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SHORT COMMUNICATION

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Effects and interaction of 7-hydroxy methotrexate and methotrexate in leukaemic cells ex vivo measured by the thymidylate synthase inhibition assay

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Abstract In high dose therapy with methotrexate (MTX) the main metabolite 7-hydroxy-methotrexate (7-OH MTX) exceeds the plasma concentration of MTX achieving about tenfold higher levels. To investigate the interaction between 7-OH MTX and MTX ex vivo, the thymidylate synthase inhibition assay was used to quantify antifolate effects in patient blast samples, measuring the inhibition of the key enzyme thymidylate synthase (TS). In 18 leukemic samples (7 ALL, 11 AML) no dose-dependent TS inhibition was observed for 7-OH MTX. However, a statistically significant increase of TS inhibition ($p < 0.05$) was observed for a 1:1 mixture of MTX and 7-OH MTX as compared to the effect of MTX alone. The half-maximal inhibitory concentrations in the short-exposure assay were 0.857 μM for MTX alone versus 0.088 μM for the 1:1 mixture with 7-OH MTX, respectively ($p \leq 0.05$). This interaction was not observed with an excess of 7-OH MTX. Similar results were obtained in long exposure experiments. We conclude that there is a dose-dependent interaction between 7-OHMTX and MTX, despite the lack of TS inhibitory effects of the metabolite alone.

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Introduction

From the date of publication of the first report of an antifolate induced remission in leukaemia [10] there has been a remarkable progress so that today about 80% of the children treated for acute lymphoblastic leukaemia (ALL) achieve 5 year survival [17]. Methotrexate (MTX) has always presented a mainstay in these protocols [3] and is used in high-dose schedules at up to 8 g/m^2 [16] and in lower doses of 20 mg/m^2 every week in maintenance therapy for all. In high-dose schedules the metabolite 7-hydroxy-methotrexate (7-OH MTX) can be found in plasma [14].The concentration of this metabolite exceeds the level of MTX soon after the end of infusion [6, 15, 22].

The effect of this metabolite and its interaction with MTX in high dose therapy is still a matter of debate. Johns et al. [13] already showed that 7-OH MTX is only a weak inhibitor of the dihydrofolate reductase in a cell free assay and Fabre et al. [7] found no effect on Ehrlich ascites tumors cell in vitro up to concentrations of 50 μM, although it has been shown that 7-OH MTX enters the cells and, like other antifolates, can be stored inside the cell as polyglutamates [21]. In contrast, 7-OH MTX showed a stronger inhibition of cell growth in a K-562 chronic myeloid cell line than MTX itself [6]. Furthermore, 7-OH MTX dose-dependently inhibits colony formation in a human Burkitt's lymphoma and a human granulocytic progenitor cell line in vitro. This effect was neutralised by adenosine indicating an inhibition of the de novo purine-synthesis [9]. Moreover, rats showed intolerable toxicity with plasma level of 7-OH MTX of 1 mM, a concentration observable in osteosarcoma therapy [5]. Interestingly, the tolerated dose in that animal model was lower for 7-OH MTX than for MTX

[12]. Experiments with two human leukaemic cell lines published recently indicate that 7-OH MTX can provoke distinct modalities of antifolate resistance compared to MTX [11].

To further investigate the effect of 7-OH MTX and its interaction with MTX the thymidylate synthase inhibition assay (TSIA) was used. This indirect toxicity assay provides a validated method to measure antifolate effects in primary patient samples. The TSIA is based on the inhibition of the thymidylate synthase (TS). This enzyme converses deoxy-uridine monophosphate (dUMP) to deoxy-thymidine monophoshate (dTMP) and H_2O. In this radiometric assay, tritium release can be measured as an indication of dTMP formation, and thus TS activity is a good parameter for the cytotoxicity of antifolates [18, 20]. It has also been shown that this assay provides clinically relevant estimates for the activity of antifolates in patients when used ex-vivo [19].

Simulations of MTX and 7-OH-MTX plasma concentrations of the standard dose of 5 g/m^2 MTX as a 24 h infusion (based on the pharmacokinetic parameters from the study of Wolfrom et al. [23]) show that the 7-OH MTX levels exceed the MTX plasma concentrations soon after the end of infusion. Approximately, a ten to one ratio of 7-OHMTX to MTX is achieved after 36 h with this schedule. At that time the essential rescue leucovorin is applied and overcomes the effect of the antifolates.

Consequently, the effect of 7-OH MTX itself was measured in the TSIA in long and short-term exposure. Furthermore, a 1:1, 5:1 and 16:1 ratio of 7-OHMTX and MTX was analysed for both short-term and long-term exposure to determine a possible interaction between these two antifolates.

Materials and Methods

Patients

Patients and/or their parents gave informed consent to the investigations done with their diagnostic samples. Thirty-three samples from leukaemic patients were prepared for testing. Five samples did not achieve the required amount of living blasts and were not worked up. Five samples had a ratio less than two between blanks (culture medium without substance as 0% TS values) and controls (cells without substance as 100% TS activity values) and were therefore considered as not evaluable. Five experiments showed results deviating from the rest and were repeated. These repetitions were included in the evaluation and part of the 18 remaining samples. Eleven samples were diagnosed as AML (one patient was included with both primary AML-M4 and a sample after relapse) and seven samples were ALL derived (Table 1). The availability of the patient-derived leukaemic cells limited the combination experiments to the ones described below.

Table 1 Description of the patient' samples.

Patient No.	Diagnosis	% Blasts
# 1	T-cell ALL	95%
# 2	AML M5	86%
# 3	AML M4	84%
# 4	Common ALL	94%
# 5	AML-M2	82%
# 6	AML-M5	84%
# 7	ALL	98%
# 8	Common ALL	96%
# 9	ALL(t)	94%
# 10	AML	ND
# 11	AML-M4	ND
# 12	AML-M5	96%
# 13	AML-Relapse	ND
# 14	AML-M4/rel.	91%
# 15	AML M4	ND
# 16	T-cell ALL	96,5%
# 17	ALL	ND

Chemicals

7-OH MTX was purchased from Schircks (Jona, Switzerland, purity greater than 99%). MTX was obtained as a gift by Pharmachemie (Haarlem, Netherlands) and of pharmaceutical quality, i.e. in accordance with the requirements of the European Pharmacopoeia. Stock solutions were prepared by dissolving the substances in water. 5-[^{3}H]dCyt was purchased from Moravek Biochemicals (Brea, CA, USA). Fetal calf serum (FCS), penicillin, streptomycin, fungizone, gentamycin, and l-glutamine were obtained from Flow Laboratories (Irvine, UK), bovine serum albumin from Organon Technika (Oss, Netherlands). Insulin, transferrin, sodium selenite were obtained from Sigma (Zwijndrecht, Netherlands).

Thymidylate synthase inhibition assay (TSIA)

The TSIA was drawn out as described by Rots et al. [18]. In brief, 135 µl cell suspension (10^6 cells/ml) was incubated at 37°C with 15 µl of antifolate solution. Blanks without cells served as reference (0% TS activity) as well as controls incubated with 15 µl of culture medium (100% TS activity). A short-term exposure assay indicates incubating for 3 hours with antifolates followed by three washing steps and an 18 h drug free period. A long-term exposure assay was done with 21 h of incubation with antifolates. For 7-OH MTX final concentrations from 0.01 µM to 1 mM were used in the short-term exposure and 0.001 µM to 16 µM in the long-term exposure assay, respectively. MTX concentrations ranged from 0.156 µM to 40 µM in the short-term exposure and 0.0039 µM to 1 µM in the long-term exposure assay, respectively. As combination a ratio of 1:1 was used in short and long-term exposure ranging from 0.0005 µM to 500 µM and 0.0002 µM to 16 µM, respectively. Moreover, a 5:1 ratio was tested for

short-term exposure (same MTX concentrations as 1:1 and five-fold more 7-OH MTX) and a 16:1 ratio was used in long-term exposure (MTX concentrations as 1:1 and 16 times more 7-OH MTX). The ratios 1:1, 5:1 and 16:1 were chosen because they reflect the ratios in patients shortly after the end of infusion, at about 48 h and in the terminal phase after administration of 5 g/m^2 MTX as a 24 h-infusion.

5-[^{3}H]-2'-deoxycytidine (final concentration: 1 µM; 9.2510^{10} Bq/mmol) was added four hours after the start of the assays. After incubation for 21 h all vials were put on ice, 150 µl 40% ice-cold trichloroacetic acid and 750 µl of 10% activated charcoal were added. After vortexing, vials were left on ice for 30 min and centrifuged (800 g, 30 min, 4°C). 450 µl of aqueous phase was transferred in a scintillation vial, and radioactivity was quantified.

Additional cytospins were made of controls and analysed to confirm a percentage of leukemic blasts of at least 80%. Cell counts were done for the validation of the assays.

Evaluation and Statistics

To calculate TS acitivity the measured activity of blanks were subtracted and the activity of the controls fixed as 100%. Sample TS acitivity was then calculated. Further the concentration of drug needed to inhibit 50% of the TS (TSI(50%)) was calculated by the two flanking concentration points of the dose response curve, assuming linearity.

To analyse differences in the inhibition of TS activities by the 7-OH MTX concentrations Friedman test was applied as provided by SigmaStat 2.03.

TSI(50%) values were examined by The Friedman test followed by an all pairwise multiple comparison by Dunn's method as included in SigmaStat 2.03.

Results

7-OH MTX

With short-term exposure conditions for 7-OH MTX no dose related inhibition of TS activity was observed in any of the patient samples. The median of the measured TS activity varied around 100% of the untreated controls showing no inhibition of the TS (data not shown). The Friedman analysis showed no differences between the inhibition of TS in the different concentrations ($p = 0.429$) confirming the absence of a dose response curve.

Similar results were obtained for the long-term exposure assay. No dose response curves were seen and all medians of TS activity over the examined concentration range were around 100% (Fig. 1) and did not differ significantly ($p = 0.166$). These results were similar in AML and ALL patient samples.

MTX and 7-OH MTX

In contrast to 7-OH MTX itself dose response curves are evident for MTX and for the 1:1 and the 16:1 mixtures of the two compounds, respectively. Thus, TSI(50%) values were calculated for these experiments.

In the short-term exposure assay an increase of inhibition of TS activity for the 1:1 concentration ratio compared to MTX was observed. The TSI(50%) was

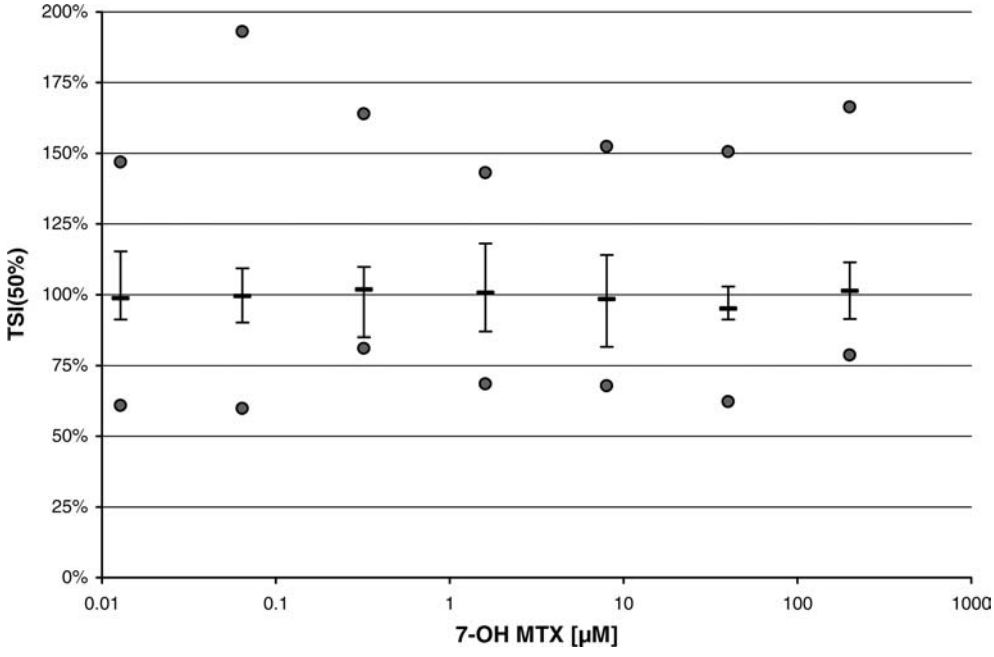

Fig. 1 TS inhibition in response to 7-OH MTX long-term exposure. The line represents medians, the *bars* 25% and 75% percentiles and the points minimal and maximal values. No significant difference in inhibition of TS could be observed ($p = 0{:}166$; $n = 16$)

0.857 μM for MTX alone vs. 0.088 μM for the 1:1 mixture with 7-OH MTX, respectively ($p \leq 0.05$). A higher concentration of 7-OH MTX (concentration ratio of 5 to 1) cancelled this effect, showing a significantly lower TS inhibition as compared to that of the 1:1 condition ($p \leq 0{,}01$) but an effect similar to MTX itself (Fig. 2). The median TSI(50%) value was 0.699 μM for the 5:1 ratio.

Similar results were obtained with the long-term exposure assay. The median TSI (50%) was 0.031 μM for MTX alone, 0.004 μM for the 1:1 mixture and 0.028 μM for the 16:1 mixture. Thus, the TSI (50%) values of the experiments with the 1:1 concentration ratio were significantly lower ($p \leq 0.05$) compared with 16:1 ratio or MTX alone. These results were seen with both AML and ALL patient samples (Fig. 3). No difference could be seen between MTX alone and the experiments with the 16:1 excess of 7-OH MTX.

Discussion

The experiments were conducted in order to better understand the effect of 7-OH MTX and its interaction with MTX with respect to inhibition of TS activity and cytotoxicity. Our results show that 7-OH MTX itself does not influence the TS activity and is therefore probably not itself cytotoxic to AML or ALL blasts at the cellular level. The effects were similar with both the short-term and the long-term exposure assay. Under the conditions of the long-term exposure assay polyglutamylation of MTX and 7-OH MTX is also important for the cytotoxicity and reflects the situation in-vivo.

It has been shown that the Glu_4-polyglutamate of 7-OH MTX is a strong inhibitor of both TS and AICAR transformylase similar to the inhibitory potency of the MTX polyglutamates [22]. Therefore, the results of the experiments with 7-OH MTX in the long-exposure assay are surprising because the conditions of the long exposure experiments should allow formation of the 7-OH MTX polyglutamates. However, formation of polyglutamates is highly variable and could not be quantified in our experiments. Additionaly, different experimental conditions could be the reason for the somewhat conflicting results.

In clinical practice 7-OH MTX appears only concomitantly with MTX. At the end of the 24 h infusion of 5 g/m^2 of MTX, the parent drug exceeds the plasma concentration of the metabolite approximately by a factor 3 and the excess of 7-OH MTX subsequently increases (≅ 60 μM respectively, ≅ 20 μM). Soon after the end of the 24 h infusion, 7-OH MTX exceeds the level of MTX reaching a ten-fold surplus of 7-OH MTX at the beginning of the leucovorin rescue at 36 h after the start of the infusion [22]. The data presented here suggest an enhanced inhibition of the TS activity for MTX in the presence of 7-OH MTX when tested at equimolar level. This enhancement disappears in case of a 5 to 16 fold surplus of 7-OH MTX but does not change into a decreased effect of MTX itself. In order to explain the interaction between 7-OH MTX and MTX at least two different mechanism may be involved. A conceivable mechanism is a synergism at equimolar level where 7-OH MTX inhibits the *de novo* purine synthesis [2] while a surplus of the metabolite diminishes the polyglutamylation of MTX or lowers the inhibition of TS by MTX [8].

High-dose therapy with MTX is monitored by measuring plasma MTX concentrations using immunoassays which cannot quantify 7-OH MTX [1]. Only some specialised laboratories can distinguish between MTX and 7-OH MTX in plasma using high-performance liquid chromatography (HPLC) or capillary electrophoresis. Our results indicate that quantification of 7-OH MTX can be useful in situations with unexpected toxicity after MTX administration. Another argument supporting the need for monitoring both MTX and 7-OH MTX are the findings of Fotoohi et al. [11] showing

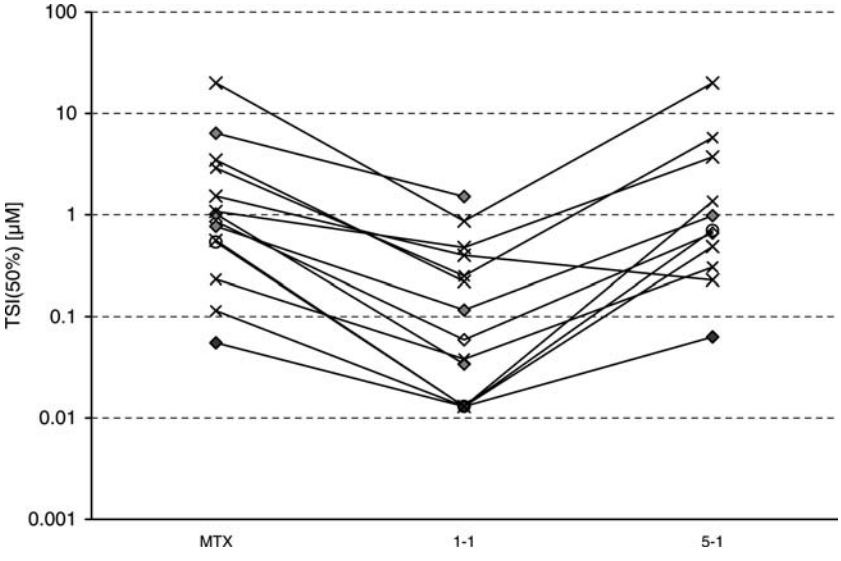

Fig. 2 Half-maximal inhibitory concentrations for the AML (*crosses*) and ALL (*filled diamonds*) patient samples under short-term exposure. 1–1 mixture has significant lower TSI(50%) values than MTX or 5–1 mixture

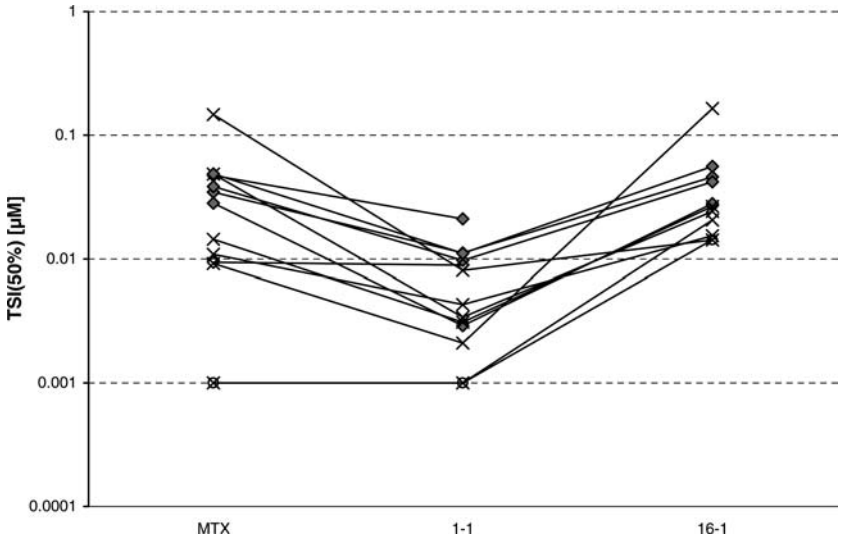

Fig. 3 Half-maximal inhibitory concentrations for the AML (*crosses*) and ALL(*filled diamonds*) patient samples under long-term exposure conditions. 1–1 mixture has significant lower TSI (50%) values than MTX or 16–1 mixture

that 7-OH MTX might play an important role in the development of antifolate resistance. This could mean that patients with high 7-OH MTX concentrations are more susceptible to MTX resistance.

In conclusion, our data show that MTX induced inhibition of TS activity is not weakened by 7-OH MTX until leucovorin rescue is administered 36 h after the start of the infusion, which overcomes 7-OH MTX and MTX effects. In a higher excess of 7-OH MTX—due to an individually higher metabolism rate or due to a different infusion regimen—an antagonism cannot be excluded as reported in one case by Erttmann et al. [4].

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