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ORIGINAL ARTICLE

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Interactions of carboxypeptidase G_2 with 6*S*-leucovorin and 6*R*-leucovorin in vitro: implications for the application in case of methotrexate intoxications

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Abstract Carboxypeptidase G_2 (CPG_2) is used when unexpected toxicity or renal failure occurs during high-dose methotrexate therapy. Leucovorin is also administered to antagonise the effects of methotrexate on purine anabolism. To investigate the effects of CPG_2 on leucovorin rescue, we incubated the enzyme with both stereoisomers and analysed the degradation. A method for separating the stereoisomers of leucovorin, the internal standard aminopterin and the degradation products by capillary electrophoresis with 2.6-dimethyl-β-cyclodextrin as a chiral selector has been developed. The active 6*S*-leucovorin is degraded much faster than the inactive 6*R*-isomer. The maximum observed degradation velocity was 31 μM/min for 6*S*-leucovorin and 20 μM/min for 6*R*-leucovorin, respectively, with an initial concentration of each stereoisomer of 250 μM. Similar results were obtained at lower concentrations of leucovorin isomers. Thus, the selectivity of CPG_2 for methotrexate in comparison to leucovorin is not as high as anticipated in the literature as only the active 6*S*-leucovorin and not the mixture of the diastereomers should be taken into account. We conclude that the protective effects of leucovorin are antagonized by CPG_2. Therefore, CPG_2 should be administered to patients with caution.

Keywords Carboxypeptidase G_2 · Methotrexate · Leucovorin · Capillary electrophoresis · Stereoisomers

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Introduction

Methotrexate (MTX, Fig. 1) is a drug used in many high-dose therapy protocols for the treatment of leukaemia or solid tumours [1]. The main metabolite found in plasma is 7-hydroxy-MTX (7-OH-MTX), whereas 4-amino-4-deoxy-N^{10}-methylpteroic acid (DAMPA) is usually only found in low quantities [8]. MTX and 7-OH-MTX can be stored inside the cell as polyglutamates [6]. MTX and its polyglutamates are inhibitors of dihydrofolate reductase, an enzyme responsible for the formation of tetrahydrofolate, which is the carrier for the transfer of carbon units. Inhibition of dihydrofolate reductase results in reduced thymine production necessary for DNA synthesis [14]. Leucovorin (folinic acid, (6*S*)-5-formyltetrahydrofolic acid; Fig. 2) rescue is usually used to prevent MTX toxicity in high-dose therapy beginning 36 h after starting the MTX infusion. In addition, forced diuresis as well as alkalinization of the urine are essential supportive care procedures for high-dose MTX.

In rare but life-threatening cases the renal clearance of high-dose MTX is strongly reduced leading to severe neutropenia, mucositis, nephrotoxicity, elevation of liver enzymes in plasma and neurotoxicity [23]. Precipitation of MTX and/or 7-OH-MTX in the renal tubules is part of the pathophysiological process. In case of intoxication, high-dose leucovorin (up to 150 mg every 6 h) is administered to antagonise the effects [4, 9]. Furthermore, haemodialysis or haemoperfusion can be used to decrease elevated MTX plasma concentrations [2, 16]. Carboxypeptidase G_2 (CPG_2) provides an experimental option in cases of reduced MTX clearance. The enzyme hydrolyses the glutamic acid moiety of the antitumour drug leading to the pharmacodynamic inactive metabolite DAMPA [12]. Accordingly, also other antifolates such as 7-OH-MTX, but also folates such as leucovorin are cleaved by CPG_2 although the affinity for MTX is higher than for the other related substances [18].

Early investigations with CPG_2 had the aim of providing an alternative to leucovorin rescue, especially for

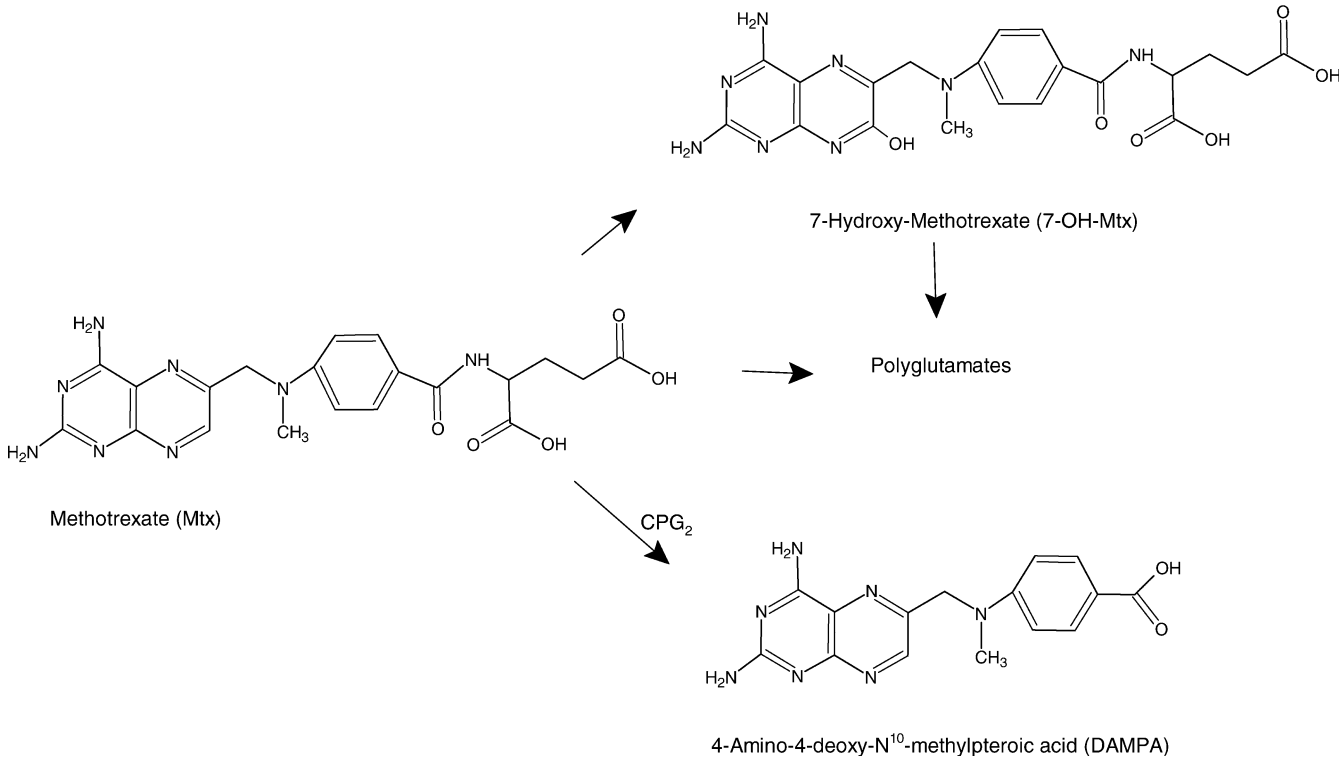

Fig. 1 MTX and its metabolites

high-dose MTX protocols in brain tumours, to selectively reduce the systemic exposure to MTX while maintaining the cytotoxic effect in the CNS [7]. More recently it has become used increasingly in cases of delayed MTX clearance and MTX toxicity [13].

As mentioned above, leucovorin can also be inactivated by CPG_2 by releasing the glutamic acid moiety from the molecule. In contrast to MTX, leucovorin represents a pair of diastereomers. The active 6*S*-leucovorin is accompanied by the pharmacodynamically inactive 6*R*-leucovorin. In the literature, a K_m of 120 μ*M* for the reaction of leucovorin with CPG_2 is reported, whereas the K_m of MTX is 8 μ*M* [18]. However, for leucovorin the Km was calculated for the mixture of diastereomers without taking into account that only the 6*S*-isomer is active. Therefore, the K_m values cited above might be misleading when considering that only one of the isomers is pharmacologically active. Thus, we incubated leucovorin with CPG_2 in vitro and examined the products by chiral capillary electrophoresis (CE) with the aim of investigating possible stereoselectivity in the degradation.

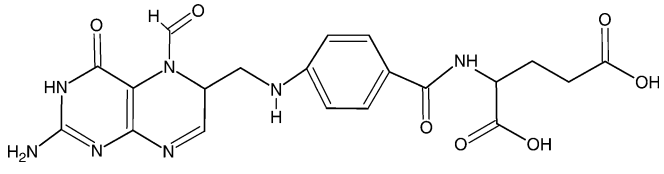

Fig. 2 Leucovorin

Materials and methods

Chemicals and solutions

All chemicals were of analytical grade unless otherwise stated. Leucovorin was purchased from Sigma-Aldrich (Deisenhofen, Germany), Carboxypeptidase from CAMR (Salisbury, UK), 6*S*-leucovorin and aminopterin from Sigma-Aldrich (Deisenhofen, Germany).

The Tris-buffer, pH 7.3, 0.1 *M* , for the incubation experiments was prepared by dissolving 26.8 mg $ZnCl_2$ and 12.2 g Tris-hydroxymethyl-aminomethane in 1 l purified water and adjusting the pH to 7.3 by adding 0.1 *M* phosphoric acid. Leucovorin was dissolved in the Tris-buffer and diluted to achieve final concentrations of 500 μ*M* and 60 μ*M* in the incubation mixture. CPG_2 was also dissolved in the Tris-buffer to a concentration of 10 U/ml. Aminopterin, used as the internal standard, was dissolved in water to a final concentration of 0.1 g/l.

The running buffer for capillary electrophoresis was prepared by adjusting a solution of 90 m *M* $NaHPO_4$ to pH 7.0 with Na_2HPO_4. For the separation of the isomers, 50 m *M* 2.6-dimethyl-β-cylodextrin was added. All solutions for capillary electrophoresis were filtered through a 0.45 μm filter.

Capillary electrophoresis

A Beckman P/ACE 5510 equipped with a diode-array detector set to a 300 nm was used. Fused-silica capillaries with an effective total length of 270 mm and an

extended light path at the detection window were used (Agilent Technologies, Waldbronn, Germany). Capillaries were rinsed with 0.1 *M* NaOH for 2 min, phosphate buffer, pH 7 for 2 min, and 30 s with the running buffer. Samples were applied to the capillary by pressure injection (3448 Pa) for 6 s. Separation was carried out with an applied voltage of 8 kV resulting in a current of about 60 μA.

Quantification

Quantification was done using aminopterin as the internal standard. Calibration using five different concentrations of leucovorin in the range 31–250 μ*M* of the diastereomers was used. Typical parameters of the calibration were $y = 0.0175x + 0.0002$, $r^2 > 0.993$. Precision and accuracy were determined with two different concentrations of the analyte both in one series and on consecutive days. Precision at 200 μ*M*, measured in one series, was better than 1.0% for both 6*R*-leucovorin and 6*S*-leucovorin with an accuracy of 2.7% and 3.3%, respectively ($n = 5$). At 50 μ*M*, precision was 1.2% and 2.4%, with accuracies of 6.9% and 7.1% for 6*R*-leucovorin and 6*S*-leucovorin, respectively ($n = 5$).

Precision measured on consecutive days at 200 μ*M* was better than 0.5% for both 6*R*-leucovorin and 6*S*-leucovorin with an accuracy of 2.4% and 2.7%, respectively ($n = 4$). At 50 μ*M*, precision measured on consecutive days was 1.2% and 3.0%, with accuracies of 3.9% and 6.3% for 6*R*-leucovorin and 6*S*-leucovorin, respectively ($n = 4$).

Incubation

Leucovorin in Tris-buffer (500 μl) was heated to 37°C. A solution containing 10 U/ml CPG_2 (50 μl), tempered to 37°C was added after removing 50 μl of the leucovorin solution for capillary electrophoresis. At certain time points, 50 μl amounts were sampled from the mixture and transferred into a vial containing 50 μl ice-cold acetonitrile and 20 μl of the aminopterin solution as the internal standard. After centrifugation for 10 min, the supernatant was injected into the capillary electrophoresis system.

Results

Although 6*S*-leucovorin and 6*R*-leucovorin are a pair diastereomers, separation was not achieved without adding a chiral selector to the running buffer. Figure 3 shows electropherograms of leucovorin and the internal standard after incubation. By using the pure 6*S*-leucovorin the migration order of the diastereomers was determined. The proposed product of the reaction of CPG_2 with (6*R*,*S*)-leucovorin is *N*-(6*R*,*S*)-5-formyl-5,6,7,8-tetrahydropteroic acid (FTHPA, Fig. 4), a molecule similar to leucovorin but lacking the glutamate moiety. The enantiomers of this compound were separated at baseline under these conditions. With decreasing 6*S*-leucovorin concentrations, the amount of *S*-FTHPA increased, assuming that the *S*-FTHPA migrated after *R*-FTHPA.

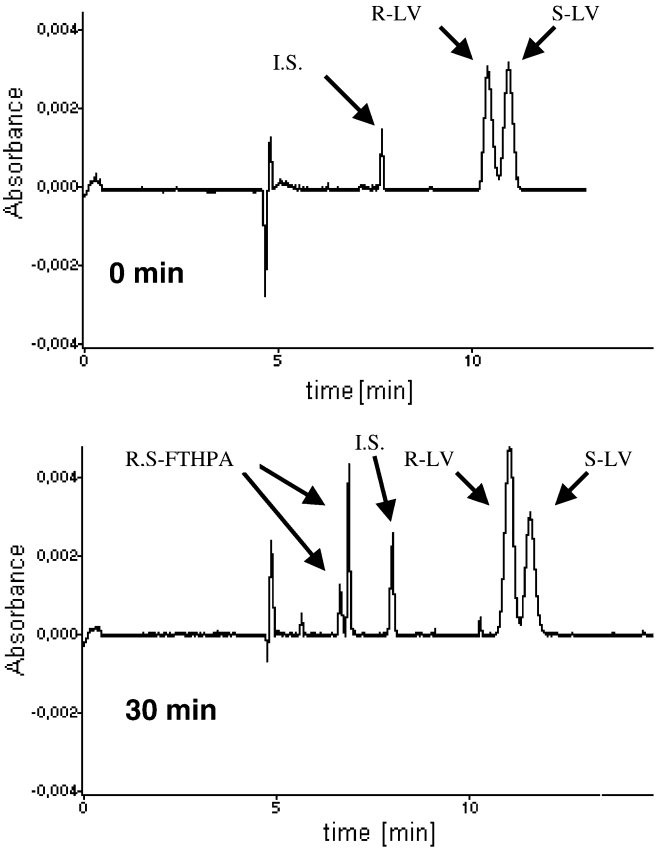

Fig. 3 Electropherogram of a sample of leucovorin before and after incubation for 30 min with CPG_2. The experimental conditions are given in the text (*LV* leucovorin, *I.S.* internal standard aminopterin)

The results of the incubation experiments with 250 μ*M* 6*R*-leucovorin and 6*S*-leucovorin are shown in Fig. 5. A significantly faster degradation of the active *S*-leucovorin in comparison to *R*-leucovorin was observed ($p \leq 0.001$) as early as 1 min after the beginning of incubation. The remaining concentrations at that time point were 235.5 ± 10.3 μ*M* 6*R*-leucovorin and 224 ± 10.3 μ*M* *S*-leucovorin ($n = 6$). The degradation velocity was estimated using the derivative $v_c = \Delta c/\Delta t$ (Fig. 5, lower graph). For 6*S*-leucovorin, velocities

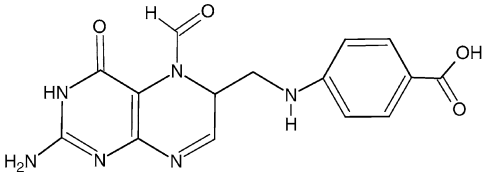

Fig. 4 *N*-(6*R*,*S*)-5-Formyl-5,6,7,8-tetrahydropteroic acid (FTHPA)

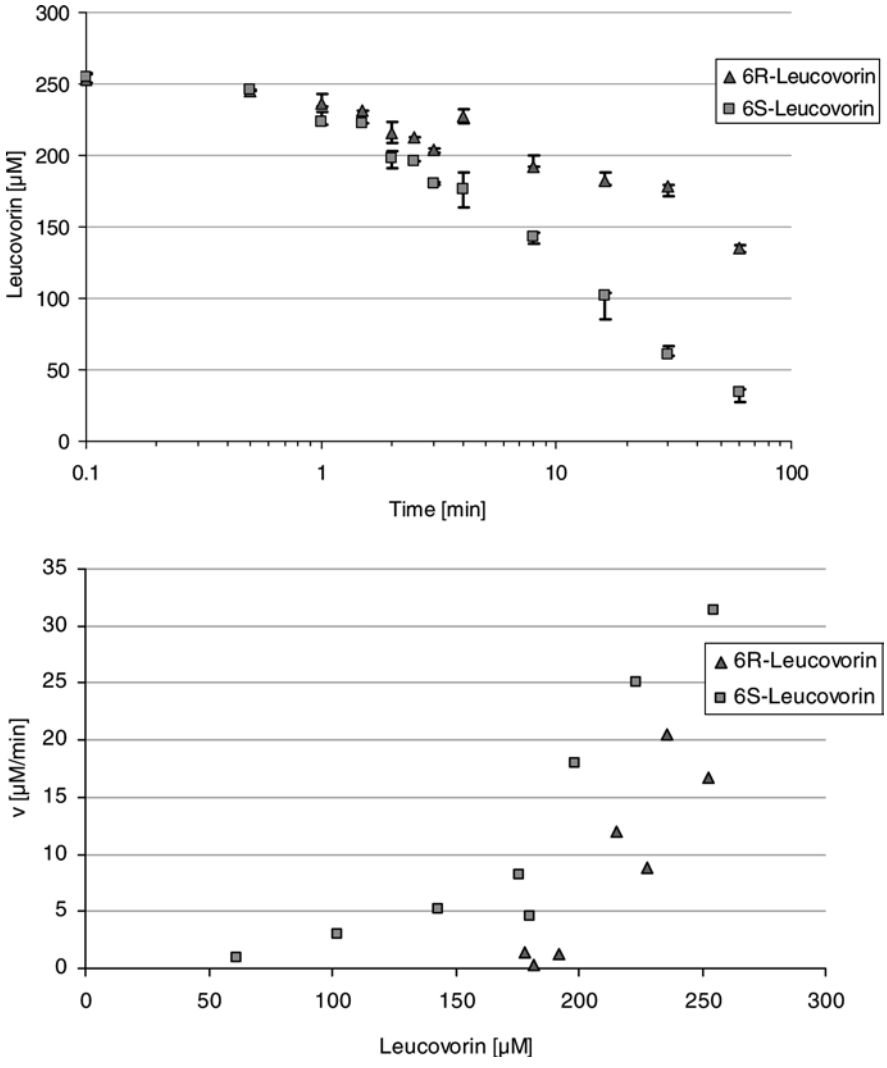

Fig. 5 Degradation of 6*S*-leucovorin and 6*R*-leucovorin by CPG_2. Racemic leucovorin (500 µ*M*) was incubated with CPG_2 and the concentrations of the isomers were quantified by capillary electrophoresis ($n = 6$). The *upper graph* shows medians and 25th and 75th percentiles, and the *lower graph* shows the same data expressed as degradation velocity versus concentration

higher than 30 µ*M*/min were observed whereas the maximum observed velocity for 6*R*-leucovorin was 21 µ*M*/min. Unfortunately, calculations of V_{max} and K_m were not possible because V_{max} was not reached in the concentration range investigated.

A second series ($n = 3$) of samples containing 30 µ*M* of *R*-leucovorin and *S*-leucovorin showed similar results with a clearly faster degradation of the active 6*S*-leucovorin (Fig. 6). The concentration of 30 µ*M* corresponds to the plasma concentrations achieved in patients

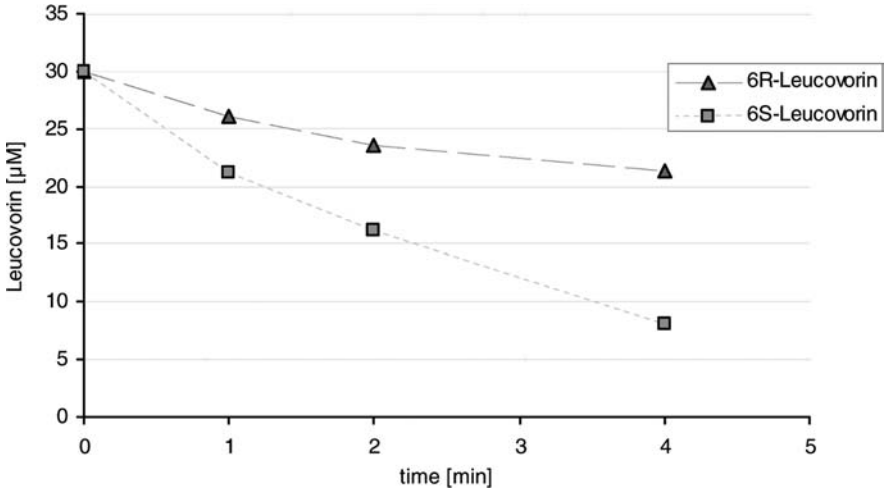

Fig. 6 Degradation of leucovorin diastereomers by CPG_2 after incubation of 60 µ*M* racemic leucovorin. The values shown are the means of three experiments

receiving standard leucovorin rescue after high-dose MTX. Qualitatively, similar results were observed in this concentration range. However, the quantification could not be done very precisely because the sensitivity of the method was not sufficient for concentrations in the range lower than 30 μ*M*.

Discussion

The separation of the diastereomers 6*S*-leucovorin and 6*R*-leucovorin using 2,6 dimethyl-*β*-cyclodextrin has been described by Cellai et al. [5] and, more recently, by Suss et al. [21]. In the work presented here, we modified the conditions to also separate its degradation products.

Although we could not confirm the structure of the degradation product *R*,*S*-FTHPA (Fig. 4) by nuclear magnetic resonance spectroscopy or mass spectroscopy, we are confident that the peaks correspond to FTHPA for the following reasons. First, with decreasing 6*S*-leucovorin concentrations the second peak of the first pair of peaks at 6 min increased. Second, the diode array spectra of the peaks from 200 to 400 nm were very similar to the spectra of leucovorin and aminopterin, indicating that the substance contains the same chromophore, the pteridine ring (data not shown). Third, the peaks migrated after the other folic acid derivatives aminopterin (internal standard) and the leucovorin diastereomers. At pH 7, all the analytes are negatively charged due to their carboxylic acid function and experience a force driving them against the electroosmotic flow towards the anode at the injection site of the capillary. However, while aminopterin and leucovorin contain two carboxylic acid functions, FTHPA contains only one. Thus, the migration of the peaks between the electroosmotic flow and the other analytes is in agreement with the structural formula of the proposed product.

From the incubation experiments, one can draw the conclusion that the K_m value of 120 μ*M* reported in the literature for the degradation of leucovorin by CPG_2 (vs 8 μ*M* for MTX) is misleading. The factor of 20 between the K_m values could lead to the conclusion that the amount of 6*S*-leucovorin degraded in vivo is negligible.

In our experiments, the affinity of the enzyme for the active 6*S*-isomer was higher than for the inactive 6*R*-isomer. Due to the limited amount of drugs and enzyme available, we could not determine the K_m exactly. However, with a reaction velocity of higher than 30 μ*M*/min one can conclude that 6*S*-leucovorin is inactivated to a certain amount, even in the presence of MTX.

For the application of CPG_2 in the clinic to decrease elevated MTX concentrations, other points also have to be considered. Figure 7 shows plasma concentrations versus time in a 15-year-old female osteosarcoma patient experiencing MTX toxicity after administration of 12 g/m^2 as a 4-h infusion. CPG_2 was administered 54 h after starting the MTX infusion. It is evident that CPG_2 rapidly decreases the elevated MTX concentrations in plasma. However, the plasma concentration measurements with the EMIT assay (diamonds in Fig. 7) overestimate the true MTX levels in comparison to the data provided by the selective capillary electrophoresis method [17]. The EMIT assay, as well as the fluorescence polarization immunoassay (FPIA) mostly used routinely in the clinic crossreacts with DAMPA, the product of the cleavage of MTX by CPG_2 [3]. Only chromatographic methods such as HPLC [15] or capillary electrophoresis [17] can distinguish between MTX, its metabolites and its degradation products.

Table 1 shows the solubility of several folate derivates at different pH values. The risk of developing renal failure is due to MTX and metabolites precipitating in the kidneys, especially when urine alkalization is not done thoroughly. DAMPA is much less soluble than MTX due to the lack of the glutamate moiety. In

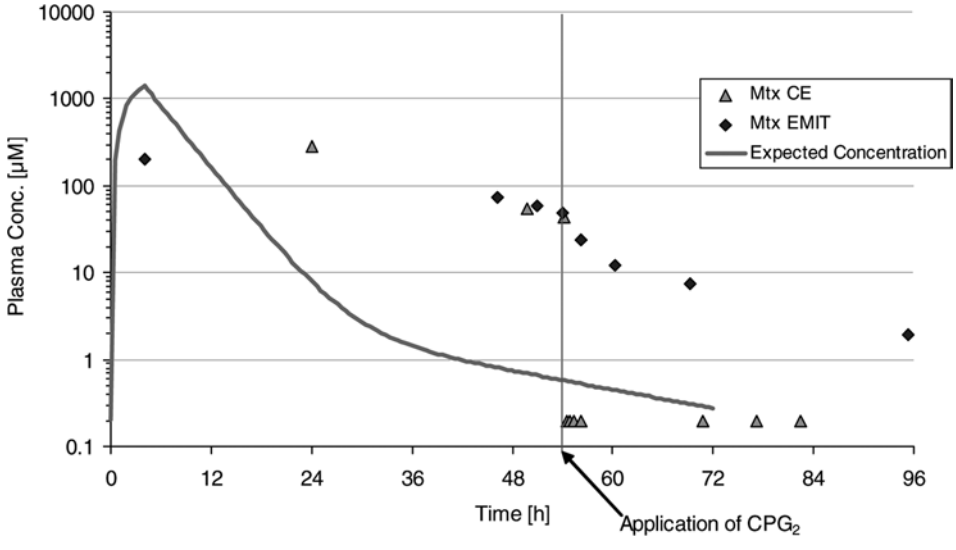

Fig. 7 Plasma concentrations in a patient experiencing MTX toxicity in comparison to the expected concentrations (*solid line*) calculated based on data from reference 10. Plasma concentrations were measured with both the immunoassay (MTX EMIT) and capillary electrophoresis (MTX CE)

Table 1 Solubility of MTX and its metabolites in aqueous solutions at different pH values. Data taken from references 8, 11 and 19

	pH 5 (μ*M*)	pH 6 (μ*M*)	pH 7 (μ*M*)
MTX	859	3414	19,912
7-OH-MTX	276	786	3,291
DAMPA	153	307	2,607

this respect, it is important to consider that the volume of distribution of DAMPA is smaller (0.13 l/kg in rhesus monkeys [22]) than that of MTX because DAMPA is not actively transported into cells and does not accumulate inside cells as a polyglutamate. Thus, when administering CPG_2 the risk of formation of precipitates in the kidneys is increased.

Furthermore, one has to keep in mind that the solubilities of the degradation products of 6*S*-leucovorin and 6*R*-leucovorin, *R*-FTHPA and *S*-FTHPA, are also decreased due to the lack of the glutamate moiety. Unfortunately, to our knowledge solubility data for these substances are not available. However, it is known that the inactive 6*R*-leucovorin shows a much longer plasma half-life than the active isomer, because it cannot be metabolized to the respective 5-methyl-derivative and is not transported inside the cell [20]. Therefore, substantial amounts of 6*R*-leucovorin are circulating and their degradation product 6*R*-FTHPA could be problematic for the kidneys. Possibly, the same has to be considered for the metabolite 7-OH-MTX and its degradation to less-soluble products.

Due to the complex situation after administration of CPG_2, drug monitoring of MTX and the degradation products should be applied. However, as explained above chromatographic or electrophoretic techniques should be used, and these are only available in a few hospitals.

For these reasons and from our experiments we would recommend that CPG_2 should only be used in cases of complete failure of MTX clearance. Several studies have shown that MTX toxicity can be treated with high-dose leucovorin and continuous thymidine infusions with the aim of restoring the intracellular thymidylate pool [4, 22]. If CPG_2 is administered, treatment should be started as early as possible and repeated administrations should be avoided due to the risk of allergic reactions. With CPG_2, it is especially important to keep the pH of the urine high and to administer leucovorin, which should preferably be given as the pure 6*S*-leucovorin. The sodium salt is preferable to the commonly used calcium folinate to reduce the amount of calcium administered.

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