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ORIGINAL ARTICLE

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# Maximum tolerated doses of methotrexate and 7-hydroxy-methotrexate in a model of acute toxicity in rats

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**Abstract** *Purpose*: After more than 50 years of methotrexate (MTX) treatment of acute lymphoblastic leukaemia (ALL), it is currently believed that as long as dose escalations are followed by adequate leucovorin rescue guided by monitoring MTX serum concentrations, hydration and urinary alkalinization, high-dose MTX (HD-MTX) can be tolerated without life-threatening toxicity. However, our recent experimental animal studies of the major metabolite of MTX, 7-OH-MTX, indicate that this concept may have some limitations. Animals with levels of 7-OH-MTX of 1 m*M*, which is below the levels routinely found in patients on HD-MTX, demonstrate intolerable toxicity and some animals die within 8 h. Electron microscopy indicates that endothelial cell and platelet functions are perturbed. Since animal data are lacking, and interspecies differences not known, we wanted to investigate the maximum tolerated doses of MTX and 7-OH-MTX in a rat model of short-term effects. The maximum tolerated dose was chosen instead of $LD_{50}$ for reasons of animal welfare. *Methods*: We infused MTX and 7-OH-MTX into anaesthetized male Wistar rats and monitored the animals for 8 h. The drugs were given as a bolus plus continuous infusion. The dose-finding ranges were 1.8–11.3 g/kg MTX and 0.1–1.2 g/kg 7-OH-MTX. *Results*: The maximum tolerated dose was between 3 and 5 g/kg for MTX and lower than 0.1 g/kg for 7-OH-MTX. The mean serum concentrations of MTX and 7-OH-MTX in animals that did not survive the 8-h period were 21.9 and 1.6 m*M*, respectively. The animals that received the highest MTX or 7-OH-MTX doses and concentrations died after sudden reductions in heart rate and blood pressure. *Conclusions*: We demonstrated a lower maximum tolerated dose of 7-OH-MTX than of MTX in rats after 8 h. The 7-OH-MTX concentrations were in the therapeutic range after HD-MTX. If the rat/human interspecies differences are not large, our data may indicate that HD-MTX regimens should not be further dose intensified, due not so much to the effects of MTX as to those of 7-OH-MTX.

**Key words** 7-Hydroxymethotrexate · Methotrexate · Maximum tolerated dose · Rat

## Introduction

A number of innovations have contributed to the important role still played by methotrexate (MTX) more than 50 years after the introduction of antifolate treatment. Most important have been the refinement of dose schedules, leucovorin rescue, combination therapy, lack of long-term toxicity [1] and pharmacokinetic monitoring [12]. High-dose MTX (HD-MTX), guided by serum concentration measurements, has improved the outcome in children with acute lymphoblastic leukaemia (ALL) [12, 13] and in patients with osteosarcoma [11, 16]. The pharmacokinetic and pharmacodynamic rationale for HD-MTX in ALL involves control of kinetics by dosage, compliance, absorption, distribution, metabolism and excretion and control of dynamics with information on lymphoblast lineage, chromosome number, MTX polyglutamate accumulation and inhibition of de novo purine synthesis [12]. Recognition of genetic abnormalities influencing the lymphoblasts' sensitivity to chemotherapy [13] and adjusting dosages on the basis of pharmacokinetic characteristics in

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individual patients may improve existing therapeutic regimens further [15].

Published evidence thus seems to support the conclusion that as long as dose escalations are followed by adequate leucovorin rescue guided by monitoring of MTX serum concentrations, hydration and urinary alkalinization, HD-MTX can be tolerated without life-threatening toxicity. However, our recent experimental animal studies of the major metabolite of MTX, 7-OH-MTX, indicate that this concept may have some limitations. Animals with 7-OH-MTX serum concentrations of 1 m*M*, which is below the levels routinely found in patients on HD-MTX [3, 9], experience intolerable toxicity and some animals die within 8 h [19]. Electron microscopy indicates that endothelial cell and platelet functions are perturbed [19].

Historically, most chemotherapeutic agents have been used without an understanding of their mechanism of action [1]. These drugs were introduced into the clinic 50 years ago without extensive preclinical documentation. Therefore, no published experimental animal data are available to address the important questions arising from the animal studies: Is there an interspecies difference between rats and humans with respect to high antifolate concentrations and acute life-threatening toxicity? What is the relative contribution of MTX and 7-OH-MTX to the acute toxicity? Can these toxic effects be counteracted by leucovorin? If the interspecies difference is not excessive, is it reasonable to suppose that HD-MTX regimens may be close to life-threatening toxicity, not so much due to the effects of MTX as to those of 7-OH-MTX?

Since all these questions cannot be addressed in patient populations, answers have to be sought in animal investigations. However, today animal welfare considerations demand that investigations must be designed with a minimal number of animals and minimal distress to the animals. The aim of the present investigation was therefore to establish maximum tolerated doses and concentrations of MTX and 7-OH-MTX after 8 h, employing a minimal number of animals and MTX and 7-OH-MTX dosing guided by our previous data on toxicity [19]. Specifically, we report here studies comparing maximum tolerated doses of MTX and 7-OH-MTX in a model for testing of the short-term effects in anaesthetized rats.

## Materials and methods

### Drugs and chemicals

MTX was kindly provided by Nycomed, Oslo, Norway. All solvents and compounds were of analytical grade. Methanol was from Labscan, Dublin, Ireland, and tetrahydrofuran was obtained from Rathburn Chemicals, Walkerburn, UK. Chemicals used in the synthesis were from Aldrich Chemical Company, USA. All samples containing MTX and 7-OH-MTX were stored at −80 °C in darkness for up to 4 weeks before analysis by high-performance liquid chromatography (HPLC).

### Synthesis of 7-OH-MTX

Synthesis of 7-OH-MTX was essentially as described previously [19], except for the collection of the methotrexate-dimethylester precipitate, which was done by centrifugation. The yellow crystals were washed three times with distilled water before lyophilization. In the third step, 7-OH-MTX was synthesized by dissolving 7-cyanomethotrexate dimethylester in tetrahydrofuran and water at 4 °C and then adding 2 *M* NaOH. Pale-yellow 7-OH-MTX crystals precipitated and were collected by centrifugation, which was followed by washing three times with acetone.

The identity of the compound was confirmed by nuclear magnetic resonance (NMR), and the purity was checked by HPLC with diode-array detection (Waters 996 PDA HPLC system) [4]. The 7-OH-MTX synthesized coeluted on HPLC and showed the same UV spectrum as 7-OH-MTX previously synthesized in our laboratory [19].

### Animals and operation

Male Wistar rats weighing 200–300 g were anaesthetized with a combination of fentanyl and midazolam. In the supine position, rats had their right external jugular vein cannulated for administration of drug and for blood sampling. The right carotid artery catheter was connected via a two-way stopcock to a transducer for blood pressure (BP) measurement. To maintain the body temperature, the rats were placed on a hollow Plexiglas plate maintained at 40 ± 1 °C with circulating water from a thermostat-controlled waterbath. The body temperature was continuously monitored with rectal digital thermometers to prevent hypothermia. Electrocardiograms (ECG) and BP were monitored using a four-channel Gould Windograf.

### Experiments

Maximum tolerated doses have replaced $LD_{50}$ in many toxicity protocols. In carcinogenicity testing the maximum tolerated dose is defined as the dose that permits 90% of the animals to survive a chronic dosing. We have used this term for the dose at which 90% of animals survive in a rat model of short-term effects. The study was designed to determine the maximum tolerated dose during an 8-h study period. MTX and 7-OH MTX were given as a bolus plus continuous infusion. The dose-finding ranges were 1.8–11.3 g/kg for MTX and 0.1–1.2 g/kg for 7-OH-MTX. The infusion rate was calculated using previous clearance data from our laboratory [18] to achieve steady-state serum levels of MTX and 7-OH-MTX similar to those obtained in humans after HD-MTX [3, 9]. MTX and 7-OH-MTX were dissolved in 150 m*M* NaCl solution and MTX solutions were adjusted to pH 8 with 1 *M* NaOH, while the 7-OH-MTX solutions attained pH 7.5 when dissolved. All solutions were sterile-filtered before administration to the rats. To minimize surgical intervention the same catheter was used to administer the drugs and to obtain the blood samples.

Two groups of control rats were used. One group received NaCl solution (150 m*M*, 30 ml/kg), the second group received mannitol solution (930 mOsm, 30 ml/kg) for the first 2 h of the experiment. The mannitol solution had the same osmolarity as the solution with the highest MTX concentration. For the first 10 min a continuous bolus infusion with either MTX (1 g/kg) or 7-OH-MTX (0.1 g/kg) was given with a syringe. During the next 110 min the drug solutions were continuously administered using a peristaltic pump. All groups received the same volume (30 ml/kg) but with varying concentrations of MTX and 7-OH-MTX. Blood samples (0.250 ml) for HPLC analysis were taken at 2, 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360 and 480 min. The catheter was flushed with heparinized NaCl solution (150 m*M*, 10 IU) both before and after sampling to avoid clotting.

When the 2-h administration of drug or NaCl solution (150 m*M*) was stopped, the animals were hydrated with $NaHCO_3$ (5 ml/kg per hour, 0.06 *M*) mixed with NaCl solution (150 m*M*) to

prevent dehydration. At 8 h, all surviving animals underwent laparotomy and exsanguination.

### Analysis and calculations

Accurate determination of MTX and 7-OH-MTX in serum and urine was performed by reverse-phase HPLC as reported previously [4]. Since blood samples were drawn immediately after death, no post-mortem redistribution of MTX or 7-OH-MTX took place. Serum concentrations of 7-OH-MTX were analyzed according to a three-compartment open model. The pharmacokinetic parameters were obtained by means of regression analysis in a semilogarithmic dataset and refer to the triexponential equation:

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

Total clearance, $CL_T$, was calculated from the equation $CL_T = \text{dose/AUC}$. The biological half-life of drug was calculated from $t_{1/2} = \ln 2/k_e$, where $k_e$ is the elimination rate constant, i.e. the slope of regression line of the $\alpha$, $\beta$, and $\gamma$ phases.

## Results

Animals receiving 7-OH-MTX and MTX were compared with groups receiving the same infusion volumes of NaCl solution (150 m*M*) or mannitol for control of volume and osmolarity respectively. The first control group ($n = 5$) receiving 30 ml/kg NaCl solution (150 m*M*), and the second control group ($n = 5$) receiving 30 ml/kg mannitol, survived for the entire 8-h experimental period. Table 1 shows that the maximum tolerated dose of MTX was between 3 and 5 g/kg and that of 7-OH-MTX was probably lower than 0.1 g/kg.

Rats receiving 7.6 and 11.3 g/kg MTX died during the first 3 h, while all rats receiving 0.9 g/kg and 1.2 g/kg 7-OH-MTX died before 6 h. The time interval from sudden reduction in heart rate (HR) and BP to death was never more than 2 min. Venous blood gas analysis showed no difference between the control groups and experimental groups (data not shown).

**Table 1** Estimation of maximum tolerated doses of MTX and 7-OH-MTX in anaesthetized rats. Rats were given increasing doses of MTX and 7-OH-MTX as a bolus plus continuous infusion. Rats that were alive after 8 h were scored as surviving

| Infusion | Surviving/total | % Surviving |
|---|---|---|
| NaCl | 5/5 | 100 |
| Mannitol | 5/5 | 100 |
| MTX (g/kg) | | |
| 1.8 | 5/5 | 100 |
| 3.0 | 5/5 | 100 |
| 5.0 | 2/5 | 40 |
| 7.3 | 1/6 | 16 |
| 11.3 | 0/3 | 0 |
| 7-OH-MTX (g/kg) | | |
| 0.1 | 4/5 | 80 |
| 0.2 | 4/5 | 80 |
| 0.6 | 4/5 | 80 |
| 0.8 | 1/5 | 20 |
| 0.9 | 0/5 | 0 |
| 1.2 | 0/4 | 0 |

### Serum levels

A concentration versus time curve after short-time bolus infusion of 0.1 g/kg 7-OH-MTX, followed by continuous infusion of different concentrations of 7-OH-MTX during the next 110 min is shown in Fig. 1. The mean serum concentration of 7-OH-MTX at the time of death was 1.58 ± 1.52 m*M* (±SD), which is below the concentration some patients achieve after HD-MTX [3, 9]. The serum levels for animals receiving a bolus infusion of 1 g/kg MTX followed by 110 min with different concentrations of MTX are shown in Fig. 2. The mean serum concentration of MTX at the time of death was 21.9 ± 21.4 m*M* (±SD) Serum concentrations of 7-OH-MTX at the 0.1 g/kg dose declined triphasically (Fig. 1).

### BP and ECG findings

The mean systolic BP of control rats was 130 mmHg and the diastolic pressure was 80 mmHg. The HR was 450–550 strokes/min during the observation period. During the experiments, no arrhythmia was observed in the different control groups. The ECG of animals that had received at least 5 g/kg MTX or 0.2 g/kg 7-OH-MTX had one or more of the following ECG findings: partial atrioventricular (AV) block in the short period of time prior to death, atrial flutter, atrial fibrillation, second-degree AV block every 2–3 s before a third-

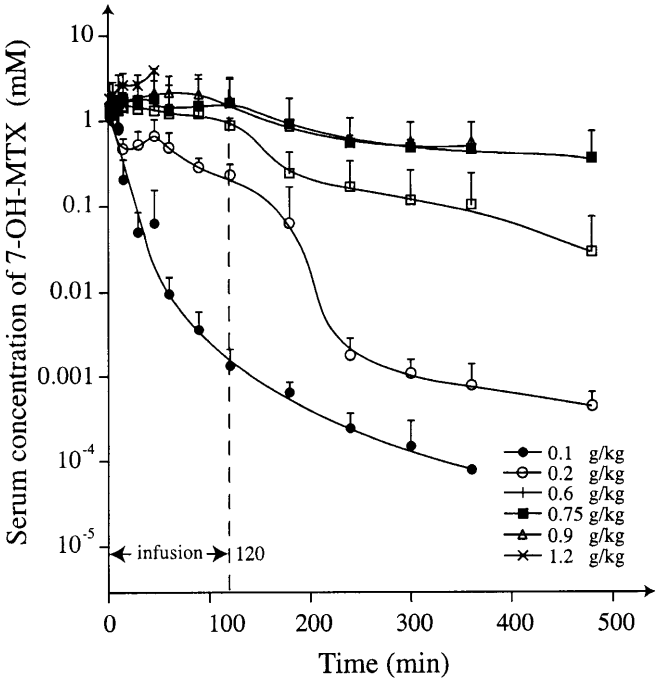


**Fig. 1** Serum concentrations of 7-OH-MTX versus time after short-time bolus infusion of 100 mg/kg 7-OH-MTX, followed by continuous infusion of different concentrations of 7-OH-MTX during the next 110 min. For animals receiving 0.1 g/kg the serum concentrations at 8 h were below the detection limit. Data are mean values ± SEM, $n = 2$–5 in each group

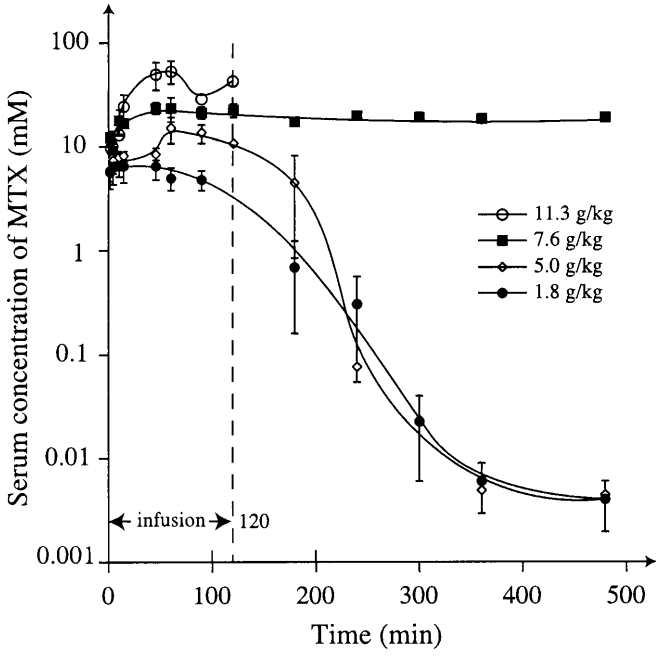


**Fig. 2** Serum concentrations of MTX versus time after short-time bolus infusion of 1 g/kg MTX, followed by continuous infusion of different concentrations of MTX during the next 110 min. Data are mean values ± SEM ($n = 2$–5) except at the doses 11.3 and 7.6 g/kg

degree total AV block occurred, and sequences with sinus arrhythmia.

### Pharmacokinetics

The pharmacokinetics of 7-OH-MTX have been calculated as both a two- and a three-compartment model [10, 17, 18]. Following 10 min i.v. infusion of the lowest concentration (0.1 g/kg) of 7-OH-MTX, the metabolite was eliminated in a triphasic manner with the following mean elimination half-life values (min, ±SD, $n = 4$): $t_{1/2}\alpha = 1.6 \pm 1.1$, $t_{1/2}\beta = 12.2 \pm 3.3$, $t_{1/2}\gamma = 62.3 \pm 33$. Total clearance ($CL_T$) was 13.3 ± 4.8 ml/min per kg. The pharmacokinetic variables were compatible with those previously observed [18]. Elimination half-lives were significantly prolonged in animals receiving doses higher than 0.6 g 7-OH-MTX and 5.0 g/kg MTX (Figs. 1 and 2).

## Discussion

Acute and chronic toxicity of MTX limit therapeutic dose intensity and may lead to discontinuation. The mechanisms of both the acute and the chronic toxicity of MTX may or may not be related to its cytostatic actions, and can be schematically categorized according to its effects on folate metabolism, purine metabolism, pyrimidine metabolism, adenosine metabolism, homocysteine/methionine/polyamine metabolism and methylene-tetrahydrofolate reductase gene mutation, and whether or not the toxicity can be reversed by folinic acid administration [2, 21].

Since the therapeutic and adverse effects of MTX treatment are the net result of the actions of MTX and its major metabolite 7-OH-MTX, it is of importance to compare the actions of the two compounds. If it could be shown that 7-OH-MTX is the culprit or the major modulator of toxicity, the development of antifolates without toxic metabolites would be of interest. Alternatively, the formation of 7-OH-MTX after MTX administration could be inhibited by pharmacological agents [6].

Previous studies have indicated that 7-OH-MTX could be more toxic than MTX in a rat model designed to test acute toxicity [5, 18, 19]. The present data confirm this in a toxicity test using a minimal number of animals [7, 8, 14, 20], defining the relevant dose levels for future mechanistic studies, thus further contributing to the use of limited numbers of animals. We demonstrated that the maximum tolerated dose for 7-OH-MTX is more than ten times less than that of the parent compound. Furthermore, the MTX/7-OH-MTX serum concentration ratio at the time of death was 14.

Our data also suggest that the mechanism(s) for the acute lethality are different from the anticancer mechanism(s), and not related to the severe tissue damage to the liver and kidney due to the short time between dosing and death. Firstly, in the systems tested for anticancer effects, MTX was effective at doses and concentrations lower than those of 7-OH-MTX, while the maximum tolerated dose and serum concentrations were lower for 7-OH-MTX than for MTX. Secondly, leucovorin did not antagonize the acute lethal effects of MTX, indicating a mechanism different from the antifolate-mediated anticancer effects (Fuskevåg et al., in press). Thirdly, observations of the animals and the ECG recordings were compatible with cardiac death. Disturbance in AV conductance, possibly as a result of endothelial damage and intravascular platelet aggregation, observed in kidney and liver vasculature, could have been responsible for the cardiac death.

Children with ALL at the end of MTX infusion at doses in the range 12–33 g/m$^2$ show 7-OH-MTX serum concentrations in the range 2.1 ± 2.0 to 3.5 ± 1.2 m$M$ (±SD) [3]. These concentrations are higher than those obtained in this study after administration of 0.9 g/kg 7-OH-MTX to five rats that died after 90–410 min with post-mortem 7-OH-MTX concentrations of 0.6–3.2 mM.

We have demonstrated that 7-OH-MTX is more toxic than MTX in a rat model of acute toxicity. Further studies should be directed at the mechanism(s) of acute toxicity and at the possibility that very high levels of 7-OH-MTX could be limiting for dose increments in HD-MTX regimens.

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