Cancer Chemother Pharmacol (1991) 28: 377–383
034457049100120B

Cancer Chemotherapy and Pharmacology

Inhibition of 7-hydroxymethotrexate formation by amsacrine*

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Received 21 January 1991/Accepted 14 April 1991

Summary. The inhibition of methotrexate (MTX) biotransformation to 7-hydroxymethotrexate (7-OH-MTX) by 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide (mAMSA) was studied in bile-drained rats in vivo and in incubates of isolated rat hepatocytes and rat-liver homogenate in vitro. In vivo, i.v. administration of 10 mg/kg mAMSA prior to [^{3}H]-MTX infusion (50 mg/kg) led to a significant alteration in 7-OH-MTX kinetics. 7-OH-MTX peak concentrations and AUC in bile and serum were reduced by 75% and the recovery of MTX as 7-OH-MTX in bile and urine decreased by 70%, whereas MTX pharmacokinetics remained unaltered. In suspensions of isolated hepatocytes, 10 μM mAMSA led to a 54% decrease in 7-OH-MTX formation. However, the hepatocellular influx and efflux of MTX was not perturbed by mAMSA. Preincubation of rat-liver homogenates with 1.25–10 μM mAMSA reduced the formation of 7-OH-MTX by up to 73%. mAMSA appeared to inhibit MTX hydroxylation competitively, exhibiting a K_i of 3 μM. Due to its inhibition of the MTX-oxidizing system, mAMSA may be beneficial in combination chemotherapy with MTX by reducing 7-OH-MTX-associated toxicity and, possibly, enhancing the cytotoxic effects of MTX.

Introduction

High-dose methotrexate (HD-MTX) anticancer therapy (1–34 g/m^2) [35] results in up to millimolar serum concentrations of the major extracellular metabolite of MTX 7-hydroxymethotrexate (7-OH-MTX) [5]. In man, 7-OH-MTX levels surpass serum MTX concentrations at 3–10 h after the cessation of MTX infusions [7], and are up to 140 times higher than the corresponding serum MTX concentrations at 12–48 h postinfusion [7, 8, 20, 21, 50].

Aldehyde oxidase metabolizes the parent drug to 7-OH-MTX in rabbit tissues [33], whereas the identity of the MTX-oxidizing system in the rat and man remains unknown. The metabolite's cytotoxicity to proliferating cells is 40–200 times lower than that of the parent compound [14, 25, 28, 43]. Like MTX, the 7-hydroxy metabolite can be converted intracellularly to the polyglutamated forms by folylpolyglutamate synthetase [24]. Although 7-OH-MTX cytotoxicity is increased several-fold by polyglutamylation [18, 49], the inhibition of dihydrofolate reductase, the main target enzyme, is minute as compared with that of equivalent intracellular levels of MTX polyglutamates [48]. In view of its considerably lower cytotoxicity, 7-OH-MTX may thus reduce MTX cytotoxicity [27, 42] by interfering with cellular drug entry [23] and subsequent synthesis of the more potent MTX polyglutamates [23, 24, 38, 44].

7-OH-MTX is 3–5 times less soluble than MTX and has been shown to induce MTX-associated renal failure by precipitating in the renal tissue of primates [30, 31]. Furthermore, recent reports have shown that at high biliary concentrations, 7-OH-MTX may precipitate in the alkaline rat bile in vivo and in vitro [10, 11].

Using a rat model, we have investigated several compounds that could potentially interact with the production of 7-OH-MTX. The vinca alkaloid vindesine has been found to suppress hepatocellular MTX uptake and, subsequently, 7-OH-MTX production in the rat in vitro [12]. Interference at the level of MTX catabolism may be another way to reduce 7-OH-MTX production. Gormley et al. [29] have found that 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (mAMSA), an acridine derivative that is effective in the treatment of human leukemias and malignant lymphomas [39, 41], is a potent aldehyde oxidase inhibitor. Hence, we investigated the capability of mAMSA to reduce the formation of 7-OH-MTX in rats in vivo, in isolated rat hepatocytes in suspension, and in rat-liver homogenate.

* This study was financially supported by the Norwegian Cancer Society and the Erna and Olav Aakre Foundation for Cancer Research

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Materials and methods

Drugs and solutions. L-Glutamyl-3,4-[^{3}H]-MTX (sp. act., 48.7 Ci/mmol; purity, 99.8% as determined by HPLC) was purchased from New England Nuclear (Boston, Mass., USA). Formulated MTX (purity, 99% as determined by HPLC) was a gift from Nycomed A/S (Oslo, Norway), and 7-OH-MTX was generously donated by Dr. F. M. Sirotnak (Memorial Sloan-Kettering Cancer Center, New York, N. Y., USA). Formulated mAMSA (Amsakrin) was a gift from Bristol-Myers Scandinavia Ltd. (Oslo, Norway). Collagenase (300 IU/mg, type I), bovine serum albumin (fraction V, defatted), and *N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) were obtained from Sigma Chemical Company (St. Louis, Mo., USA). Methanol and tetrahydrofuran (both HPLC-grade) were supplied by Rathburn Chemicals (Walkerburn, UK). All other reagents were of analytical grade. All samples containing MTX and 7-OH-MTX were stored under protection from light at −20° C for a maximum of 2 weeks.

Experimental procedures in rats in vivo. Male Wistar rats weighing 270–330 g (Charles River, Wiga GmbH, Sulzseld, FRG) were used for all experiments. For the in vivo experiments, 12 rats were randomly allocated to 2 groups. The animals were anesthetized during the experiments and their right external jugular vein and bile duct were cannulated [9] prior to immediate transfer of the rats to restraining cages. The experiments were carried out at ambient temperatures, and the body temperatures of the animals were not regulated.

On the day of experimentation, the mAMSA lactate was dissolved in 50 mg/ml glucose to a final concentration of 1 mg/ml (pH 5). MTX solutions were prepared by dissolving the drug in 50 mg/ml glucose to a concentration of 5 mg MTX/ml and adding [^{3}H]-MTX to a final activity of 7.7 μCi/ml (pH 7).

The + mAMSA group received brief infusions (10 min) of 10 mg/kg mAMSA through a central venous catheter, whereas control rats (– mAMSA) were given the diluent (pH 5). The infusions were terminated at 5 min prior to the administration of [^{3}H]-MTX (50 mg/kg, 10 min). The venous catheters were flushed with heparinized (10 IU/ml) saline immediately after the infusions and following each subsequent blood-sampling period.

Venous samples of 200 μl were drawn from the catheters both prior to and immediately after drug administration and, subsequently, at 2, 5, 10, 15, 30, 45, 60, 90, and 120 min after cessation of the MTX infusion. Bile samples were obtained prior to and during the period of MTX administration (10 min), at 15-min intervals for the initial 60 min thereafter, and at 30-min intervals for the last 60 min. Voided urine was collected via funnels during the procedures; after the animals had been killed, the urinary bladders were aspirated to ensure complete collection. pH was measured in samples of bile and urine. Venous blood samples for blood-gas and hematocrit analyses were drawn from the venous catheters at the end of the experiments. All animals received maintenance fentanyl anesthesia and were hydrated and alkalinized as previously described [9].

Experimental procedures in isolated rat hepatocytes. Rat-liver cells were prepared essentially according to the method of Berry and Friend [4, 47]. Following collagenase perfusion, the livers were perfused for 1 min with a collagenase-free solution at 20° C. Hepatocytes were separated from nonparenchymal cells by centrifugation [3], and the cells were washed twice in the incubation medium. Cell viability was assessed by trypan blue exclusion [26], and cell counts were obtained using a hemocytometer. The mean viability was 94 ± 1.8 at the start of the studies and 89.2 ± 1 at the termination of experiments 3.5 h later.

Freshly isolated rat hepatocytes were incubated in a medium containing 137 mM NaCl, 5.37 mM KCl, 0.81 mM $MgSO_4$, 4 mM $CaCl_2$ 0.34 mM KH_2PO_4, 20 mM HEPES buffer, and 2% bovine serum albumin. The solution was adjusted to pH 7.4 with NaOH. After 30 min agitation at 37° C in 100-ml capped flasks, hepatocyte suspensions (5 ml, 1.7×10^6 cells/ml) were preincubated with mAMSA for 30 min prior to the addition of [^{3}H]-MTX. MTX and mAMSA solutions were added at concentrations 100-fold that in the final incubate (MTX, 10–200 μM; mAMSA, 1.25–10 μM). Control and experimental cell suspensions were incubated in parallel and were always taken from the same rat-hepatocyte suspension.

At 3 h after MTX addition, 4.5 ml of the hepatocyte suspension was pipetted into 10-ml conical polyethylene tubes, which had been preweighed twice on a Mettler AE 163 electronic balance. The hepatocyte sample was immediately centrifuged at 140 *g* for 1 min. The supernatant was siphoned off, and the cell pellets were subsequently washed twice with ice-cold isotonic saline (0° C) and then centrifuged (140 *g*, 1 min). After freeze-drying overnight (18 h), tubes containing the cell pellets were weighed twice. The dry weight of the hepatocytes was calculated as the difference in the mean weight of tubes with and those without pellets. The variability in duplicate weights of pellet-containing tubes was 0.78% of the mean cell-pellet weights and, hence, was negligible. The total sample recovery was 101.6%, and the loss of MTX and 7-OH-MTX during the washing procedures constituted only 2.9% of total recovery.

For cell-transport experiments, hepatocyte suspensions (5 ml, 1.6×10^6 cells/ml) were incubated with agitation for 30 min at 37° C prior to preincubation with 1.25, 2.5, 5, and 10 μM, mAMSA or diluent for 30 min. Then, the MTX-influx study was initiated by the addition of [^{3}H]-MTX to a final concentration of 10 μM, and 250-μl aliquots were removed from the incubates at 0.5, 1, 1.5, 2, 2.5, 3, 5, and 10 min thereafter. At 30 min after MTX addition, the residual 3 ml of the hepatocyte suspensions containing 0, 2.5, and 10 μM mAMSA was washed twice in ice-cold isotonic saline (0° C) and then centrifuged at 140 *g* for 1 min. The cell pellet was resuspended in drug-free incubation medium (37° C) and incubated at 37° C. At 5, 10, 15, 20, 25, and 30 min after the washings, 250 μl aliquots of the incubation media were removed. The samples obtained during the influx and efflux studies were immediately pipetted into 1 ml ice-cold isotonic saline (0° C) overlying a mixture of dinonyl phtalate and dibutyl phtalate (1 : 3, v/v; 250 μl) in 1.5-ml polyethylene microcentrifuge tubes. The cells were separated from the medium by centrifugation in less than 10 s [1]. The procedure for determination of the cell-associated radioactivity has been described in detail elsewhere [1].

Experimental procedures in rat-liver homogenate extract. Five rats received ether anesthesia prior to laparatomy and rapid removal of the liver. The liver was quickly rinsed in ice-cold 0.25 M sucrose solution (0° C), weighed, minced with scissors, and homogenized with a Potter-Elvehjem homogenizer in 1 : 4 g/ml 0.25 M sucrose solution (0° C). The procedures were carried out at 4° C and all reagents and equipment were kept on ice. Following centrifugation of the crude homogenate at 7,600 *g* for 15 min, the supernatant was removed and used for incubation experiments. Cell-free portions (5 ml) were pipetted into 100-ml flasks. After 10 min incubation with agitation at 37° C, the homogenate was preincubated with mAMSA for 5 min prior to the addition of MTX. MTX and mAMSA solutions were added at concentrations 100-fold those in the final incubate (MTX, 10–200 μM; mAMSA, 1.25–10 μM). At 10, 20, 30, 45, and 60 min after MTX addition, 400-μl aliquots were removed from the incubation medium and immediately placed on ice. MTX recovery was 102.5%. The protein content of the cell-free system (homogenate) was 26.64 mg/ml according to Bradford's method using albumin as a reference standard [6].

Analytical procedures. Analyses of MTX, 7-OH-MTX, and polyglutamates 1–3 in serum, bile, urine, hepatocytes, supernatants, and homogenate were performed using reverse-phase high-pressure liquid chromatography (HPLC), fraction sampling, and determination of radioactivity as reported elsewhere [9], except that cell pellets were resuspended in 0.5 ml isotonic saline and vortex-mixed for 2 min prior to precipitation with 2 M perchloric acid (PCA) and subsequent injections of the supernatants onto the HPLC column. The assay detects MTX and its major extracellular metabolites 7-OH-MTX and 2,4-diamino-N^{10}-methylpteroic acid (DAMPA), with no interference by MTX polyglutamates 1–3 or mAMSA.

Calculations and statistics. MTX serum concentrations were analyzed according to a two-compartment open model. Pharmacokinetic parameters were obtained by linear regression analysis in a semilogarithmic data set and refer to the biexponential equation

$c = Ae^{-\alpha t} + Be^{-\beta t}$.

Total clearance, C_T, was calculated by the equation

$C_T = \text{Dose}/(AUC_0 + A/\alpha + B/\beta)$,

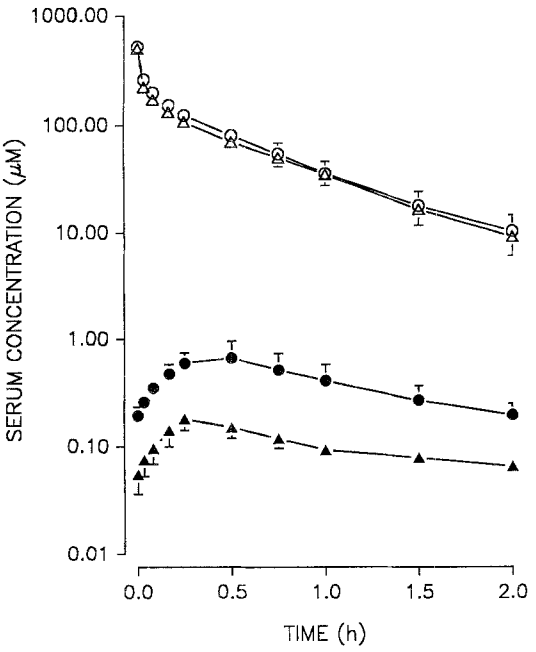

Fig. 1. Serum concentrations of MTX (*open symbols*) and 7-OH-MTX (*closed symbols*) vs time following brief infusions of 50 mg/kg [^{3}H]-MTX. MTX and 7-OH-MTX in animals pretreated with 10 mg/kg mAMSA are denoted by *triangular symbols*, whereas those of controls are denoted by *circular symbols*. All animals were anesthetized and subjected to biliary drainage. Data represent the mean ± SEM ($n = 6$)

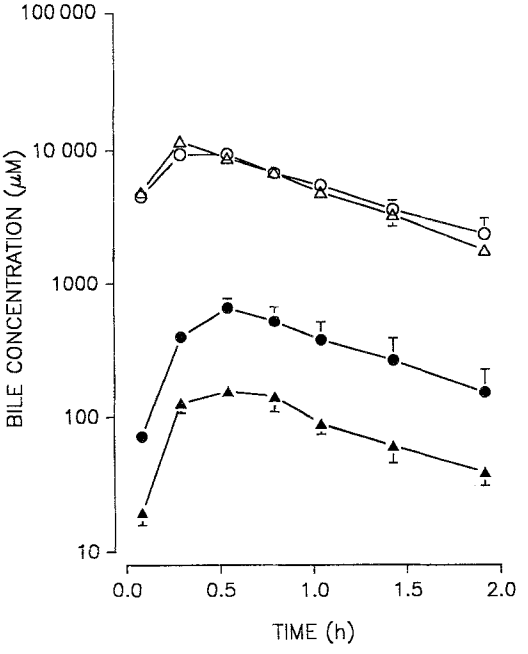

Fig. 2. Biliary concentrations of MTX and 7-OH-MTX vs time following brief infusions of 50 mg/kg [^{3}H]-MTX in the presence and absence of pretreatment with 10 mg/kg mAMSA (symbols and procedures as described in Fig. 1)

where AUC_0 is the area under the curve during drug infusion (10 min) as calculated using a triangular area and A and B are the zero-time intercepts of the extrapolated lines of the α- and β-phases, respectively. Biliary clearance was calculated by the equation

$C_B = (\text{Bile flow} \times C_B)/C_S$,

where C_B and C_S are the corresponding concentrations in bile and serum, respectively. The central volume of distribution, V_c, was obtained by dividing the dose by the sum of A and B, and the apparent volume of distribution in the post-distributional phase, V_β, was calculated by dividing total clearance by β. The AUCs for 7-OH-MTX concentrations in serum (S-AUC_{7OH}) and bile (B-AUC_{7OH}) were calculated using the trapezoidal rule. The microcomputer program Enzfitter (Elsevier-Biosoft, London, UK) was used for analysis of the Michaelis-Menten kinetics of the hydroxylation processes.

Statistical analyses of the in vivo data were performed using the nonparametric Mann-Whitney U-test (Microstat; Ecosoft Inc., Indianapolis, Ind., USA). The in vitro data were analyzed by one-way analysis of variance and estimation of least significant distance (Statgraphics; STSC, Rockville, Mass., USA). Statistical significance was defined as $P < 0.05$. All results were expressed as mean values ± SEM.

Results

MTX hydroxylation in vivo

Following brief infusions of 50 mg/kg MTX (10 min) in both groups of animals, the serum MTX concentration-decay curves exhibited a rapid initial phase (10 min) and a considerably slower second phase (Fig. 1). The pharmacokinetic variables of MTX were not affected by mAMSA pretreatment (Table 1). Serum 7-OH-MTX concentrations reached peak levels at 15–30 min after cessation of the MTX infusion (Fig. 1), with similar elimination half-lives being found for the two groups (66.3 and 80.6 min, $n = 6$). Mean serum 7-OH-MTX peak levels (0.18 ± 0.04 vs 0.67 ± 0.29 μM) and S-AUC_{7OH} values (Table 1) were reduced 4-fold in mAMSA-treated animals as compared with controls.

Table 1. Pharmacokinetic variables of MTX and 7-OH-MTX in rats infused with 50 mg/kg [^{3}H]-MTX over 10 min

Drug	Pharmacokinetic parameters	−mAMSA	+mAMSA
MTX	$t_{1/2\alpha}$ (min)	2.74 ±0.43	2.43 ±0.24
	$t_{1/2\beta}$ (min)	26.9 ±3.1	28.0 ±3.6
	V_c (ml/kg)	276 ±18	329 ±40
	V_β (ml/kg)	413 ±13	483 ±47
	C_T (ml min^{-1} kg^{-1})	11.3 ±1.1	12.9 ±2.1
	C_B[a] (ml min^{-1} kg^{-1})	8.2 ±1.3	9.4 ±1.9
	C_{NB}[b] (ml min^{-1} kg^{-1})	3.1 ±0.3	3.5 ±1.1
7-OH-MTX	$t_{1/2}$ (min)	66.3 ±12.6	80.6 ±9.5
	S-AUC_{7OH}[c] (μM × min)	0.049±0.017	0.013±0.002*
	B-AUC_{7OH}[d] (μM × min)	44.5 ±12.2	9.4 ±1.7*

At 5 min prior to the administration of MTX, 10-min infusions of mAMSA (10 mg/kg, +mAMSA) or diluent (glucose, −mAMSA) had been terminated. All animals were anesthetized and subjected to biliary drainage during the experiments. Data represent the mean ± SEM ($n = 6$)

[a] $C_B = (\text{Bile flow} \times c_B)/c_S$ (see Materials and methods)

[b] C_{NB} is nonbiliary clearance ($C_{NB} = C_T - C_B$)

[c] AUC for 7-OH-MTX concentrations in serum

[d] AUC 7-OH-MTX concentrations in bile

* $P < 0.05$ between the groups

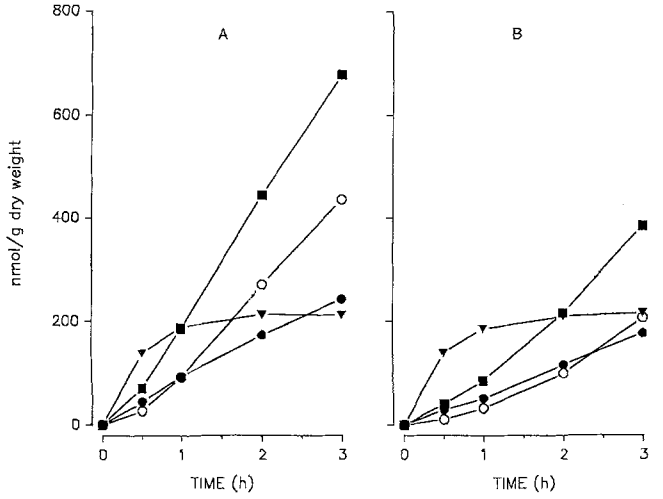

Fig. 3 A, B. MTX hydroxylation in isolated rat hepatocytes. Hepatocyte suspensions were preincubated with **A** 50 mg/ml glucose solution (control) or **B** 10 μM mAMSA for 30 min prior to the addition of 10 μM [^{3}H]-MTX. The incubates were sampled at 0.5, 1, 2, and 3 h after MTX addition, and the formation of 7-OH-MTX as well as the intra- and extracellular distribution of MTX and 7-OH-MTX were assessed. Data represent the means of 4 experiments. *Open circles*, extracellular 7-OH-MTX; *filled circles*, intracellular 7-OH-MTX; *filled squares*, total 7-OH-MTX formed; *filled triangles*, intracellular MTX

In bile, MTX and 7-OH-MTX concentrations reached peak levels within 30 min after termination of the MTX infusion, which were followed by monophasic elimination profiles showing similar half-lives ranging from 30 to 39 min (Fig. 2). Biliary clearance of MTX (C_B) was not influenced by mAMSA pretreatment and constituted 73% of the total body clearance (C_T) in both groups (Table 1). In contrast, the biliary peak levels (164 ± 23 vs 633 ± 101 μM) and AUC (B-AUC$_{7OH}$) for the metabolite were reduced 4- to 5-fold by pretreatment with mAMSA (Table 1).

The cumulative recovery of MTX in bile and urine (summarized in Table 2) shows that the biliary and renal systems eliminated 45%–47% and 22%–26% of the MTX dose, respectively. mAMSA-treated rats demonstrated 3–4 times lower biliary (0.71% vs 2.83%, $n = 6$) and urinary (0.019% vs 0.055%, $n = 6$) elimination of 7-OH-MTX as compared with controls. Meanwhile, there was no alteration in the biliary or urinary recovery of MTX related to mAMSA pretreatment.

During the experiments, mAMSA-treated animals secreted mean biliary volumes (3.25 ± 0.25 ml) that were significantly larger than those observed in control rats (2.57 ± 0.15 ml), whereas mean urinary volumes of 0.85 and 1.04 ml, respectively, were similar in the two groups. Biliary pH was equivalent in the groups (8.17 and 8.24, $n = 6$) and remained constant throughout the 2-h experiments; urinary pH was 6.1 and 6.4 ($n = 6$) at the end of the experiments. On termination of the experiments, venous pH and hematocrit values were similar in the two groups, being 7.34–7.37 ($n = 6$) and 0.34 ($n = 6$), respectively.

Table 2. Percentage of the cumulative biliary and urinary recovery of MTX 7-OH-MTX in rats at 2 h after the i.v. infusion of 50 mg/kg [^{3}H]-MTX over 10 min

		−mAMSA	+mAMSA
Bile	MTX	43.9 ±1	44.6 ±4.7
	7-OH-MTX	2.83 ±0.57	0.71 ±0.15*
Urine	MTX	25.7 ±3.2	21.5 ±5.8
	7-OH-MTX	0.055±0.01	0.019±0.004*

At 5 min prior to the administration of MTX, 10-min infusions of mAMSA (10 mg/kg, +mAMSA) or diluent (glucose, −mAMSA) had been terminated. All animals were anesthetized and subjected to biliary drainage during the experiments. Data represent the mean ± SEM ($n = 6$)

* $P < 0.05$ between the groups

Table 3. Inhibition of MTX hydroxylation in isolated rat hepatocytes by incubation (3 h) in various concentrations of mAMSA at 37° C

mAMSA (μM)	% of MTX hydroxylated	% decrease in total 7-OH-MTX formation
0	7.65±0.47	–
1.25	7.13±0.32	6.7
2.5	6.35±0.15*	16.9
5	5.14±0.13**	32.8
10	3.54±0.13***	53.6

The amount of 7-OH-MTX formed was expressed as a percentage of the total concentration of [^{3}H]-MTX (10, 25, 50, 100, and 200 μM) added to the incubates. Data represent the mean ± SEM ($n = 5$)

* $P < 0.05$ between 0 and 2.5 μM mAMSA; ** $P < 0.05$ between 2.5 and 5 μM mAMSA; *** $P < 0.05$ between 5 and 10 μM mAMSA

MTX hydroxylation in isolated rat hepatocytes

To assess intra- and extracellular levels of MTX and 7-OH-MTX, hepatocyte suspensions with or without 10 μM mAMSA were sampled at 0.5, 1, 2, and 3 h after MTX addition. The distribution of 7-OH-MTX in the hepatocyte suspensions and the intracellular appearance of MTX are shown in Fig. 3A. Intra- and extracellular 7-OH-MTX levels were significantly lower in the mAMSA-containing incubates (Fig. 3B). The proportion of 7-OH-MTX correlated inversely with increasing mAMSA concentrations in the hepatocyte suspensions (Table 3). The hydroxylation process was significantly decreased (16.9%) at 2.5 μM mAMSA, and at 10 μM mAMSA it was reduced by 54%.

MTX hydroxylation in rat-liver homogenate

The MTX hydroxylation process was linear for at least 1 h after the addition of MTX to the medium. We assessed the rate of 7-OH-MTX formation at 30 min after MTX addition. In control incubates, 7.3% of the added MTX was converted to 7-OH-MTX without a proportional reduction in 7-OH-MTX formation at increasing MTX concentrations from 10 to 200 μM. The hydroxylation process was significantly inhibited (22.9%) at 1.25 μM mAMSA (Table 4). A 72.9% reduction in MTX hydroxylation was

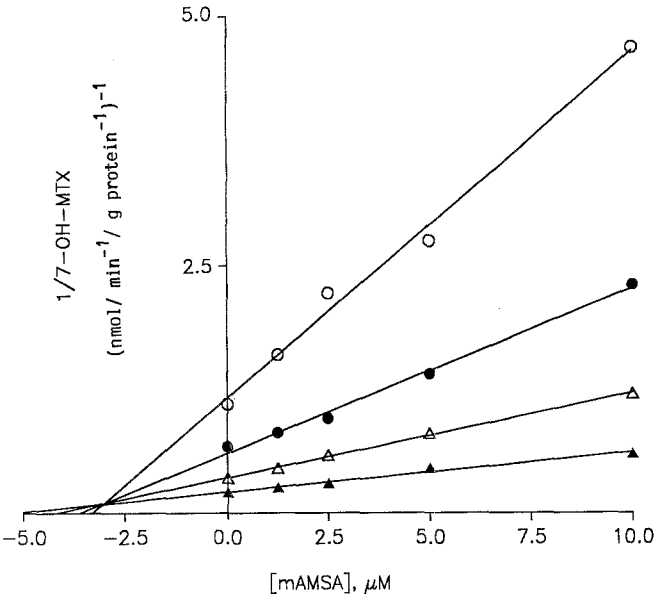

Fig. 4. Dixon plot analysis of 7-OH-MTX formation in the presence of mAMSA in the 7,600 *g* supernatant of crude rat-liver homogenate. Batches of the supernatant were preincubated with 0–10 μM mAMSA at 5 min prior to the addition of [^{3}H]-MTX. At 30 min after MTX addition, incubation medium was sampled to assess the rate of 7-OH-MTX formation relative to mAMSA concentrations. *Open circles*, 10 μM [^{3}H]-MTX; *closed circles*, 25 μM [^{3}H]-MTX; *open triangles*, 50 μM [^{3}H]-MTX; *closed triangles*, 100 μM [^{3}H]-MTX

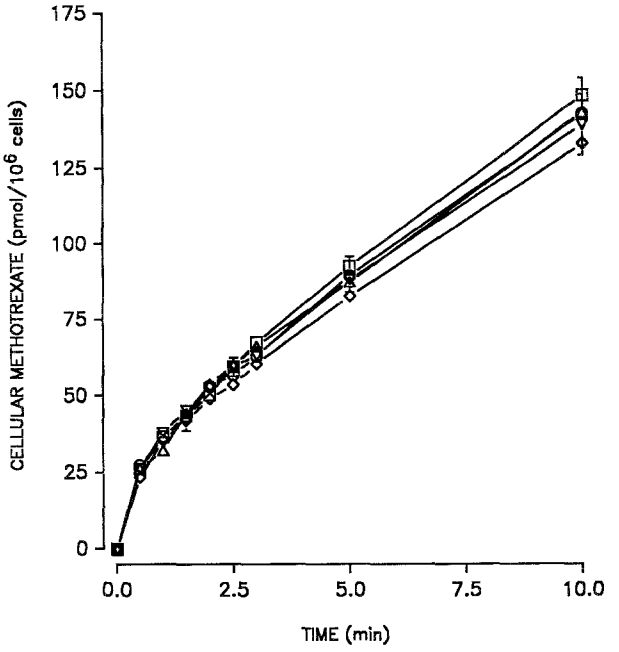

Fig. 5. Cellular accumulation of MTX vs time in the presence or absence of mAMSA. Rat hepatocytes in suspension were exposed to 10 μM [^{3}H]-MTX after preincubation with mAMSA at increasing concentrations (1.25–10 μM) or to the diluent (glucose). Data represent the mean ± SEM ($n = 3$). *Open circles*, Control; *open triangles*, 1.25 μM mAMSA; *open squares*, 2.5 μM mAMSA; *open inverse triangles*, 5 μM mAMSA; *open diamonds*, 10 μM mAMSA

Table 4. Inhibition of MTX hydroxylation by mAMSA in rat-liver homogenate

mAMSA (μM)	% of MTX hydroxylated	% decrease in total 7-OH-MTX formation
0	7.34±0.74	–
1.25	5.66±0.52*	22.9
2.5	4.54±0.47	38.1
5	3.04±0.43**	58.6
10	1.99±0.33***	72.9

Batches of the 7,600-*g* supernatant of the crude liver homogenate were incubated with 10, 25, 50, 100, and 200 μM [^{3}H]-MTX at various concentrations of mAMSA for 30 min at 37° C. The amount of 7-OH-MTX formed was expressed as a percentage of the total concentration of MTX initially added to the incubation medium. Data represent the mean ± SEM ($n = 5$)

* $P < 0.05$ between 0 and 1.25 μM mAMSA; ** $P < 0.05$ between 1.25 and 5 μM mAMSA; *** $P < 0.05$ between 2.5 and 10 μM mAMSA

seen following preincubation with 10 μM mAMSA. Double reciprocal plots (not shown) gave an apparent V_{max} of 7.2 ± 0.2 nmol min^{-1} g protein^{-1}, whereas the calculated value (Enzfitter) was 19 ± 5.3 nmol min^{-1} g protein^{-1}. The Dixon plot shown in Fig. 4 yielded an apparent K_i value of 2 μM. The similar concentrations of MTX polyglutamates 1–3 found in mAMSA-incubated and control homogenate portions (not shown) may indicate that the action of folylpolyglutamate synthetase is not inhibited by mAMSA.

MTX transport in isolated rat hepatocytes

MTX influx and efflux studies were carried out in hepatocyte suspensions. The cell-associated [^{3}H]-MTX was assessed as a function of time after the addition of MTX. The content of [^{3}H]-MTX in the liver cells remained unaltered regardless of the presence (1.25–10 μM) or absence of mAMSA (Fig. 5). At 30 min following the removal of MTX from the medium, mean intracellular [^{3}H]-MTX levels were equivalently reduced (32%–35%) in incubates with and those without mAMSA (not shown). Thus, the hepatocellular transport of MTX was not perturbed by this agent.

Discussion

As part of a series of experiments aimed at outlining strategies for reducing unwanted toxicity and improving the therapeutic cytotoxicity and response rates of the established anticancer agent MTX, the present study evaluated the effect of mAMSA on the formation of 7-OH-MTX in the rat. mAMSA is a possible candidate for combination chemotherapy with MTX, since these drugs are active single agents in the treatment of leukemia and display different modes of action. A dose of 10 mg/kg mAMSA equals approximately 50 mg/m^2 in the rat [19] and corresponds roughly to the i.v. clinical doses of 50–120 mg/m^2 [41, 45]. Following the infusion of 10 mg/kg mAMSA in bile-drained rats, Cysyk et al. [17] reported serum

mAMSA levels that were equivalent to the in vitro concentrations used in the present study.

In both groups of rats, MTX was eliminated biphasically from serum during the 2-h experimental period. However, a third serum-MTX elimination phase has previously been detected in rats observed for periods of >6 h [9, 11, 52]. The pharmacokinetic variables of MTX, which were similar in the two groups, were consistent with our previous findings in rats that had received 50 mg/kg [^{3}H]-MTX [11]. Hence, mAMSA-induced perturbations of MTX hydroxylation had no effect on MTX pharmacokinetics, possibly due to the moderate conversion (3%) of the parent drug to 7-OH-MTX in this species. Consequently, the impact of the MTX/mAMSA interaction resides in the modulation of 7-OH-MTX effects.

7-OH-MTX peak levels, the AUC_{7OH} in serum and bile, and the amount of MTX recovery as 7-OH-MTX in urine and bile were substantially reduced by pretreatment with 10 mg/kg mAMSA. The 26% higher bile flow observed in mAMSA-treated animals may be explained by osmotic choleresis [16, 37], as both mAMSA and MTX are actively secreted into bile, resulting in substantial biliary levels of parent compounds and metabolites [10, 13, 17].

The mAMSA-inhibited biotransformation of MTX to 7-OH-MTX appeared to be competitive. Consistently, mAMSA has been reported to be a highly potent (K_i value, 0.06 μM) and competitive inhibitor of *N*-nicotinamide oxidation by purified rabbit aldehyde oxidase [29]. Since mAMSA itself is not metabolized by aldehyde oxidase [29], the exact mechanism whereby mAMSA acts as a competitive inhibitor remains unresolved.

Whereas aldehyde oxidase catabolizes MTX in the rabbit liver [33], the identity of the enzyme responsible for 7-OH-MTX formation in the rat is not known [32, 34]. In an attempt to identify the hydroxylating enzyme in the rat, Yu et al. [54] investigated the influence of the xanthine oxidase inhibitor allopurinol, the aldehyde dehydrogenase inhibitor cyanamide, and the glutathione-depleting agent phorone on the production of 7-OH-MTX after i. v. administration of 9 mg/kg MTX. These compounds, which did not include an aldehyde oxidase inhibitor, had no effect on metabolite formation [54]. Exhibiting a calculated K_i value of 3 μM, which is consistent with the value of 2.5 μM previously established in crude rabbit-liver homogenate [40]. mAMSA is a potent inhibitor of MTX hydroxylation in the rat. As mAMSA has been proposed to be the most potent inhibitor of purified aldehyde oxidase [29], our findings may indicate that MTX hydroxylation in the rat is catalyzed by this enzyme. Since the experiments were not carried out in a purified enzyme system, the K_i value can only be considered a gross index.

As a model for MTX hydroxylation studies, the rat appears to be a more appropriate choice than the rabbit. In contrast to man and the rat, several types of rabbit tissue besides the liver convert MTX to 7-OH-MTX [15, 46]. Moreover, rat-liver tissue biotransforms MTX at a rate that is in better agreement with data previously obtained in humans [32, 34].

7-OH-MTX has been proposed as a mediator of MTX-associated clinical toxicity. Renal failure due to the precipitation of the metabolite in renal tissues of mammals has been reported by Jacobs et al. [30, 31]. Additionally, in our laboratory, 7-OH-MTX has been demonstrated to precipitate at high metabolite concentrations in rat bile in vivo and in vitro [10, 11], and it has been suggested that the metabolite plays a role in the clinically acute hepatotoxicity frequently encountered after high-dose MTX (HD-MTX) therapy [2, 36, 51, 53]. Moreover, 7-OH-MTX may reduce the overall cytotoxic effect of MTX [22–24, 27, 38, 42]. Consequently, the combination of mAMSA and MTX may prove to be advantageous in diminishing MTX-associated renal and hepatic toxicity while possibly enhancing the cytotoxic effect during HD-MTX therapy. Myelosuppression, the major MTX-associated toxicity, could possibly increase as a result of reduced MTX hydroxylation. However, in contrast to 7-OH-MTX-associated renal and liver toxicity, the hematological toxicity of MTX may be successfully counteracted by leucovorin rescue.

In conclusion, mAMSA is a potent competitive inhibitor of 7-OH-MTX formation in rat liver. The question as to whether mAMSA may increase the therapeutic index of MTX by reducing the production of 7-OH-MTX and, hence, modulating the clinical toxicity or cytotoxicity of the parent drug cannot be answered without further investigation.

Acknowledgements. Two of the authors (R. M. B. and E. S.) are Fellows of the Norwegian Cancer Society. All good advice and competent technical assistance rendered by Mr. A. Bessessen, Ms. R. Jæger, Ms. I. Karlsen, and Dr. B. Klemetsdal are gratefully acknowledged.

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