

High dose methotrexate chemotherapy: pharmacokinetics, folate and toxicity in osteosarcoma patients

Laila Holmboe,^{1†} Anders M. Andersen,² Lars Mørkrød,^{1,4} Lars Slørdal^{5,6} & Kirsten Sundby Hall³

Departments of ¹Medical Biochemistry, ²Pharmacology and ³Oncology, Norwegian Radium Hospital, Oslo University Hospital, ⁴Institute of Clinical Biochemistry, Faculty of Medicine, University of Oslo, Oslo, ⁵Department of Laboratory Medicine, Children's and Women's Health, Norwegian University of Science and Technology, St Olavs University Hospital, Trondheim and ⁶Department of Clinical Pharmacology, St Olavs University Hospital, Trondheim, Norway

WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- High dose methotrexate (HD MTX) is generally well tolerated, although unpredictable acute toxicities occur frequently.
- Low overall toxicity in paediatric patients and increased liver toxicity in adults has been reported, but there are no reports addressing the relationship between acute liver toxicity and gender in patients treated with HD MTX.
- Previous studies in animals suggested involvement of the 7-hydroxy-methotrexate metabolite in acute liver toxicity, but this has not been investigated in humans and the aetiology of acute liver toxicity remains unclear.

WHAT THIS STUDY ADDS

- Survival has increased dramatically over the past decades for patients with malignancies such as osteosarcoma and since these patients are frequently children or adolescents at the time of high dose methotrexate, identification of risk factors for toxicity increases the possibility for tailoring treatment.
- Our study presents a detailed analysis of acute toxicity, folate concentrations and pharmacokinetics of both methotrexate and its major extracellular metabolite 7-hydroxy-methotrexate and identifies several factors that are highly correlated with acute liver toxicity.

AIMS

To investigate the relationships between pretreatment folate concentrations, MTX pharmacokinetics and acute toxicities following high dose methotrexate (HD MTX) therapy.

METHODS

MTX and its major extracellular metabolite 7-OH-MTX were measured in eight serum samples per HD MTX cycle in 65 consecutive osteosarcoma patients (288 cycles) and AUC (area under the blood concentration–time curve) was calculated. Pretreatment concentrations of folate in serum (S) and erythrocytes (ER) were determined. Hepatic, renal and haematological toxicities, assessed by routine laboratory parameters, as well as mucositis were graded according to National Cancer Institute Common Terminology Criteria for adverse events (CTCAE v.3.0). Dermatitis and pleuritis were reported as occurred or not.

RESULTS

S- and ER-folate pretreatment concentrations increased significantly with increasing number of HD MTX cycles ($P < 0.001$). ER-folate pretreatment concentrations were higher among males (median 610 nmol l⁻¹, 95% CI 550, 680) compared with females (median 465 nmol l⁻¹, 95% CI 430, 520, $P < 0.001$), but showed no correlation with MTX or 7-OH-MTX pharmacokinetics. We found correlations between alanine aminotransferase peak concentration (ALAT_{max}) and clearance of MTX ($P < 0.001$), gender ($P < 0.001$), age ($P < 0.001$) and 7-OH-MTX concentrations ($P < 0.001$), the latter being the main factor influencing ALAT_{max}.

CONCLUSION

Our results suggest that 7-OH-MTX is involved in the development of HD MTX hepatic toxicity and that young female patients are most affected.

Correspondence

Dr Laila Holmboe MD, Department of Nuclear Medicine, Oslo University Hospital, Ullevål N-0407 Oslo, Norway.
Tel.: +47 2211 9355
Fax: +47 2211 9029
E-mail: laila.holmboe@uus.no

[†]Present address: Department of Nuclear Medicine, Oslo University Hospital, Ullevål, Norway.

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Introduction

High dose methotrexate (HD MTX), defined as $>1 \text{ g m}^{-2}$, is given in combination with doxorubicin and a platinum agent in most osteosarcoma protocols [1]. The use of leucovorin (LV; 5-formyl-tetrahydrofolic acid) rescue [2] has facilitated the escalation of MTX doses to high plasma concentrations with enhanced anticancer as well as cytotoxic effects. Dihydrofolate reductase is the primary drug target for MTX, although polyglutamated forms of antifolates inhibit other enzymes involved in intracellular folate metabolism [3], thus interrupting normal cellular metabolism and inducing cell death. It is believed that the mechanism of action is responsible both for drug efficacy and toxicity. MTX is hydroxylated to 7-OH-MTX mainly in hepatocytes where concentrations of 7-OH-MTX exceed those of the parent compound shortly after infusion of HD MTX [4]. MTX and 7-OH-MTX may precipitate in renal tubules [5–8] and contribute to renal toxicity [9], but their solubility in urine is enhanced by adequately hydrating and alkalinizing the patient. Routine monitoring of plasma MTX concentrations with early detection of abnormal clearance has permitted countermeasures, such as adjustments of LV doses and intensified hydration regimes, to prevent excessive host toxicity [10, 11]. Despite LV rescue, hydration and urinary alkalinization, HD MTX is associated with toxicities affecting the liver, kidneys, skin, lungs, bone marrow and gastrointestinal system, particularly the oral mucosa. In osteosarcoma patients with normal kidney function, HD MTX-induced renal toxicity occurs in approximately 2% of patients in clinical trials [12, 13]. As MTX is primarily cleared renally, MTX-induced renal toxicity can be life-threatening, because it delays MTX excretion, resulting in sustained, elevated plasma MTX concentration and enhancement of other adverse events.

Since MTX targets several critical events in folate metabolism and competes with endogenous folate both for cellular uptake and for the formation of polyglutamates, it might be expected that pretreatment serum (S-) and/or erythrocyte (ER-) folate could predict subsequent toxicity of the drug. Serum or plasma folate describes extracellular concentrations and current changes in folate intake, whereas ER-folate reflects body storage levels at the time of erythropoiesis when folate enters the erythroblasts and is trapped intracellularly [14]. In the present study, both S- and ER-folate concentrations were determined prior to HD MTX cycles.

Previous studies of HD MTX pharmacokinetics have generally relied on limited sampling strategies, with three to five serum samples per cycle, while measuring only the parent compound by immunoassay techniques. In the present study a total of eight serum samples were collected per patient for each cycle of chemotherapy, one prior to the HD MTX infusion and additional samples at 2, 4, 6, 12, 24, 48 and 72 h after the start of the infusion.

Herein, we present a detailed analysis of MTX and 7-OH-MTX concentrations from 288 HD MTX cycles, with corresponding toxicity data.

Methods

Patients

Sixty-five consecutive patients with high grade osteosarcoma treated with HD MTX at the Norwegian Radium Hospital between September 1994 and April 2003 were included. Patients were defined as paediatric (<15 years) or adults (≥ 15 years), and the patient characteristics are summarized in Table 1. In two patients, osteosarcoma occurred in previously irradiated areas (bilateral retinoblastoma and Hodgkin's lymphoma). Concomitant drugs with known interference with MTX were not administered during the HD MTX treatment cycles. The patients did not have a history of renal, hepatic or cardiac disease and none of the patients had underlying Epstein Barr, herpes or cytomegalovirus infection. One patient was hepatitis C virus antibody positive. Informed consent was obtained and the study was approved by the Regional Ethics Committee (REK, Oslo, Norway) and the local Institutional Review Board.

Chemotherapy

Patients <40 years were treated according to current institutionally approved protocols, including SSG (Scandinavian Sarcoma Group) VIII [15], ISG (Italian Sarcoma Group)/SSG I [16, 17], ISG/SSG II [18] and ISG/SSG XIV [19]. All protocols were multi-drug regimens consisting of HD MTX,

Table 1

Patient characteristics

Characteristic	Number of patients (n = 65)
Gender	
Male	39
Female	26
Age	
<15 years	18
≥ 15 years	47
Median	18
Range	(9–51)
Tumour	
Extremity localized	46
Non-extremity localized	19
Newly diagnosed	59
Recurrent disease	6
Without overt metastases at diagnosis	43
With metastases at diagnosis	22
Number of MTX cycles received	
1–4	38
5–9	15
10	12

cisplatin, doxorubicin, ifosfamide and, in some patients, etoposide. The number of HD MTX cycles planned to be given in each treatment protocol varied from 2 to 10 cycles. For patients who could not follow the ongoing protocol due to toxicity, disease progression or age ≥ 40 years, protocols were modified. Actual body surface area at each cycle was determined from height and weight by standard nomograms for adults and for children and the methotrexate dose was adjusted accordingly. Patients were treated with MTX as a 4 h infusion with LV rescue started 24 h after initiation of MTX and continued until blood samples showed that MTX concentrations were below $0.2 \mu\text{mol l}^{-1}$ [20]. Eighteen paediatric and 47 adult patients received a median of five and three HD MTX cycles, respectively. Ablative surgery was performed 9–12 weeks after initiation of treatment. Postoperatively, patients received adjuvant chemotherapy for up to 30 weeks.

Blood samples and analyses

Eight blood samples (one before the MTX infusion and at 2, 4, 6, 12, 24, 48 and 72 h after start of infusion) per patient per cycle were collected in serum tubes (Vacutainer, Brand, UK) and centrifuged at 1300 *g* for 12 min. Samples were stored at -20°C . MTX and 7-OH-MTX concentrations were measured in each serum sample using the HPLC method developed by Lawson *et al.* [21] and modified as described by Seidel *et al.* [22]. Briefly: serum samples (200 μl) were deproteinized by addition of 40 μl perchloric acid (2 mol l^{-1}) and centrifuged prior to injection of 25–50 μl of the clear supernatant onto the chromatographic system. Patient samples collected up to 24 h after the start of infusion were manually diluted 1:10 in serum from healthy volunteers before preparation. The within and between run coefficients of variation (CVs) were 4% and 8% at the relevant concentrations of both compounds (40 μM of MTX and 2 μM of 7-OH-MTX). At low concentrations the within run CV increased 8% at 0.1 μM and 15% at 0.05 μM . The lower limit of quantification (signal to noise ratio 5:1) was $0.02 \mu\text{mol l}^{-1}$ for both MTX and 7-OH-MTX. Lack of interference from endogenous substances or drugs was verified by visual inspection of all chromatograms, including chromatograms from samples taken just before the start of MTX infusion. No interference was observed from relevant folates or other study drugs. S-folate and ER-folate were measured in pre-treatment samples using time-delayed immunofluorescence and a semiautomated analyzer (AutoDelfia, Wallac, Turku, Finland). The detection limit was 1.9 nmol l^{-1} (calculated from the mean value $- 2\text{SD}$ of the null response). Reproducibility (total CV) for both analytes was $<10\%$. Crossreactivities (at the 50% displacement level) with folinic acid and methotrexate were 0.04% and 2.4% respectively. In the same pretreatment samples methotrexate concentrations were not detectable by HPLC. In blood samples before infusion and 4, 12, 24, 48 and 72 h subsequent to start of MTX infusion, alanine aminotransferase (ALAT), alkaline phosphatase (ALP), gamma-

glutamyl transferase (γGT), total bilirubin, creatinine, platelet and white blood cell (WBC) counts were analyzed by routine methods and data were extracted from patient files.

Toxicity

The National Cancer Institute Common Terminology Criteria for Adverse Events v.3.0 (CTCAE) score system was applied for grading hepatic, renal, haematologic and mucosal toxicity, while occurrence of dermatitis and pleuritis was categorized as absent or present [23].

Calculations

Individual pharmacokinetic parameters were calculated using PK Solutions 2.0 (Summit Research Services, CO, USA). Drug exposure during HD MTX cycles was expressed as $\text{AUC}(0,72 \text{ h})$ (area under the blood concentration–time curve) calculated by the summation of integrals defined by the drug concentrations and the respective time spans over which they had been obtained (trapezoidal rule). MTX clearance ($\text{l m}^{-2} \text{ h}^{-1}$) was calculated as $\text{CL}_{\text{MTX}} = \text{MTX dose} / \text{AUC}_{\text{MTX}}$. For comparison of 7-OH-MTX concentrations between cycles the ratio $\text{AUC}_{7\text{-OH-MTX}} : \text{AUC}_{\text{total}}$ (where $\text{AUC}_{\text{total}} = \text{AUC}_{\text{MTX}} + \text{AUC}_{7\text{-OH-MTX}}$) was calculated. The first treatment cycle was used as baseline (cycle₁) for calculating the intra-individual cycle-to-cycle relative differences ($\Delta_{\text{cycle}} = \text{cycle}_n - \text{cycle}_1$) of pharmacokinetic parameters and of folate pretreatment concentrations (Δ S-folate and Δ ER-folate). A two-way analysis of variance (ANOVA) was used to identify cycle-to-cycle and interindividual variation in pharmacokinetic and toxicity-related parameters. Interindividual variation was expressed as 95% confidence intervals for each cycle. While CL_{MTX} was normally distributed, all other parameters (MTX and 7-OH-MTX peak values, ratio $\text{AUC}_{7\text{-OH-MTX}} : \text{AUC}_{\text{total}}$, S-folate, ER-folate, WBC and platelet nadirs, creatinine, ALAT, γGT , ALP and total bilirubin peak values) had to be log transformed to exhibit a normal distribution. Patients were grouped by gender and age (paediatric or adults) and differences between groups were assessed by Student's *t*-test for normally distributed data and Mann-Whitney test (M-W) elsewhere. *P* values were corrected for multiple comparisons by means of Bonferroni's rule. The level of significance was set to 0.05. Bivariate associations between markers of toxicity and folate concentrations, age, gender, cycle number and pharmacokinetic parameters were evaluated by two-sided Spearman's rank correlation coefficients (ρ_{sp}). The first treatment cycle was used as baseline and relationships between toxicity markers (dependent variables) and folate concentrations, age, gender, cycle number and pharmacokinetic parameters (independent variables) were investigated by backward multiple linear regression analysis. Table tests were performed as Chi squared test with Yates' correction. Data were analyzed with the Excel AnalyzeIt XP and SPSS version 13 (SPSS, Inc., Chicago, IL, USA).

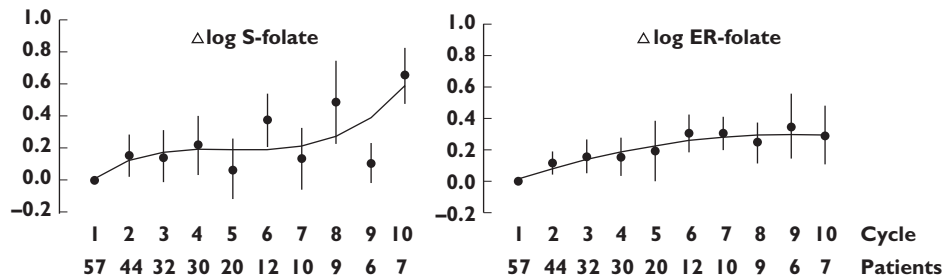


Figure 1

Serum (S-) and erythrocyte (ER-) folate concentrations were measured prior to therapy and before each cycle of high dose methotrexate. Relative cycle-to-cycle differences (Δ) are shown using first cycle pretreatment concentrations as baseline. Interindividual variation in 65 patients at each cycle is illustrated as 95% confidence intervals of mean relative differences. Polynomial regression curves are fitted to the experimental data to indicate dependency of cycle number

Results

With a median follow-up of 88 months, the overall survival rate at 5 years for the population of 65 patients was 62%. There was no significant difference in survival between genders.

Folate concentrations

In pretreatment samples from 227 HD MTX cycles, ER-folate concentrations ranged from 150 to 2050 nmol l⁻¹ with a median of 565 nmol l⁻¹ and 95% CI 210, 1390 (reference range 315–835 nmol l⁻¹). ER-folate was higher in males (median 610 nmol l⁻¹, 95% CI 550, 680) compared with females (median 465 nmol l⁻¹, 95% CI 430, 520, M-W, $P < 0.001$).

S-folate, ranged from 3 to 55 nmol l⁻¹ with a median of 11.3 nmol l⁻¹ and 95% CI 4, 45 (reference range was 5.8–23 nmol l⁻¹) and was slightly higher in males (median 11.9 nmol l⁻¹, 95% CI 10.4, 14.0) compared with females (median 9.7 nmol l⁻¹, 95% CI 7.9, 14.0, M-W, $P = 0.02$).

Figure 1 depicts intra- and interindividual relative differences in S-folate and ER-folate concentrations. Variations in ER- and S-folate concentrations between cycles within patients (CV 22.6% and 7.1% respectively) were not significantly greater than variation between patients (CV 15.7% and 3.3% respectively). Both S- and ER-folate concentrations increased significantly with increasing number of HD MTX cycles ($\rho_{sp} = 0.26$ and 0.13; $P < 0.001$ and 0.05, respectively).

Pharmacokinetics

There were sufficient samples available in 288 HD MTX cycles to enable calculation of pharmacokinetic parameters. Following a 4 h continuous HD MTX infusion, MTX was eliminated from the plasma in a biphasic manner with α and β half-lives ($t_{1/2,\alpha}$ and $t_{1/2,\beta}$) while 7-OH-MTX elimination fitted with a monophasic course (Figure 2). The biphasic elimination curves for MTX and the monophasic curves described for 7-OH-MTX were apparent from the chosen

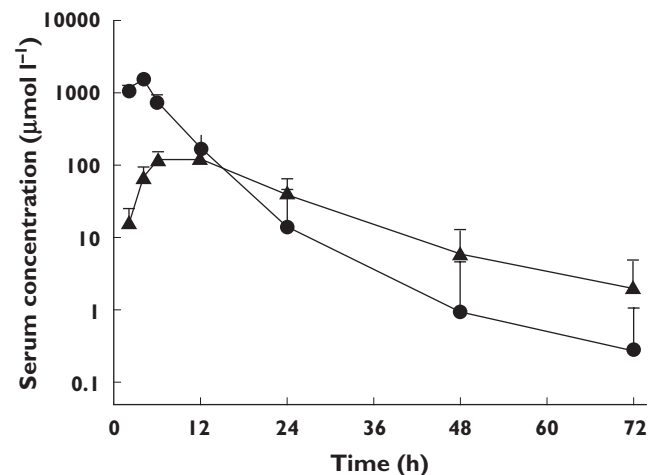


Figure 2

Methotrexate (●) and 7-hydroxy-methotrexate (▲) concentration–time data from 288 high dose methotrexate cycles in 65 patients. Data are presented as mean + 1SD

sampling strategy with a total of eight serum samples over 72 h. However, a calculation based on the two latest time points for both compounds would increase the estimated terminal half-life for 7-OH-MTX from 10 h to 15 h, and increase the estimated terminal half-life for MTX by a factor of almost two, which may suggest that the elimination pharmacokinetics in serum for MTX and 7-OH-MTX are most adequately described by tri- and bi-phasic processes, respectively. On the other hand, this is entirely based on single measurements at the 72 h time point, when analytical variability is at its most disadvantageous, and we have not pursued this. Thus, elimination kinetics are described as bi- and monophasic for MTX and its 7-hydroxylated metabolite. The estimated values for CL_{MTX} , the ratio $AUC_{7-OH-MTX} : AUC_{total, MTX}$, $t_{1/2,\alpha}$, $t_{1/2,\beta}$ and 7-OH-MTX $t_{1/2,\beta}$ are listed in Table 2. Analyses were based on observed, and not extrapolated, AUCs. This may underestimate the AUCs by a magnitude of less than 0.1% (median) for MTX and less

Table 2

Pharmacokinetic parameters of methotrexate (MTX) and 7-hydroxy-methotrexate (7-OH-MTX)

High dose methotrexate cycles (n = 288)	Mean	Median	Quartiles (Q-, Q+)	Range
MTX dose (g m ⁻²)	12		(12, 12)	8–16
Peak MTX (μM)	1509		(1325, 1683)	722–2512
Peak 7-OH-MTX (μM)	130		(101, 160)	27–253
MTX clearance (l m ⁻² h ⁻¹)*	2.9		(2.49, 3.19)	1.10–4.95
Ratio AUC _{7-OH-MTX} : AUC _{total}		0.20	(0.16, 0.24)	0.03–0.43
MTX t _{1/2,α} †		2.49	(2.23, 2.85)	1.67–6.49
MTX t _{1/2,β} ‡		8.38	(7.55, 9.30)	5.63–21.74
7-OH-MTX t _{1/2,β} ‡		10.16	(8.44, 12.14)	6.40–27.11

*MTX dose/AUC_{MTX}. †at 4–6–12 h. ‡at 24–48–72 h.

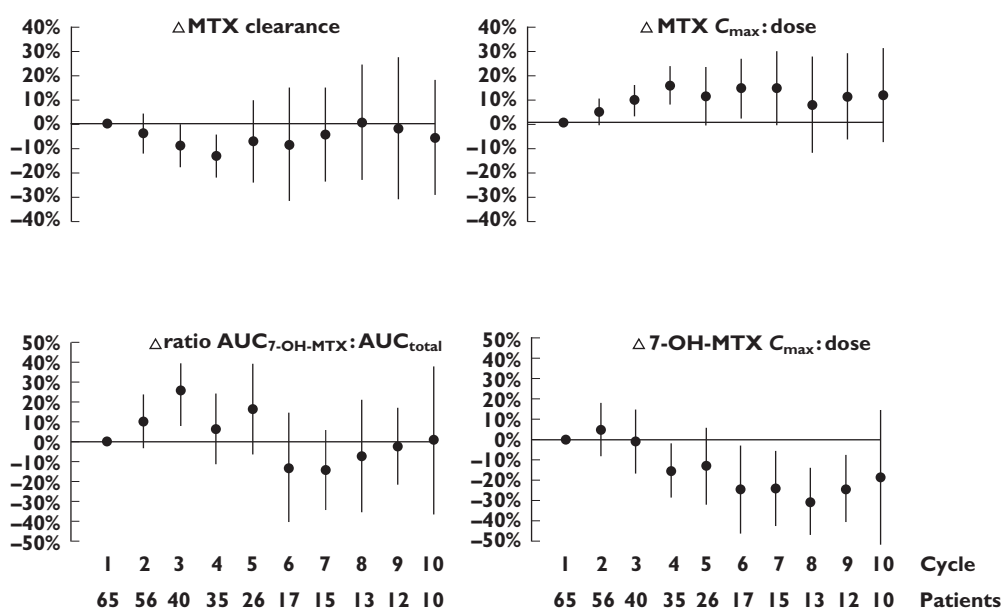


Figure 3

Cycle-to-cycle relative differences (Δ) in methotrexate clearance and peak concentrations (MTX_{C_{max}} : MTX dose) and in 7-hydroxy-methotrexate ratio (AUC_{7-OH-MTX} : AUC_{total}) and peak concentrations (7-OH-MTX C_{max} : dose) obtained in 288 infusions of high dose methotrexate using concentrations at first cycle as baseline. The points represent mean relative differences and the bars indicate the 95% confidence intervals illustrating interindividual variation at each cycle

than 2% (median) for 7-OH-MTX, which was considered negligible. There was up to nine-fold difference in the 7-OH-MTX concentrations between patients, and although 7-OH-MTX peak concentrations were significantly correlated with MTX dose, neither MTX dose nor MTX peak value could predict the ratio AUC_{7-OH-MTX} : AUC_{total}.

Variations in 7-OH-MTX peak concentrations and ratio AUC_{7-OH-MTX} : AUC_{total} between cycles in individual patients (ANOVA; CV 26.3% and 26.5% respectively) were of the similar magnitude as the variations in levels between patients (ANOVA; CV 20.9% and 21.0% respectively). Variations in CL_{MTX} and MTX peak concentrations between cycles within the same patient (ANOVA; CV = 16.0% and 15.6% respectively) were not significantly different com-

pared with concentrations between patients (ANOVA; CV = 10.6% and 10.1% respectively). Figure 3 shows relative differences in CL_{MTX} and MTX peak concentrations, the ratio AUC_{7-OH-MTX} : AUC_{total} and 7-OH-MTX peak concentrations. Interindividual variation was increased after cycle number 4 for all pharmacokinetic parameters, most likely due to the declining number of patients receiving additional therapy cycles. In a multiple backward regression model there was a significant trend of increasing MTX peak concentrations ($p_{sp} = -0.26, P < 0.001$), decreasing 7-OH-MTX peak concentrations ($p_{sp} = -0.23, P < 0.001$) and CL_{MTX} slightly decreasing with increasing cycle number ($p_{sp} = -0.22, P < 0.001$). The ratio AUC_{7-OH-MTX} : AUC_{total} increased during cycles 1–4 and then decreased. There was no

Table 3

High dose methotrexate (HD MTX) toxicity in 65 osteosarcoma patients

	No toxicity	CTCAE* grade 1	CTCAE grade 2	CTCAE grade 3	CTCAE grade 4
Alanine aminotransferase	≤ULN†	>ULN –2.5 × ULN	>2.5–5.0 × ULN	>5.0 –20 × ULN	>20 × ULN
Cycles (%)	2	14	25	42	17
Patients	0	7	6	26	26
Alkaline phosphatase	≤ULN	>ULN –2.5 × ULN	>2.5–5.0 × ULN	>5.0 –20 × ULN	>20 × ULN
Cycles (%)	97	3	0	0	0
Patients	58	7	0	0	0
Gamma-glutamyl transferase	≤ULN	>ULN –2.5 × ULN	>2.5–5 × ULN	>5.0 –20 × ULN	>20 × ULN
Cycles (%)	27	43	23	7	0
Patients	12	18	23	12	0
Bilirubin	≤ULN	>ULN –1.5 × ULN	>1.5–3.0 × ULN	>3.0–10 × ULN	>10 × ULN
Cycles (%)	41	35	24	0	0
Patients	13	21	31	0	0
Creatinine	≤ULN	>ULN –1.5 × ULN	>1.5–3.0 × ULN	>3.0–6.0 × ULN	>6.0 × ULN
Cycles (%)	95	3	2	0	0
Patients	54	8	3	0	0
Leucocyte nadir	≥3.0 × 10 ⁹ l ⁻¹	<LLN‡ –3.0 × 10 ⁹ l ⁻¹	<3.0–2.0 × 10 ⁹ l ⁻¹	<2.0–1.0 × 10 ⁹ l ⁻¹	<1.0 × 10 ⁹ l ⁻¹
Cycles (%)	32	5	30	27	6
Patients	6	1	14	30	14
Thrombocyte nadir	≥75 × 10 ⁹ l ⁻¹	<LLN (75 × 10 ⁹ l ⁻¹)	<75–50 × 10 ⁹ l ⁻¹	<50–25 × 10 ⁹ l ⁻¹	<1.0 × 10 ⁹ l ⁻¹
Cycles (%)	53	20	7	10	10
Patients	9	14	5	15	22
Mucositis					
Cycles (%)	64	13	8	15	0
Patients	18	9	8	30	0

*Common Terminology Criteria Adverse Events v 3.0. †Upper level of normal. ‡Lower level of normal.

correlation between CL_{MTX}, ratio AUC_{7-OH-MTX} : AUC_{total}, MTX or 7-OH-MTX peak concentrations and age, gender or pre-treatment folate concentrations, respectively.

Toxicity

Table 3 summarizes the hepatic, renal, haematological and oral mucosal toxicities encountered. There were no life-threatening adverse events or therapy related deaths during the HD MTX treatment cycles. Most patients had transient elevations in ALAT concentrations. Peak values (at 4 h) were significantly higher among female patients (median 10.0 × ULN, 95% CI 7.6, 13.9) compared with male patients (median 5.5 × ULN, 95% CI 4.6, 7.1, M-W, *P* < 0.001) and were significantly higher among paediatric patients (median 10.5 × ULN, 95% CI 8.0, 11.5) compared with adults (median 4.8 × ULN, 95% CI 4.2, 6.6, M-W, *P* = 0.02). The relationship between ALAT peak values and pharmacokinetic parameters was studied in a backward linear multiple regression analysis with log ALAT as an independent variable and log CL_{MTX}, log MTX peak concentration, log ratio AUC_{7-OH-MTX} : AUC_{total}, log 7-OH-MTX peak concentration, age and gender as dependent variables. We found significant positive correlations between log ALAT and log ratio AUC_{7-OH-MTX} : AUC_{total} (*P* < 0.001), age (*P* < 0.001), gender (*P* < 0.001) and log CL_{MTX} (*P* < 0.001). Age contributed with a factor of 2.5 for the youngest patients relative to the oldest and gender contributed with a factor of 1.7 for female (1.0 for male). Comparing cycles with high ratio AUC_{7-OH-MTX} : AUC_{total}

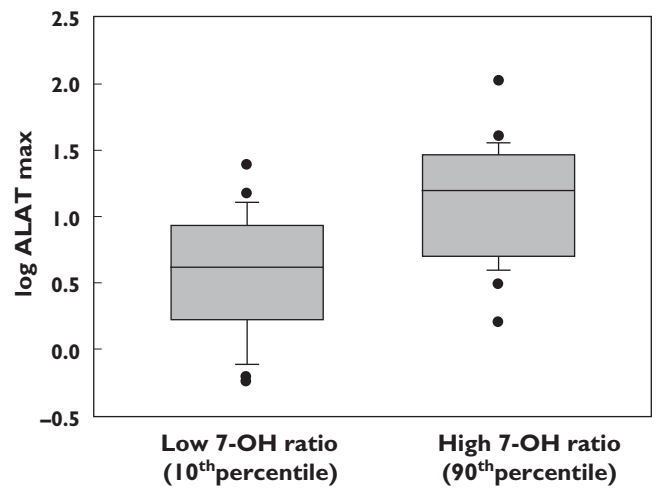


Figure 4

Box plot showing significant (*P* < 0.001) difference in ALAT peak value between cycles having a high (≥90th percentile) 7-hydroxy-methotrexate ratio (AUC_{7-OH-MTX} : AUC_{total}) and cycles having a low (≤10th percentile) 7-hydroxy-methotrexate ratio

(≥90th percentile) with low ratio (≤10th percentile), this variable contributed with a 3.3 fold increase in peak ALAT concentrations (Figure 4). Comparing cycles with low CL_{MTX} (≤10th percentile) with high CL_{MTX} (≥90th percentile), this variable contributed with a 1.3 fold increase in peak ALAT

concentrations. These analyses indicated that high relative concentrations of 7-OH-MTX, young age, female gender and low CL_{MTX} were among the contributing factors for hepatocellular damage, assessed as increases in ALAT concentrations after MTX infusion. γ GT peak values were significantly higher among female patients (median $3.1 \times ULN$, 95% CI 2.7, 4.0) than among male patients (median $1.4 \times ULN$, 95% CI 1.3, 1.7, M-W, $P < 0.001$), but was not correlated with age.

Renal toxicity grade 1–2 developed in 13 patients with creatinine elevations after the first cycle in 11 patients and after the fourth cycle in two patients. Five of these patients continued therapy according to protocol (four patients < 40 years), while five patients were excluded from further HD MTX cycles due to renal toxicity (four patients > 40 years), and the remaining three patients (all < 40 years) did not receive further HD MTX due to other causes.

Platelet nadirs did not correlate with gender, but were slightly higher among paediatric patients (median $172 \times 10^9 l^{-1}$, 95% CI 128, 169) compared with adults (median $125 \times 10^9 l^{-1}$, 95% CI 106, 136, M-W, $P < 0.001$).

WBC nadirs were slightly lower (median $2.2 \times 10^9 l^{-1}$, 95% CI 1.8, 2.7) in patients with low baseline S-folate concentrations, compared with patients with high concentrations (median $2.8 \times 10^9 l^{-1}$, 95% CI 2.5, 3.2, M-W, $P = 0.005$), using the same cut-off value for S-folate as described by Sterba *et al.* [24], thus dividing patients by low (≤ 10 nmol l^{-1}) and high folate concentrations (> 10 nmol l^{-1}).

There was a significant correlation between mucositis grade and age. Mucositis in paediatric patients occurred in 32 of 124 HD MTX cycles: grade 0 in 92 cycles, grade 1 in nine cycles, grade 2 in 13 cycles and grade 3 in 10 cycles. Among adults mucositis occurred in 85 of 171 HD MTX cycles: grade 0 in 86 cycles, grade 1 in 31 cycles, grade 2 in 13 cycles and grade 3 in 31 cycles (chi-squared test with Yates' correlation = 16.4; $P < 0.001$). There was no correlation between mucositis and gender, pretreatment folate concentrations, MTX dose, pharmacokinetic parameters, number of MTX cycles or renal toxicity. Dermatitis developed after 7% of cycles (18 patients) and pleuritis after 1% (three patients, all female).

Discussion

Acute toxicities in HD MTX therapy are often unexpected and not typically dose dependent. Being a folate analogue, MTX competes with natural folates both for cellular uptake and metabolism, and it could be expected that patients with poor pretreatment folate status would be more likely to develop toxicity. In our study S- and ER-folate concentrations increased significantly with increasing number of HD MTX cycles. These findings are in agreement with those reported by Sterba *et al.* [24], who suggested that increasing folate baseline concentrations could originate from repetitive LV administration. We also found lower initial

folate concentrations in females compared with males, but except for slightly lower WBC nadirs in patients with low baseline folate concentrations, there was no correlation between folate concentrations and the markers of acute toxicity that we studied. Publications concerning the impact of pretreatment folate status on HD MTX related acute toxicity are limited. In a case report, severe encephalopathy was described in association with the first but not the second cycle of HD MTX, where pretreatment folate plasma concentrations were low before the first cycle and then 10-fold higher before the second cycle [25].

MTX targets several critical events in folate metabolism and baseline folate concentrations could be possible predictors of *in vivo* MTX pharmacobiological action. In our study approximately 2200 serum samples were analyzed allowing a detailed analysis of pharmacokinetics, but there were no significant correlations between pretreatment folate concentrations (227 HD MTX cycles) and pharmacokinetic variables.

Acute hepatic toxicity related to HD MTX occurs frequently and we observed elevated liver transaminases, bilirubin and γ GT in 98%, 59% and 73% of the cycles with peak values up to 105, 2.9 and 7.9 times normal, respectively. The pathophysiology has remained unclear, but in our study we found that ALAT peak values were correlated with 7-OH-MTX exposure and were 3.3 fold increased at the 90th percentile compared with the 10th percentile of $AUC_{7-OH-MTX} : AUC_{total}$.

In contrast to earlier reports of lower overall toxicity in paediatric patients [26] and reports of increased liver toxicity in adults [27], we found significant positive correlations between acute liver toxicity and young age. The highest ALAT concentrations were apparent in paediatric patients (paediatric : adult ratio for $ALAT_{max}$ was 2.5:1.0) and although the paediatric patients received a higher number of HD MTX infusions, there was no correlation between number of cycles and toxicity.

We also found significant correlations between the maximum ALAT concentration and gender (female : male ratio for $ALAT_{max}$ was 1.7:1.0). There are, however, no previous reports addressing the relationship between acute liver toxicity and gender in patients treated with HD MTX. It is notable, though, that Becker *et al.* [28] found significantly greater risk of liver toxicity in females after low dose MTX, and suggested that hormonal interactions or differences in MTX pharmacodynamics between genders might be involved.

Polymorphisms in folate-metabolizing enzymes and transport proteins may modify the toxicity and drug elimination of folate antagonists [29–31]. Baggott *et al.* [32] described a bimodal distribution of 7-OH-MTX production in a group of 29 patients with rheumatoid arthritis receiving low dose MTX (one cycle per patient) and suggested the existence of two phenotypes in the metabolism of MTX to 7-OH-MTX. Although we found large differences in 7-OH-MTX concentrations between patients, a unimodal

distribution of $AUC_{7-OH-MTX}$, with intermediate concentrations of the metabolite was displayed in the majority of patients.

While Erttmann *et al.* [4] reported that 7-OH-MTX concentrations decreased with repeated cycles in osteosarcoma patients, Borsi *et al.* [33] described that increasing number of cycles did not affect 7-OH-MTX concentrations in children with ALL. In our study, however, a biphasic trend was apparent. From the first to the fourth cycle 7-OH-MTX concentrations increased significantly, but after the fifth cycle 7-OH-MTX concentrations decreased. Our suggestion is that this could possibly be caused by increased activity of renal and hepatic ATP-binding cassette transporter proteins, thus increasing elimination of 7-OH-MTX.

Since both MTX and cisplatin are included in the osteosarcoma treatment protocols, it is likely that repetitive exposure to these nephrotoxic drugs would alter MTX clearance. The effect of repeated HD MTX administration on the pharmacokinetics of MTX in our study revealed the same trend as described by Crews *et al.* [34]. As the number of HD MTX infusions increased, CL_{MTX} decreased slightly, but we found no association between HD MTX cycle number and creatinine elevation. Renal toxicity (creatinine CTCAE grade ≥ 1) was seen in 5% of the cycles, and of the 65 patients in our study, five were excluded from further cycles due to MTX toxicity. It should be noted, however, that serum creatinine is not a sensitive marker of nephrotoxicity, and osteosarcoma patients today are monitored by measurement of glomerular filtration rate.

To our knowledge, this is the first report of an association between 7-OH-MTX concentrations, age, gender and acute hepatocellular damage in humans. Putative mechanisms remain obscure, but it is noteworthy that the 7-hydroxylated MTX metabolite formed in the liver has regained some cytotoxic activity and possesses lesser solubility at relevant pH compared with the parent compound. Thus, the association may reflect a direct toxic effect of 7-OH-MTX on hepatic parenchyma cells.

Competing Interests

There are no competing interests to declare.

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