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THE EFFECT OF VINDESINE ON METHOTREXATE HYDROXYLATION IN THE RAT

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Abstract—The effect of vindesine (VDS) on methotrexate (MTX) disposition was studied in bile-drained rats administered VDS prior to [^{3}H]MTX, and in isolated rat hepatocytes and rat liver homogenate concomitantly incubated with MTX and VDS at 37°. *In vivo*, 7-hydroxylation was reduced by 0.65 mg/kg VDS. In VDS-treated animals, biliary recovery of the MTX dose (50 mg/kg) as 7-hydroxymethotrexate (7-OH-MTX) (1.75 ± 0.2%, mean ± SEM) was significantly reduced compared to controls (2.83 ± 0.57%). *In vitro*, hydroxylation of MTX (10–200 μM) in hepatocytes was reduced by 14.3 and 66.4% (means) at 12.5 and 100 μM VDS, respectively. With increasing VDS concentrations up to 100 μM, a reduction in intracellular MTX accumulation could account for the decreased MTX hydroxylation. Experiments in a cell free system gave no evidence of inhibition of 7-OH-MTX formation by VDS. *In vitro* MTX transport studies demonstrated that VDS inhibited the hepatocellular influx of MTX, as (1) the accumulation of MTX corresponded inversely to increasing VDS concentrations and (2) the MTX efflux was not increased by VDS. The apparent K_i for VDS inhibition of MTX influx was 57 μM. We suggest that VDS, by reducing the 7-OH-MTX formation in liver cells, may have implications for combination chemotherapy regimens which include MTX.

High-dose methotrexate (HD-MTX§) infusions are currently used in several anti-neoplastic therapy regimens [1], and millimolar serum concentrations of 7-OH-MTX, MTXs major extracellular metabolite, have been measured in humans after the largest doses [2]. 7-OH-MTX concentrations surpass the plasma MTX levels 3–10 hr after cessation of MTX infusions [3], and are 10–140-fold higher than the corresponding serum MTX concentrations 12–48 hr post infusion [3–5].

In mammals, MTX is hydroxylated by hepatic aldehyde oxidase, and 7-OH-MTX was initially considered a detoxification product [6, 7]. The 7-hydroxylated metabolite, although 40–200-fold less cytotoxic than the parent compound [8–10], may limit MTX toxicity towards tumor cells [11, 12] by reduction of the cellular MTX entry and synthesis of the more potent MTX polyglutamates [4, 13, 14]. Moreover, 7-OH-MTX, 3–5-fold less soluble than MTX at physiological pH, has been rendered a possible mediator of nephrotoxicity through precipitation in renal tubules [15, 16]. Furthermore, we have recently reported precipitations of 7-OH-MTX in the alkaline rat bile *in vivo* and *in vitro*, suggestive of an involvement in MTX-induced acute hepatotoxicity [17, 18].

In two clinical trials, VDS has been found to affect MTX and 7-OH-MTX disposition kinetics when administered concomitantly with MTX [19, 20]. Although the cause of this interference has remained unresolved, VDS has been postulated to interact with methotrexate metabolism [21].

As inhibition of MTX hydroxylation would constitute a logical strategy for circumvention of 7-OH-MTX-associated clinical toxicity following HD-MTX therapy, this study was undertaken to investigate MTX and 7-OH-MTX disposition kinetics in VDS pretreated rats *in vivo*, and to elucidate, in two *in-vitro* systems, the mechanism involved.

MATERIALS AND METHODS

Drugs and chemicals. L-Glutamyl-3,4-[^{3}H]MTX (sp. act. 48.7 Ci/mmol, purity 99.8% by HPLC) was purchased from New England Nuclear (Boston, MA, U.S.A.). Formulated MTX (purity 99% by HPLC) was a gift from Nycomed A/S, Oslo, Norway. 7-OH-MTX was a gift from Dr F. M. Sirotnak, Memorial Sloan-Kettering Cancer Center, New York, U.S.A. Formulated VDS was obtained from Eli Lilly & Co. (Indianapolis, IN, U.S.A.). Collagenase 300 units/mg (type I), bovine albumin (fraction V, defatted) and HEPES were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Methanol and tetrahydrofuran (both HPLC grade) were from Rathburn Chemicals (Walkerburn, U.K.). All other reagents were of analytical grade.

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§ Abbreviations: MTX, methotrexate; 7-OH-MTX, 7-hydroxymethotrexate; DAMPA, 2,4-diamino-N^{10}-methylpteroic acid; HD-MTX, high-dose methotrexate; HPLC, high-pressure liquid chromatography; VDS, vindesine (desacetyl vinblastine amide) sulfate; VCR, vincristine sulphate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; k_i, inhibition constant; T_{max}, maximum rate of transport.

All samples containing MTX and 7-OH-MTX were stored, protected from light, at −20° for a maximum of 2 weeks.

In vivo *experiments*. Male Wistar rats weighing 270–310 g (Charles River, WIGA GmbH, Sulzseld, F.R.G.) were used for all experiments. During the *in vivo* experiments, 12 rats, randomly allocated to two groups, were anesthetized and had their right external jugular vein and bile duct cannulated [22]. The solution of formulated VDS, 1 mg/mL in isotonic saline, was diluted in 50 mg/mL glucose to a final concentration of 0.065 mg/mL (pH 7.7). MTX solutions were prepared by dissolving the drug in isotonic saline to a concentration of 5 mg/mL MTX and adding [^{3}H]MTX to a final activity of 7.7 μCi/mL (pH 7.0). One group (+VDS) received short-time infusions (10 min) of 0.65 mg/kg VDS, whereas control rats (−VDS) were administered the diluent at identical pH, through central venous catheters. The infusions were terminated 5 min prior to [^{3}H]-MTX administration (50 mg/kg i.v., 10 min). The venous catheters were flushed with heparinized saline (10 I.U./mL) immediately after infusions, and after each subsequent blood sampling.

Blood and bile samples for MTX and 7-OH-MTX analyses were obtained as evident from Figs 1 and 2. Voided urine was collected during procedures. Upon killing the animals, the urine bladders were aspirated to assure complete collection. pH was measured in bile and urine samples. 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 described previously [22].

Isolated rat hepatocytes. Rat liver cells were prepared essentially by the method of Berry and Friend [23], and Seglen [24]. Following collagenase perfusion, the livers were perfused for 1 min with a collagenase-free solution at 20°. Hepatocytes were separated from non-parenchymal cells by centrifugation [25], and the cells were washed twice in the incubation medium. The cell viability was assessed by trypan blue exclusion [26], and cell counts were by hemocytometer counts. Cell viability was 93.1 ± 0.8% (mean ± SE) at the start and 86.0 ± 1.3% at the end of the incubations 3.5 hr 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 at 37° with NaOH.

Suspensions of hepatocytes (5 mL, 1.7 × 10^6 cells/mL) were incubated with agitation at 37° in 100-mL capped flasks for 30 min prior to preincubation with VDS (12.5–100 μM). After 30 min preincubation, [^{3}H]MTX was added to final concentrations of 10–200 μM. MTX and VDS were added at 100-fold higher concentrations than the final concentrations in the hepatocyte suspensions (Table 3, Fig. 5). Control and experimental cell batches were incubated in parallel, and were always from the same rat liver hepatocyte suspension. At 3 hr post 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 weight. The hepatocyte sample was immediately centrifuged at 140 *g* for 1 min. The supernatant was siphoned off and the cell pellets subsequently washed twice with ice-cold isotonic saline (0°) followed by centrifugation at 140 *g* for 1 min.

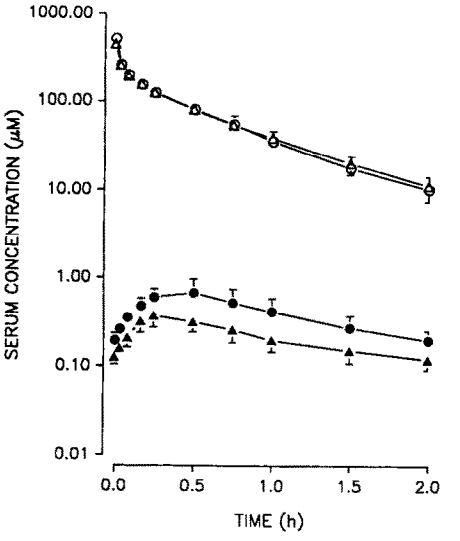

Fig. 1. Serum concentrations of methotrexate (MTX) (open symbols) and 7-hydroxymethotrexate (7-OH-MTX) (closed symbols) versus time following short-time infusions of 50 mg/kg [^{3}H]MTX. MTX and 7-OH-MTX in animals pretreated with 0.65 mg/kg vindesine (VDS) are denoted by triangular symbols whereas those of controls are denoted by circular symbols. All animals were anesthetized and bile-drained. Data are given as mean ± SE, N = 6.

After freeze drying overnight (18 hr), tubes containing pellets were weighed twice. The dry weight of the hepatocytes was calculated as the difference in mean weights of the tube with and without cell pellet. Variability in duplicate weights of pellet-containing tubes was 0.52% of the mean cell pellet weights, and hence negligible. The sample recovery was 99.9% of the total MTX added, and the loss of MTX and 7-OH-MTX during the wash procedures constituted only 2.9% of the recovery.

Rat liver homogenate. After ether anesthesia and laparatomy livers were rapidly excised from five rats. The tissue was quickly rinsed in ice-cold 0.25 M sucrose solution (0°), weighed, minced with scissors and homogenized with a Potter–Elvehjem homogenizer in 1:4 g/mL ice-cold 0.25 M sucrose solution. The procedures were carried out at 4° and all reagents and equipment were kept on ice. Following centrifugation of the crude homogenate at 7600 *g* for 15 min (0°), the supernatant was removed and used for incubation experiments. Homogenate portions (5 mL) were pipetted into 100-mL flasks. After 10 min incubation with agitation at 37° the homogenate was preincubated with VDS for 5 min prior to MTX addition. MTX and VDS solutions were added at concentrations 100-fold

Table 1. Pharmacokinetic variables of methotrexate (MTX) and 7-hydroxymethotrexate (7-OH-MTX) in rats infused 50 mg/kg [^{3}H]MTX during 10 min

Compound	Pharmacokinetic parameters	−VDS	+VDS
MTX	$T_{½(\alpha)}$ (min)	2.74 ± 0.43	2.83 ± 0.16
	$T_{½(\beta)}$ (min)	26.9 ± 3.1	29.8 ± 3.9
	V_c (mL/kg)	276 ± 18	314 ± 26
	V_β (mL/kg)	413 ± 13	461 ± 30
	Cl_T (mL/min·kg)	11.3 ± 1.1	11.0 ± 1.3
	Cl_B (mL/min·kg)	8.2 ± 1.3	8.1 ± 1.2
	Cl_{NB} (mL/min·kg)*	3.1 ± 0.3	2.9 ± 0.6
7-OH-MTX	$T_½$ (min)	66.3 ± 12.6	63.3 ± 4.2
	S-AUC_{7OH} (μM·min)	0.049 ± 0.017	0.026 ± 0.004
	B-AUC_{7OH} (μM·min)	44.5 ± 12.2	24.3 ± 2.6

Ten minute infusions of vindesine (0.65 mg/kg, +VDS) or diluent (isotonic saline, −VDS) were terminated 5 min prior to MTX infusions. All animals were anesthetized and bile-drained during experiments. Data are given as mean ± SE, N = 6.

* Cl_{NB} is nonbiliary clearance ($Cl_{NB} = Cl_T - Cl_B$).

higher than the final incubate concentrations. At 10, 20, 30, 45 and 60 min following MTX addition, 400-μL aliquots were removed from the incubation medium and immediately placed on ice. MTX recovery was 97.2%. The protein content of the incubated homogenate extract was 24.6 mg/mL according to Bradford's method, applying albumin as reference standard [27].

MTX transport experiments. For influx studies, hepatocyte suspensions (5 mL, 2.5 × 10^6 cells/mL, mean) were incubated with agitation for 30 min at 37° prior to preincubation with 12.5–100 μM VDS or diluent for 30 min. [^{3}H]MTX was added to final concentrations of 10–200 μM. Aliquots (250 μL) of the incubation medium were removed 0.5, 1, 1.5, 2, 2.5, 3, 5 and 10 min after addition of MTX. The efflux studies were initiated by washing the residual 3 mL of the hepatocyte suspensions containing 0, 25 or 100 μM VDS twice in ice-cold isotonic saline 30 min post MTX addition. The cell pellet was then resuspended in incubation medium containing VDS but not MTX (37°) and incubated at 37°. At 5, 10, 15, 20, and 30 min after washing, 250 μL aliquots of the incubation media were removed. The samples obtained during the MTX transport studies were immediately pipetted into 1 mL ice-cold isotonic saline (0°) overlying a mixture of dinonyl phtalate and dibutyl phtalate (1:3, 250 μL) in 1.5 mL polyethylene microcentrifuge tubes. The cells were separated from the medium by centrifugation in less than 10 sec [28]. Further handling procedures prior to determination of the cell-associated radioactivity have been described in detail previously [28].

Analytical methods. Analyses of MTX and 7-OH-MTX concentrations in serum, bile, urine, hepatocytes, supernatants and homogenate were performed by reverse phase HPLC, fraction sampling and determination of radioactivity as reported previously [22], with the following exception: cell pellets were resuspended in 0.5 mL isotonic saline and vortex mixed for 2 min prior to precipitation with 2 M PCA and subsequent injection of the 10,000 *g* supernatants on the HPLC. The assay

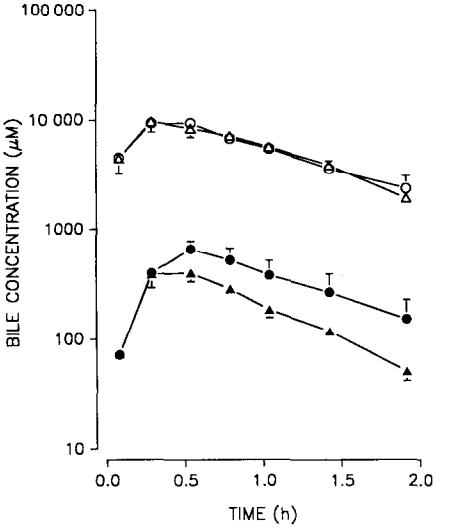

Fig. 2. Biliary concentrations of methotrexate (MTX) and 7-hydroxymethotrexate (7-OH-MTX) versus time following short-time infusions of 50 mg/kg [^{3}H]MTX with and without pretreatment with 0.65 mg/kg vindesine (VDS). Symbols and procedures as in Fig. 1.

detects MTX and its major extra-cellular metabolites 7-OH-MTX and DAMPA, with no inferference from polyglutamates 1–3 of MTX.

Calculations. The MTX serum concentrations were analysed according to a two-compartment open model. Pharmacokinetic parameters were calculated as described elsewhere [17]. The $AUC_{0\text{-}2\,hr}$ for 7-OH-MTX concentrations in serum (S-AUC_{7OH}) and bile (B-AUC_{7OH}) was calculated by the trapezoidal rule.

For analysis of the Michaelis–Menten kinetics of the MTX transport and hydroxylation processes, we applied the microcomputer program Enzfitter^R

Table 2. Percentage of cumulative biliary and urinary recovery as methotrexate (MTX) and 7-hydroxy-methotrexate (7-OH-MTX) in rats administered 50 mg/kg [^{3}H]MTX i.v. (10 min)

		−VDS	+VDS
Bile	MTX	43.9 ± 1.0	42.1 ± 4.5
	7-OH-MTX	2.83 ± 0.57	1.75 ± 0.20*
Urine	MTX	25.7 ± 3.2	24.1 ± 3.4
	7-OH-MTX	0.055 ± 0.010	0.061 ± 0.019

Ten minute infusions of vindesine (0.65 mg/kg, +VDS) or diluent (isotonic saline, −VDS) has been terminated 5 min prior to MTX infusions. The animals were anesthetized and bile-drained during experiments. Data are given as mean ± SE, N = 6.
* $P < 0.05$ between the two groups.

(Elsevier-Biosoft, London, U.K.). Statistical analyses were performed using the non-parametric Mann–Whitney U-test (Microstat^R; Ecosoft Inc., Indianapolis, IN, U.S.A.). Statistical significance was defined as $P < 0.05$. All results are expressed as mean ± SE.

RESULTS

MTX hydroxylation in vivo

Following short-time infusions of 50 mg/kg MTX (10 min), groups pretreated with VDS or diluent both demonstrated biphasic elimination profiles of MTX, with a rapid initial phase during the first 10 min and a considerably slower second phase (Fig. 1). The pharmacokinetic variables are given in Table 1. The serum MTX concentration–time profiles and pharmacokinetic variables were comparable in both groups. Serum 7-OH-MTX peak levels were 35.8% (mean, N = 6) lower and AUC (S-AUC_{7OH}) 46.9% smaller in VDS-treated compared to control rats, but these differences did not reach statistical significance.

In bile, MTX peak levels were comparable in the two groups (Fig. 2). Biliary MTX declined monophasically, with similar half-lives in both groups (35.5 and 38.9 min, means). Biliary peak levels of 7-OH-MTX were 41.4% lower (371 ± 57 vs 633 ± 101 μM, N = 6) and AUC (B-AUC_{7OH}) 45.4% smaller in VDS-pretreated compared to untreated animals (Table 1).

The cumulated MTX recovery as 7-OH-MTX in bile was significantly (mean 38.2%, N = 6) lower in the VDS treated group as compared to the controls (Table 2). There was no alteration in the biliary recovery as MTX or the urinary recovery as MTX or 7-OH-MTX by VDS pretreatment. VDS-dosed and control animals excreted equal volumes of bile (2.65 and 2.57 mL, means) and urine (1.13 and 1.04 mL, means), respectively, during the experiments. Bile pH remained constant during the experiments at 8.2 (means) in both groups, and urinary pH was similar at 6.1 and 6.4 (means) in the VDS group and control group, respectively.

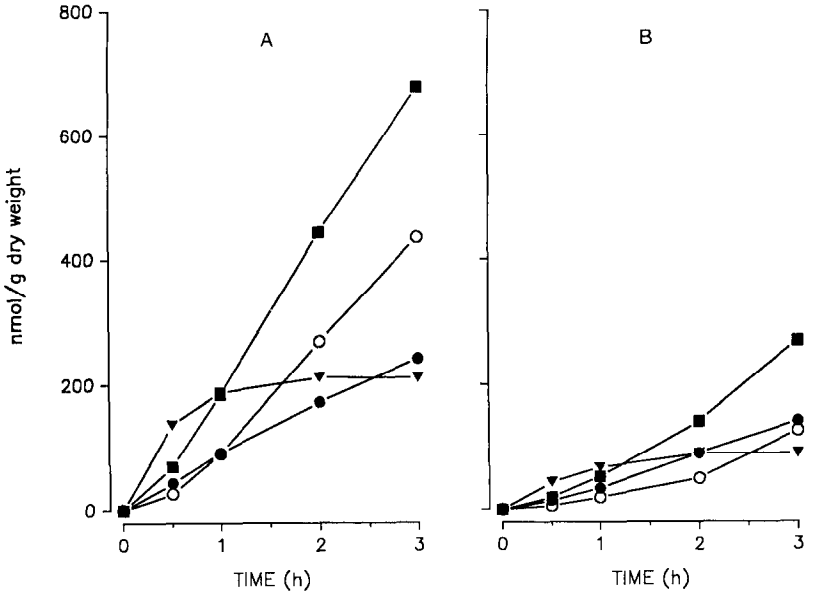

Fig. 3. Methotrexate (MTX) hydroxylation in isolated rat hepatocytes. Hepatocyte suspensions were preincubated with (A) isotonic saline (control) or (B) 100 μM vindesine (VDS) for 30 min prior to the addition of 10 μM [^{3}H]MTX. The incubates were sampled at 0.5, 1, 2 and 3 hr post MTX addition, followed by assessment of 7-hydroxymethotrexate (7-OH-MTX) formation and the intra- and extracellular distribution of MTX and 7-OH-MTX. Data are given as means of four experiments. Extracellular 7-OH-MTX (open circle); intracellular 7-OH-MTX (filled circle); total 7-OH-MTX formed (filled square); intracellular MTX (filled triangle).

Table 3. The reduction of methotrexate (MTX) hydroxylation and accumulation in isolated rat hepatocytes by incubation (3 hr) in various concentrations of vindesine (VDS)

VDS (μM)	% MTX hydroxylated	% Decrease in total 7-OH-MTX formation	% Decrease in intracellular MTX
0	14.1 ± 0.6	—	—
12.5	12.0 ± 0.6*	14.3	30.4 ± 5.3*
25	9.6 ± 0.3	31.9	44.0 ± 6.6
50	7.5 ± 0.2	47.5	59.2 ± 4.5
100	5.1 ± 0.3	66.4	73.5 ± 2.0

The amount of 7-hydroxymethotrexate (7-OH-MTX) formed was measured in per cent of the total [^{3}H]MTX, 10, 25, 50, 100 and 200 μM, added to the incubates. The concomitant reduction in intracellular MTX was assessed in per cent of accumulated drug intracellularly at the absence of vindesine in the incubates. Data are given as mean ± SE, N = 5.
* $P < 0.05$ between 0 and 12.5 μM VDS.

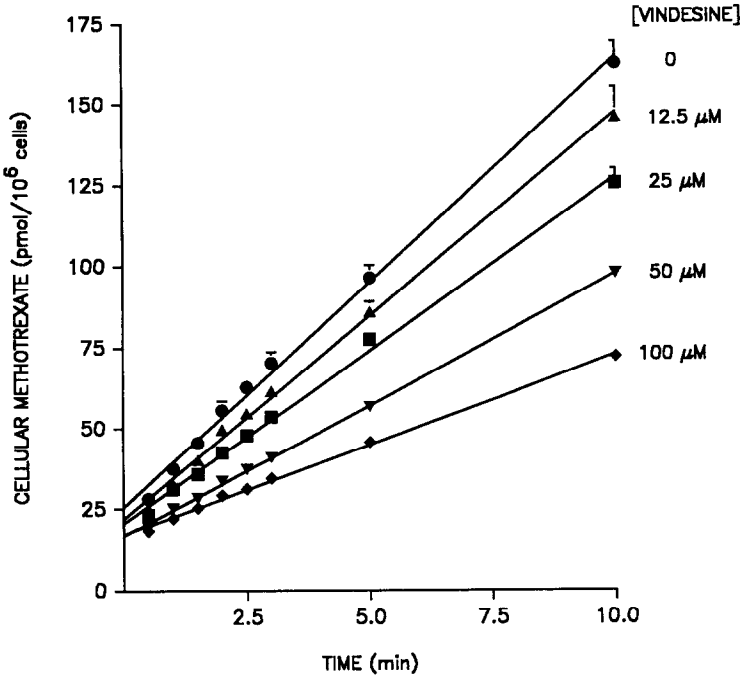

Fig. 4. The effect of vindesine (VDS) on cellular influx of methotrexate (MTX). Rat hepatocytes in suspension were exposed to 10 μM [^{3}H]MTX alone or after preincubation with VDS at increasing concentrations. Data are given as mean ± SE, N = 3.

At the end of experiments, venous pH was 7.34 and 7.32 (means) in the control and VDS group, respectively. At termination of experiments, hematocrit values were equal in the two groups (0.34–0.35, means).

MTX hydroxylation in isolated rat hepatocytes

The formation of 7-OH-MTX in the hepatocyte suspension was examined as a function of time at an extracellular concentration of 10 μM MTX (Fig. 3A). When hepatocytes were incubated concomitantly with 100 μM VDS, the intracellular levels of MTX and the formation of 7-OH-MTX were significantly and equally reduced in comparison to control suspensions (Fig. 3B). 7-OH-MTX accumulated intracellularly to the extent of 31.8 and 31.9% (means) of the intracellular MTX by 0.5 hr and 139.1 and 114.3% by 3 hr in hepatocytes incubated with VDS and diluent, respectively.

The proportion of 7-hydroxylated MTX correlated inversely to increasing concentrations of VDS (Table 3), and was uninfluenced by the MTX concentrations in the incubates. The formation of 7-OH-MTX was significantly inhibited at the lowest VDS concentration (12.5 μM), and was 66.4% reduced at VDS concentration of 100 μM. The decrease in intracellular MTX was, however, more pronounced at increasing VDS concentrations, with a 73.5%

Table 4. Inhibition of net [^{3}H]methotrexate ([^{3}H]MTX) accumulation in isolated rat hepatocytes by pretreatment with various concentrations of vindesine (VDS)

VDS (μM)	MTX (pmol/10^6 cells)	% Decrease in cellular MTX
0	162.3 ± 6.9	—
12.5	145.9 ± 9.2	10.1
25	125.3 ± 4.6*	22.8
50	97.6 ± 0.6	39.9
100	72.3 ± 2.1	55.5

The hepatocytes were incubated with 10 μM [^{3}H]MTX for 10 min. Data are given as mean ± SE, N = 3.
* $P < 0.05$ between 0 and 25 μM VDS.

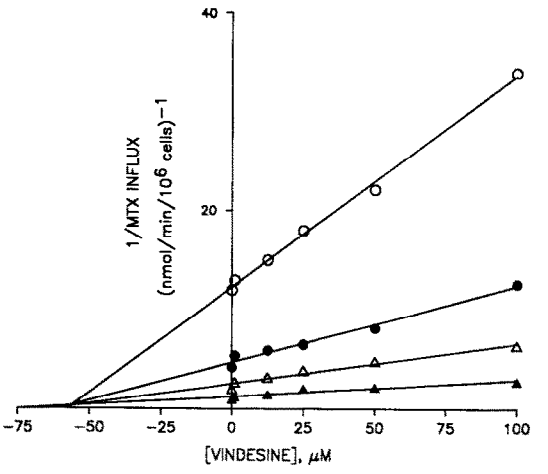

Fig. 5. Dixon plot analysis of methotrexate (MTX) influx in isolated rat hepatocytes in the absence or presence of vindesine. The hepatocyte suspension was preincubated with 0–100 μM vindesine 30 min prior to adding [^{3}H]MTX at various concentrations. Ten minutes post [^{3}H]MTX addition, hepatocyte suspensions were sampled to assess the rate of MTX influx relative to VDS concentrations: 10 μM [^{3}H]MTX (open circle); 25 μM [^{3}H]MTX (closed circle); 50 μM [^{3}H]MTX (open triangle); 100 μM [^{3}H]MTX (closed triangle).

reduction after 100 μM VDS. The rate of MTX hydroxylation was obtained at each level of MTX (10–200 μM) and VDS (0–100 μM). A Dixon plot of the data gave an apparent K_i of 41.4 μM (not shown).

The formation of MTX polyglutamates 1–3 were reduced proportionally to the intracellularly available substrate. Consequently, there were no detectable interactions with the conversion of MTX to polyglutamates (not shown).

MTX hydroxylation in liver homogenate

In liver homogenate, the 7-OH-MTX formation rate was linear for at least 1 hr of incubation with MTX. We assessed the hydroxylation rate 30 min after adding MTX to the medium. At increasing VDS concentrations (0–100 μM), there was no evidence of inhibition of MTX hydroxylation. Unaffected by the VDS concentration in the medium, 4.67 ± 0.12% (range 4.6–4.79) of added MTX was converted to 7-OH-MTX. Furthermore, there was no indication of a reduced hydroxylation rate relative to increasing MTX concentrations in the 10 to 200 μM range.

Cell transport of MTX in isolated rat hepatocytes

The influx of 10 μM [^{3}H]MTX during 10 min in the presence of increasing VDS concentrations is shown in Fig. 4. Within the initial 3 min, the MTX uptake was near linear with respect to time and more rapid than that in the period of 3 to 10 min. The MTX influx was clearly inhibited in the presence of VDS 30 sec after MTX addition (Fig. 4).

The net MTX accumulation in hepatocytes after 10 min incubation with [^{3}H]MTX demonstrated a marked inhibition by VDS (Table 4). Concentrations of 25 μM VDS inhibited MTX accumulation significantly, and 100 μM VDS reduced MTX accumulation by 55.5%.

The inhibition kinetics of MTX transport by VDS was determined by Dixon plot analysis at various [^{3}H]MTX and VDS concentrations (Fig. 5). The apparent k_i value for inhibition of MTX influx was 57 μM. The T_{max} estimated from a Lineweaver–Burk plot was practically identical with the calculated T_{max} of 5.5 nmol/min/10^6 cells (mean, N = 5) applying Enzfitter^R.

For efflux studies, hepatocytes exposed to MTX (10 μM) and VDS (0, 25 or 100 μM) for 30 min were washed twice prior to resuspension in MTX-free medium to enable detection of a potential VDS influence on MTX efflux. However, the 25.5 and 24.8% (means, N = 3) reduction in hepatocellular confinement of MTX in the presence of 25 and 100 μM VDS, respectively, was not significantly different from control suspensions.

DISCUSSION

We have recently examined the disposition kinetics of MTX and 7-OH-MTX following high-dose equivalent infusions to the Wistar rat [17, 22]. Employing this *in vivo* animal model and two *in vitro* systems, isolated rat hepatocytes and rat liver homogenate, we present the first report on the mechanism responsible for VDS interactions with the hydroxylation of MTX. The interacting agent is the latest synthesized vinca alkaloid in clinical use [29], and has been successfully tested in combination chemotherapy regimens with MTX for a wide variety of human cancers [30–32].

Rats administered 50 mg/kg [^{3}H]MTX, independent of pretreatment or not with 0.65 mg/kg VDS, demonstrated biphasic serum MTX elimination curves over the 2 hr experimental period. This is in agreement with previous studies in rats given 1–1000 mg/kg MTX [17, 33]. The pharmacokinetic variables of MTX are consistent with the previous findings in bile-drained rats administered 50 mg/kg [^{3}H]MTX [17].

The reduction of 7-OH-MTX peak concentrations and AUC_{7OH} in the serum of VDS pretreated rats was in accordance with the findings of Bore *et al.* [20], who reported disposition kinetics after

simultaneous administration of MTX and VDS to patients. The VDS dose employed in the *in vivo* experiments corresponded roughly to the 3 mg/m^2 body surface VDS dose routinely used in the clinic [19, 20, 34]. The 36–47% reductions in peak 7-OH-MTX levels were not statistically significant, presumably due to the gross interindividual variability in 7-OH-MTX levels in serum and bile. This is in accordance with the heterogeneity of MTX hydroxylation in man [3, 35, 36]. The MTX recovery as 7-OH-MTX in bile was significantly smaller in VDS pretreated than untreated animals. In view of the *in vitro* findings, the VDS pretreated rats were expected to yield lower biliary MTX levels, smaller recovery as MTX in bile, and longer serum MTX half-lives in comparison to controls. The tendencies observed were, however, minute.

Since VDS failed to inhibit MTX hydroxylation in the cell-free system or interfere with the rate of hepatocellular MTX efflux, the reduced accumulation of cellular MTX is considered to be due to influx inhibition. Consistently, VCR, the vinca alkaloid in longest clinical use, had been reported to affect cellular MTX transport in malignant cells *in vitro* [37–39], though transport data remain contradictory on whether this agent affects the MTX influx or efflux route [37, 38]. As bile is the major route of VDS excretion [34], this compound could potentially compete with MTX for hepatocyte uptake. VDS inhibits MTX uptake by an approximate k_i of 60 μM. Eadie–Hofstee and Lineweaver–Burk plot analyses indicate, however, that the MTX influx inhibition is caused by a noncompetitive interaction.

7-OH-MTX has been proposed as a mediator of clinical toxicity during HD-MTX toxicity. The metabolite may lead to renal failure and impaired drug elimination by precipitation in renal tissue [15, 16]. As the metabolite precipitates in alkaline rat bile when present at high concentrations [17, 18], 7-OH-MTX may play a role in MTX-associated acute hepatotoxicity, frequently reported during HD-MTX therapy [40–43].

Since 7-OH-MTX may reduce the overall cytotoxic effect of the parent compound [8, 11, 13] and play an important role in the development of MTX-associated side effects [16–18], the combination of VDS and MTX may be beneficial in reducing MTX-associated toxicity without diminishing the cytotoxic action of HD-MTX therapy. In this context, a VDS mediated synergistic increase of MTX cytotoxicity in malignant cell lines is noteworthy [44–46].

In conclusion, the perturbations of 7-OH-MTX kinetics observed during concomitant administration of MTX and VDS in the clinic, is not due to reduced MTX hydroxylation, but rather to inhibition of hepatocellular MTX uptake. This property of VDS may have consequences for MTX cytotoxicity and clinical toxicity during combination therapy and should be exploited further.

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