSA-MTX/10^7 cells and 0.8 pmol \([ ^{125}I ]\)HSA/10^7 cells accumulated in the cells, as shown in Fig. 1B. After 2 and 4 h, this amount increased to 4.2 pmol \([ ^{125}I ]\)HSA-MTX/10^7 cells and 3.8 pmol \([ ^{125}I ]\)HSA/10^7 cells and to 5.2 pmol \([ ^{125}I ]\)HSA-MTX/10^7 cells and 5.4 pmol \([ ^{125}I ]\)HSA/10^7 cells, respectively.

Therefore, these results suggest that MTX-HSA uptake takes place via a HSA-mediated endocytosis process in DU-145 cells.

**TS In Situ Inhibition by MTX and MTX-HSA.** Inhibition of TS was determined in intact cells by measuring the antifolate-induced inhibition of TS-catalyzed conversion of \([ ^{3}H ]\)dUMP to dTMP, during which reaction the release of \([ ^{3}H ]\)H_2O in the medium is impaired (21, 22). TS in situ inhibition by MTX and MTX-HSA in KB cells grown at low folate conditions (Table 1) showed that MTX-induced inhibition of TS is at least 3 orders of magnitude more potent than MTX-HSA-induced inhibition. Following 4 h of drug incubation, 50% TS in situ inhibition was achieved with 16.5 \( n \)M MTX. For MTX-HSA, TS IC_{50} inhibition was not observed below 50 \( \mu \)M. Four

![Fig. 1 A](image1.png) DU-145 cells were treated with 2 \( \mu \)M \([ ^{3}H ]\)MTX or 50 \( \mu \)M \([ ^{3}H ]\)MTX-HSA. After the indicated period of time, cells were harvested, and the amount of accumulated radioactivity was measured as described in “Materials and Methods.” Results are the mean of three experiments; bars represent SD. * result of one experiment; ** result is the mean of two experiments (60 min \([ ^{3}H ]\)MTX). B, DU-145 cells were treated with 9.88 \( \mu \)M \([ ^{125}I ]\)HSA-MTX or \([ ^{125}I ]\)HSA. After the indicated period of time, cells were harvested, and the amount of accumulated radioactivity was measured as described in “Materials and Methods.”
h of drug incubation followed by 20 h of incubation in drug-free medium should reveal how efficiently MTX and MTX-HSA are retained within the cell by either polyglutamylated or endocytic vesicles. MTX is efficiently retained in KB cells; 50% TS in situ inhibition was achieved with 59.3 nM MTX. For MTX-HSA, KB cells became somewhat more sensitive when incubated without any drug for another 20 h compared with the effect induced after 4 h of incubation. An IC50 TS inhibition value of 40.4 μM was determined. When cells were continuously incubated for 24 h with MTX, IC50 TS in situ inhibition was achieved with 7.3 nM MTX. MTX-HSA-induced IC50 TS in situ inhibition was found at 6.9 μM MTX-HSA after a 24-h incubation period. These results suggest that a certain period of time is required to perform the necessary processes of MTX-HSA uptake, accumulation, and release of active compound into the cytosol before inhibition of dihydrofolate reductase and, consequently, TS takes place.

### Influence of Methylamine on the Cell Cycle Effect Induced by MTX and MTX-HSA.

Materials entering lysosomes become exposed to an acidic milieu with a pH between 4 and 5 and to a collection of many different digestive enzymes that are able to degrade a large variety of complex materials (24). To determine whether MTX-HSA is lysosomally degraded to liberate MTX (and excrete it into the cytoplasm), methylamine, a compound that is taken up in lysosomes and leads to an increase of the lysosomal pH (25), was used. Because lysosomal enzymes are active at acidic pH only, methylamine will considerably decrease enzymatic activity in lysosomes. To measure the effect of MTX-HSA with and without methylamine in vitro, the human lymphoblastic leukemia cell line CCRF-CEM was selected, and, as a sensitive parameter for the efficacy of the compounds, flow cytometric cell cycle analysis was used.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Drug incubation</th>
<th>TS IC50 (nM) MTX</th>
<th>TS IC50 (nM) MTX-HSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB (LF)**</td>
<td>4 h</td>
<td>16.5 ± 0.7</td>
<td>62,190 ± 17,010</td>
</tr>
<tr>
<td>KB (LF)</td>
<td>4 h + 20 h</td>
<td>59.3 ± 21.5</td>
<td>40,350 ± 3,910</td>
</tr>
<tr>
<td>KB (LF)</td>
<td>24 h</td>
<td>7.3 ± 0.3</td>
<td>6,940 ± 1,295</td>
</tr>
</tbody>
</table>

**LF, low folate (5 nM leucovorin + 10% dialyzed FCS); DFM, drug-free medium.

whereas 20 nM MTX did, as expected. In addition, the incubation of CCRF-CEM cells with 10 μM MTX-HSA for 48 h also resulted in theaccumulation of cells in the S phase of the cell cycle and the disappearance of the G2-M peak. The fact that 7 nM MTX had no influence on the cell cycle after 48 h proves that the observed effect is based on MTX-HSA. Furthermore, these results demonstrate that an incubation period of 24 h is sufficient for MTX to exert a strong S-phase block, whereas with MTX-HSA, this effect is only observed after 48 h and with higher concentrations. These results are in accordance with the results obtained with TS inhibition, where we demonstrated that MTX-HSA requires a longer period of time and higher concentrations than MTX to exert its effect.

To verify the hypothesis that MTX is released from MTX-HSA by a lysosomal process and delivered to the cytosol, where it binds to its target enzyme (dihydrofolate reductase) and induces the observed cell cycle alterations, CCRF-CEM leukemia cells were incubated with MTX and MTX-HSA in combination with methylamine. As shown in Fig. 3, methylamine antagonized the effect on the cell cycle induced by 10 μM MTX-HSA after 53 h of incubation. This effect was specific for MTX-HSA because it was not possible to antagonize the effect on the cell cycle induced by 20 nM MTX after 30 h of incubation by simultaneously adding methylamine. These results show that a lysosomal degradation to an active compound is necessary for the antitumor activity of MTX-HSA.

**Growth-inhibitory Effect of MTX and MTX-HSA.** Growth inhibition studies on a panel of human and murine leukemia cell lines (Table 2) revealed that, for the majority of the cell lines tested, MTX is approximately 3 orders of magnitude more potent than MTX-HSA. Furthermore, the growth-inhibitory effects of MTX-HSA parallel those of MTX because cell lines that are more sensitive/resistant to MTX are also more sensitive/resistant to MTX-HSA. An increase in unconjugated MTX in tissue culture medium due to external cleavage of MTX-HSA during the experiments was not observed (data not shown). Because the concentration of unconjugated MTX in the MTX-HSA preparations after purification was approximately 0.07%, it cannot be excluded that the growth-inhibitory effect of MTX-HSA is partially caused by the unconjugated MTX in the MTX-HSA preparation. Interestingly, MTX-HSA was only 42 and 35 times less potent than MTX in the MTX-resistant cells CEM/MTX and L1210-FBP cultured in high folic acid-containing medium, respectively (both cell lines are deficient in uptake through the reduced folate carrier), which cannot be explained by the unconjugated MTX in the preparation. These results demonstrate that MTX-HSA has a growth-inhibitory effect at a high concentration and that MTX-HSA can circumvent MTX resistance caused by a MTX transport defect. MTX-HSA does not display greater sensitivity to cell lines expressing either the reduced folate carrier or folate-binding protein as antifolate transport route. However, in cell lines expressing the folate-binding protein (L1210-FBP, KB, and MDCK-PK5), we observed that blocking folate-binding protein with folic acid reduces the growth-inhibitory effects of MTX-HSA. This result suggests that folate-binding protein is involved in the effect of MTX-HSA.

**Accumulation of MTX-HSA in Tumor Tissue.** Concentrations of radioactivity in the tumor tissue were measured at 1, 3, 8, and 48 h after administration of MTX-HSA to Walker-256 carcinoma-bearing female rats at a dose level of 13.2
\[ \text{\( \mu \text{mol/kg MTX-HSA}, \) which corresponds to 6 mg/kg MTX. At 1 h after administration, the MTX-HSA concentration measured to 25 nmol/g tumor tissue, which is approximately 25 \( \mu \text{M}. \) At 3 h after administration, the concentration in the tumor reached its maximum (29 nmol/g tumor tissue), which is approximately 29 \( \mu \text{M}. \) At 8 and 48 h after administration, 19 nmol/g (approximately 19 \( \mu \text{M} \)) and 18 nmol/g (approximately 18 \( \mu \text{M} \)) MTX-HSA, respectively, were measured. These results show that MTX-HSA is trapped in the tumor tissue for up to 48 h at concentrations between 18 and 29 \( \mu \text{M}. \)"

Efficacy of MTX-HSA in Nude Mice with Human Tumor Xenografts. The therapeutic efficacy of MTX and MTX-HSA was investigated in vivo in different human tumor xenografts growing s.c. in nude mice. As depicted in Fig. 4, A–D, MTX-HSA induced dose-dependent antitumor activity in vivo. When equivalent MTX doses were administered, superiority of MTX-HSA over MTX was observed. Strong antitumor effects (T/C \( \leq 50\% \)) were seen in the breast cancers MDA-MB-231 (Fig. 4A) and ZR-75-1 (Fig. 4B), in the mesotheliomas H-Messo-1 and MSTO-211 (Fig. 4C), in the prostate cancer PC3 (Fig. 4D), in the MTX-resistant leukemia CCRF-CEM/MTX, in the colon carcinoma T84, in the kidney carcinoma Caki, and in the lung cancer Calu3. A moderate antitumor activity corresponding to a tumor growth delay (T/C = 50–75\%) was observed in the bladder carcinoma BXF 1299, in the colon cancers Colo-205 and WiDr, and in the prostate cancer DU-145. No antitumor activity of MTX-HSA was found in the kidney cancer KTC-185-GM1. In these efficacy studies, 10–20\% of the animals died in the higher dose groups treated with 15–20 mg/kg MTX-HSA and 75–100 mg/kg free MTX, which is probably due to the much longer half-life of MTX-HSA. A reversible body weight decrease of up to 20\% of the initial value was observed in the surviving animals.

All results are summarized in Table 3. Fourteen tumors from eight different tissues were tested. The strongest antitumor activities were obtained in breast and mesothelium cancers. In summary, 9 of 14 tumors (64\%) showed a clear response with tumor inhibition, stasis, or partial regression, and only 5 of 14 tumors (36\%) gave moderate response with tumor growth delay or no response.

DISCUSSION
In the present study, we examined the mechanism of action of the drug conjugate MTX-HSA in human cancer cell lines. Internalization of the conjugate was shown by radioactive-labeled MTX-HSA. There are two types of receptors as candidates for MTX-HSA uptake, the albumin-binding proteins or the folate-binding protein, where MTX of the conjugate is the
Different uptake kinetics were measured for MTX and MTX-HSA. However, similar uptake kinetics were observed with HSA and MTX-HSA, suggesting that MTX-HSA is internalized by albumin-mediated endocytosis in DU-145 cells. However, in experiments with cell lines expressing the folate-binding protein, it was also observed that blocking the folate-binding protein with folic acid reduced the growth-inhibitory effect of MTX-HSA (Table 2). These results imply that MTX-HSA is also internalized by the binding of the MTX-HSA molecule to the folate-binding protein, resulting in folate-bind-

![Fig. 3](image)

**Fig. 3** Influence of methylamine on the cell cycle effect induced by MTX and MTX-HSA on CCRF-CEM cells. Cells were incubated with 30 nM MTX for 30 h or with 10 μM MTX-HSA for 53 h, with or without simultaneous addition of 3 mM methylamine. After the incubation periods, cell cycle analysis was performed as described in “Materials and Methods.”

**Table 2** Growth inhibition by MTX-HSA compared with MTX

Cells were exposed to the indicated drug for 72 h or 6 days (DU-145 cells); IC_{50} values are given as the concentration of drug at which growth is inhibited by 50% compared with controls. Values are the mean ± SD of three to five experiments.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Folate/serum status</th>
<th>RFC(^{a})</th>
<th>FBP</th>
<th>IC_{50} (nM) MTX</th>
<th>IC_{50} (nM) MTX-HSA</th>
<th>IC_{50} MTX-HSA/MTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCRF-CEM</td>
<td>2.2 μM FA/10% FCS</td>
<td>Pos</td>
<td>Neg</td>
<td>9.1 ± 1.0</td>
<td>16,300 ± 4,090</td>
<td>1,800</td>
</tr>
<tr>
<td>CEM-7A</td>
<td>0.15 mM LV/10% dialFCS</td>
<td>Pos</td>
<td>Neg</td>
<td>1.7 ± 0.7</td>
<td>2,100 ± 210</td>
<td>1,235</td>
</tr>
<tr>
<td>CEM/MTX</td>
<td>2.2 μM FA/10% FCS</td>
<td>Neg</td>
<td>Neg</td>
<td>1,885 ± 57</td>
<td>78,330 ± 3,565</td>
<td>42</td>
</tr>
<tr>
<td>CEM-R30dm</td>
<td>2.2 μM FA/10% FCS</td>
<td>Pos</td>
<td>Neg</td>
<td>18.3 ± 2.0</td>
<td>18,375 ± 1,750</td>
<td>1,002</td>
</tr>
<tr>
<td>L1210</td>
<td>2.2 μM FA/10% FCS</td>
<td>Pos</td>
<td>Neg</td>
<td>4.6 ± 0.6</td>
<td>4,235 ± 890</td>
<td>920</td>
</tr>
<tr>
<td>L1210-FBP</td>
<td>5 mM LV/10% dialFCS</td>
<td>Neg</td>
<td>Pos</td>
<td>12.4 ± 3.7</td>
<td>7,140 ± 2,080</td>
<td>590</td>
</tr>
<tr>
<td>L1210-FBP</td>
<td>2.2 μM FA/10% FCS</td>
<td>Neg</td>
<td>Pos</td>
<td>1,940 ± 114</td>
<td>66,975 ± 10,105</td>
<td>35</td>
</tr>
<tr>
<td>KB</td>
<td>4 mM LV/10% dialFCS</td>
<td>Pos</td>
<td>Pos</td>
<td>17.1 ± 3.0</td>
<td>17,960 ± 2,290</td>
<td>1,055</td>
</tr>
<tr>
<td>KB</td>
<td>2.2 μM FA/10% FCS</td>
<td>Pos</td>
<td>Pos</td>
<td>19.6 ± 1.0</td>
<td>30,940 ± 9,685</td>
<td>1,579</td>
</tr>
<tr>
<td>MDCK-3</td>
<td>2.2 μM FA/10% FCS</td>
<td>Pos</td>
<td>Neg</td>
<td>18.7 ± 0.7</td>
<td>16,785 ± 1,035</td>
<td>998</td>
</tr>
<tr>
<td>MDCK-PK5</td>
<td>2.2 μM LV/10% dialFCS</td>
<td>Pos</td>
<td>Pos</td>
<td>16.2 ± 2.5</td>
<td>5,395 ± 1,130</td>
<td>330</td>
</tr>
<tr>
<td>MDCK-PK5</td>
<td>2.2 μM FA/10% FCS</td>
<td>Pos</td>
<td>Pos</td>
<td>36.9 ± 5.2</td>
<td>30,290 ± 9,435</td>
<td>821</td>
</tr>
<tr>
<td>DU-145</td>
<td>2.2 μM FA/10% FCS</td>
<td>Pos</td>
<td>?</td>
<td>8.0 ± 3.5</td>
<td>6,667 ± 3,512</td>
<td>833</td>
</tr>
</tbody>
</table>

\(^{a}\) RFC, reduced folate carrier; FA, folic acid; LV, leucovorin; FBP, folate-binding protein; FPGS, folylpolyglutamate synthetase; Pos, positive; Neg, negative.
Leamon and Low (26) showed previously that folic acid conjugated to proteins is taken up by cells via endocytosis after binding to the folate-binding protein in the cell membrane. The accumulation studies with DU-145 were performed in the presence of 2.2 μM folic acid, a concentration that blocks folate-binding protein. Thus, a possible folate-binding protein-mediated endocytosis is not expected to be observed in DU-145 cells under the experimental condition chosen. In conclusion, both albumin-mediated endocytosis and folate-binding protein-mediated endocytosis are possibly involved in the growth-inhibitory effect of MTX-HSA.

For CCRF-CEM cells, it was shown by Jansen et al. (17) that the dihydrofolate reductase level in the cells amounts to 2.8 ± 0.5 pmol [3H]MTX binding/mg protein, which is approximately 1.9–2.8 pmol dihydrofolate reductase/10^7 cells. The dihydrofolate reductase levels were not determined in DU-145 cells, but presumably DU-145 cells contain similar levels. Therefore, [3H]MTX, [3H]MTX-HSA, and 125I-HSA-MTX accumulated in DU-145 cells (Fig. 1A and B) at the necessary concentrations to inhibit the target enzyme dihydrofolate reductase when using this assumption. However, as shown in Fig. 1A, incubation with a higher concentration of MTX-HSA than of MTX is needed to obtain the critical intracellular concentration.

Treatment with methylamine, an inhibitor of the lysosomal proteolytic pathway (25), led to the inhibition of MTX-HSA-induced cell cycle alterations, indicating that lysosomal degradation of MTX-HSA to an active compound (MTX or MTX-lysine) is a prerequisite for its activity. Also, the divergence in time after which the effects of MTX and MTX-HSA on TS and cell cycle were observed is an indication of a slower release of an active compound out of MTX-HSA in the cytoplasm compared with the direct effect of MTX. Therefore, endocytosis is presumably responsible for cellular uptake of MTX-HSA with subsequent lysosomal degradation to an active form of MTX.

This active form exerts its action on the dihydrofolate reductase, resulting in TS inhibition, cell cycle alterations, and growth inhibitory effects.

Fig. 4: Effect of MTX-HSA and MTX treatment on MDA-MB-231 (A), ZR-75-1 (B), MSTO-211 (C) and PC3 (D) xenografts. Mice were treated with 0.9% NaCl solution, MTX-HSA, or MTX as indicated in the figure. Results are the mean of six to eight mice. Bars, SE.
inhibition of tumor cells, including MTX-resistant tumor cells. These results are in accordance with previous findings in which it was demonstrated that a MTX-HSA-monomoclonal antibody conjugate is internalized by endocytosis and transferred to the lysosomal compartment. There the action of lysosomal enzymes is distinguished from MTX in cell lines that do not express the folic acid receptors, and the effect of MTX-HSA can only clearly be demonstrated in vitro tests. Thus, these results suggest that cytotoxic concentrations of MTX-HSA are reached in the tumor tissue, which makes MTX-HSA an effective antitumor agent, even in MTX-resistant tumors.

We observed in vitro that MTX was more effective than MTX-HSA and that the effect of MTX-HSA can only clearly be distinguished from MTX in cell lines that do not express the reduced folate carrier (CEM/MTX cells and L1210-FBP cells in 2.2 µM folic acid; Table 2). However, the advantage of MTX-HSA over MTX becomes apparent in vivo. When equivalent MTX doses were administered, superiority of MTX-HSA over MTX was observed, as depicted in Fig. 4, A-D. In vivo, an important difference between MTX-HSA and MTX is their completely different pharmacokinetic behavior, the accumulation of MTX-HSA in tumor tissue because of the enhanced microvascular permeability and the EPR effect in macromolecular therapeutics: a review. J. Controlled Release, 65: 271–284, 2000. 4. Matsumura, Y., and Maeda, H. A new concept for macromolecular therapeutics: mechanism of tumoricidal accumulation of proteins and the antitumor agent smancs. Cancer Res., 46: 6387–6392, 1986.

Table 3  Effect of MTX-HSA treatment on human tumor xenografts

<table>
<thead>
<tr>
<th>Tumor cell line</th>
<th>Optimal tumor response in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZR-75-1</td>
<td>Day 28, T/C 6/19</td>
</tr>
<tr>
<td>MSTO-211</td>
<td>+ + +</td>
</tr>
<tr>
<td>CEM/MTX</td>
<td>20/10 82</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>11/10 +</td>
</tr>
<tr>
<td>Calu3</td>
<td>21/23 +</td>
</tr>
<tr>
<td>H-Messo-1</td>
<td>19/29 +</td>
</tr>
<tr>
<td>PC3</td>
<td>25/35 +</td>
</tr>
<tr>
<td>T84</td>
<td>19/37 +</td>
</tr>
<tr>
<td>Caki</td>
<td>15/47 +</td>
</tr>
<tr>
<td>BXF 1299</td>
<td>18/56 –</td>
</tr>
<tr>
<td>Colo-205</td>
<td>5/71 –</td>
</tr>
<tr>
<td>WiDr</td>
<td>13/52 –</td>
</tr>
<tr>
<td>DU-145</td>
<td>11/59 –</td>
</tr>
<tr>
<td>KTC-185-GM1</td>
<td>18/81 –</td>
</tr>
</tbody>
</table>

<sup>a</sup> Activity rating: - -, inactive, T/C > 50%; + , tumor inhibition, T/C > 25–50%; + +, tumor stasis, T/C ≈ 25%; T/T<sub>o</sub> > 75–125%; + + +, partial regression, T/C ≤ 25%, T/T<sub>o</sub> > 10–75%; and + + + +, complete remission, T/C ≤ 25%, T/T<sub>o</sub> ≤ 10%.

We thankfully acknowledge the productive cooperation with E. Frei and M. Weigand from the DKFZ (Heidelberg, Germany), and we thank I. Kathmann and L. Comijn for technical assistance.

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In Vitro and in Vivo Antitumor Activity of Methotrexate Conjugated to Human Serum Albumin in Human Cancer Cells

Katja Wosikowski, Elfi Biedermann, Benno Rattel, et al.


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