Cancer Chemother Pharmacol (1997) 40: 367–370

SHORT COMMUNICATION

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Methotrexate increases red blood cell concentrations of 6-methylmercaptopurine ribonucleotide in rats in vivo

Received: 20 October 1996 / Accepted: 15 February 1997

Abstract *Purpose*: To elucidate the effect of methotrexate (MTX) on 6-mercaptopurine (6-MP) metabolism in rats. *Methods*: Fourteen rats were given 6-MP 20 mg/kg daily for 7 days. Seven of the rats were also given MTX 20 mg/kg on days 5 and 7. Blood samples were obtained from all rats on days 0, 5 and 8, and red blood cell (RBC) lysates were analysed for thiopurine methyltransferase (TPMT) activity and the concentration of methylated 6-MP metabolites [methyl mercaptopurine ribonucleotides (MMPRP)] and 6-thioguanine nucleotides (6-TGN). *Results*: The concentration of MMPRP increased 2.4 times from day 5 to day 8 in RBCs from rats given MTX in addition to 6-MP, as against 1.2 times in rats given 6-MP alone ($P = 0.003$). 6-TGN levels increased and TPMT activity decreased from day 5 to day 8, with no difference between the 6-MP and the 6-MP plus MTX groups. *Conclusions*: Single bolus doses of MTX increase the concentration of MMPRP in rats given daily s.c. doses of 6-MP, with no effect on 6-TGN concentration or TPMT activity.

Key words Methylmercaptopurine · 6-Thioguanine nucleotide · Interaction · Metabolism

Introduction

The hypoxanthine-guanine-phosphoribosyltransferase (HGPRT)-catalysed formation of active 6-thioguanine nucleotides (6-TGN) and subsequent incorporation of these metabolites into DNA and RNA is the main mechanism of the cytotoxic action of 6-mercaptopurine (6-MP). In children with acute lymphoblastic leukaemia (ALL), red blood cell (RBC) concentrations of 6-TGN are negatively correlated to the activity of thiopurine methyltransferase (TPMT) [10]. TPMT catalyses *S*-methylation of thioinositole monophosphate (TIMP), the key intermediate in 6-MP metabolism, resulting in the formation of methyl mercaptopurine ribonucleotides (MMPRP). TPMT activity is genetically regulated [18], and the activity in the RBC reflects the activity in lymphoblasts, liver and kidneys [13, 16, 21]. Most studies on TPMT activity and 6-MP metabolites are performed with the easily accessible RBC.

MMPRP shows cytotoxic activity [20] and is a potent inhibitor of phosphoribosyl pyrophosphate (PRPP) amidotransferase, the first enzyme in purine de novo synthesis (PDNS), leading to accumulation of PRPP [2, 14]. In vitro data suggest that inhibition of PDNS by MMPRP is a crucial event in the cytotoxicity of 6-MP [5].

In Molt 4 cells, MTX inhibits two folate-dependent enzymes involved in PDNS: glycinamide ribonucleotide (GAR) formyltransferase and aminoimidazolecarboxamide ribonucleotide (AICAR) formyltransferase [4]. In mouse L1210 leukaemia cells grown in culture, PRPP amidotransferase is thought to be the primary site of action of MTX [15]. Dihydrofolate-Glu_5, which accumulates in MTX-treated cells, also inhibits PRPP amidotransferase in cultured mouse L1210 cells [15]. Inhibition of PDNS results in increased levels of PRPP, the donor of the ribose phosphate moiety of nucleotides.

The core of maintenance chemotherapy in children's ALL is daily 6-MP combined with weekly MTX. This treatment regimen is based on clinical observations showing a synergistic effect when the two drugs are combined. The biochemical basis for the synergism between 6-MP and MTX is thought to be the inhibition by both drugs of PDNS, resulting in increased PRPP levels and enhanced incorporation of 6-MP derivatives into DNA and RNA.

In order to avoid the disturbing factors of multi-drug treatment and small and/or heterogeneous populations that complicate the study of thiopurine metabolism in

The study was supported by the Erna and Olav Aakre Foundation for Cancer Research

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patients, we have studied the effect of MTX on 6-MP metabolism in rats in vivo.

Materials and methods

Chemicals

Methotrexate infusion concentrate 100 mg/ml was from Lederle, American Cyanamid Company Lederle Laboratories Division, Wayne, N.Y. *S*-Adenosyl-L-(methyl-^{14}C)-methionine (^{14}C-SAM) was from Amersham International, Aylesbury, Bucks., UK. Insta-Gel II was from Packard Instrument Company, Meriden, Conn. All other reagents were from Sigma Chemical Company, St. Louis, Mo.

Animals

Fourteen male Wistar rats weighing 250–300 g at study start were used. All rats were given 6-MP 20 mg/kg daily for 7 days. Seven of the rats were given MTX 20 mg/kg on days 5 and 7 in addition. Drugs were given s.c. in a volume of 1 ml. The 6-MP solution was made by dissolving 6-MP in water and adjusting the pH to 9 with NaOH. The 6-MP/MTX solution was made by diluting MTX infusion concentrate in an aqueous solution of 6-MP to a final concentration of 20 mg/ml of each drug. Drug solutions were filtered through Millex-GS non-pyrogenic sterile 0.22 μm filters before use. The study was approved by the local Committee for Animal Research at the University of Tromsø.

Blood samples and preparation

Blood (1 ml) was sampled from the tail vein before study start (day 0) and on days 5 and 8. Owing to problems with sampling in some of the animals, blood was also sampled from the jugular vein on day 8. There was no difference in the concentrations of metabolites or enzyme activity measured in RBC lysates from blood obtained by the two techniques. RBC lysates were prepared as previously described by Weinshilboum et al. [20]. After a cell fraction had been obtained, 200-μl aliquots of the RBC suspension was removed for 6-TGN analysis. RBC suspension and lysate were stored at −80 °C until analysis.

Analytical techniques

6-TGN was measured after the method of Lennard [9]. Briefly, 200 μl RBC-suspension was added to dithiotreitol (DTT) and H_2SO_4 and heated at 100 °C for 1 h. After cooling, NaOH and phenyl mercuric acetate (PMA) in toluene was added and the PMA-purine adduct was extracted. The parent purine, thioguanine, was back-extracted with HCl and analyzed by reversed-phase HPLC.

MMPRP was analyzed after enzymatic hydrolysis of the nucleotides with calf intestine alkaline phosphatase (CIAP) followed by reversed-phase HPLC [6]. With this method, the sum of mono-, di- and tri-phosphorylated nucleotides is measured.

TPMT activity was measured radiochemically as described by Weinshilboum et al. [19] as modified to assay rat RBC TPMT activity [17]. The method is based on formation of (^{14}C-methyl)-mercaptopurine from 6-MP and ^{14}C-SAM. Since measured TPMT activity is dependent on the sample RBC fraction [8], all samples were diluted with 0.9% NaCl to the lowest RBC fraction observed in the 42 samples; 0.195.

Statistics

Mann-Whitney U-test (Statview II, Abacus Concepts, 1984 Bonita Avenue, Berkeley, CA 94704, USA) was used to compare the difference in metabolite concentration from day 5 to day 8 in the two treatment groups.

Results

Samples from 14 rats were analyzed on days 0 and 5. On day 8, the sample from one of the 7 rats given 6-MP alone was accidentally lost during lysate preparation. Thus, the metabolite concentrations and enzyme activity on day 8 in RBCs from rats given 6-MP alone are based on six observations. For all other values $n = 7$.

The RBC concentration of MMPRP increased in all rats from day 5 to day 8. The mean concentration of MMPRP (pmol/100 μl pRBC) in rats given 6-MP alone was 868 ± 508 (SD) on day 5 and 947 ± 314 (SD) on day 8. In rats given 6-MP and MTX the mean concentration of MMPRP was 1067 ± 705 (SD) on day 5 and 2082 ± 476 (SD) on day 8. The increase in MMPRP was significantly greater ($P = 0.003$) in rats given 6-MP and MTX than in rats given 6-MP alone (2.4 vs 1.2 times increase, respectively; Fig. 1).

No free MMPR or MMP was detected in the RBC lysates, as evident from HPLC analysis before hydrolysis. The limits of detection for these two substances in spiked RBC lysate were about 50 nM. In RBC lysates prepared from blood samples, the concentration of metabolites will depend on the cell fraction of the sample.

The RBC concentration of 6-TGN increased in 12 of the 14 rats from day 5 to day 8. In 2 rats in the 6-MP plus MTX group, 6-TGN decreased from day 5 to day 8. The mean concentration of 6-TGN (pmol/100 μl pRBC) in rats given 6-MP alone was 216 ± 42 (SD) on day 5 and 241 ± 34 (SD) on day 8. In rats given 6-MP and MTX the mean concentration of 6-TGN was 168 ± 24 (SD) on day 5 and 183 ± 33 (SD) on day 8. There was no difference between the treatment groups (Fig. 2).

RBC TPMT activity tended to decrease throughout the study. In rats given 6-MP alone the RBC TPMT activity (nmol MMP/h/ml pRBC) was 31.9 ± 4.8 (SD), 26.7 ± 3.9 (SD) and 24.8 ± 3.2 (SD) on days 0 (before study start), 5 and 8, respectively. In rats given 6-MP and MTX the RBC TPMT activity was 30.8 ± 2.7 (SD), 27.1 ± 1.2 and 26.6 ± 1.5 on days 0, 5 and 8,

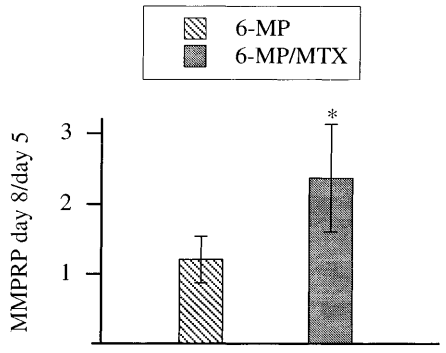

Fig. 1 Ratio of day 8 to day 5 red blood cell (RBC) methylmercaptopurine ribonucleotide (*MMPRP*) concentration in rats given 6-mercaptopurine (*6-MP*) alone or in combination with methotrexate (*MTX*). *$P = 0.003$. *Bars* mean values ± SD

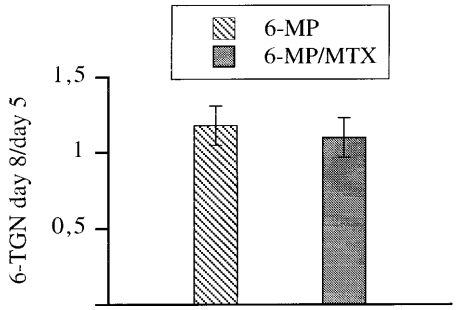

Fig. 2 Ratio of day 8 to day 5 RBC 6-thioguanine nucleotide (*6-TGN*) in rats given 6-mercaptopurine (*6-MP*) alone or in combination with methotrexate (*MTX*). *Bars* mean values ± SD

respectively. There was no difference between the treatment groups.

In the group of rats given 6-MP alone the mean weight gain from day 0 to 8 was 15 g, range 2–30 g. In the other group, given 6-MP plus MTX, 1 rat gained 4 g while the other 6 lost weight during the study. Mean weight loss was 9 g, range 3–18 g.

Discussion

6-MP and MTX are the cornerstones of maintenance treatment in children with ALL. The biochemical mechanism underlying the synergistic effect of these drugs is mainly explained on the basis of in vitro studies. We here present an in vivo study in rats, showing that the concentration of MMPRP in RBCs increases in the presence of MTX while the 6-TGN concentration is basically unaffected.

C_{max} and AUC of 6-MP in rats and humans increase in the presence of MTX [1, 7]. The increased bioavailability of 6-MP by MTX might be due to inhibition of first-pass metabolism; MTX has been shown to inhibit human xanthine oxidase (XO) in the liver and small intestine [11]. In vitro studies have shown that the presence of MTX enhances the incorporation of 6-MP into DNA and RNA because of increased levels of PRPP [3, 4]. The increased concentration of MMPRP in RBCs from rats given 6-MP and MTX observed in the present study might therefore be due to increased bioavailability of 6-MP, increased PRPP levels, or both.

In murine WEHI-3b leukemia cells treated with 6-MP, preincubation with MTX increased the intracellular concentration of TIMP, while the 6-TGN concentration was unaffected [12]. However, it should be noted that the 6-MP concentration in this study was kept constant by continuous addition of the drug. The authors did not measure the concentration of methylated 6-MP metabolites. Nevertheless, similar results were obtained in the present in vivo study: increased MMPRP (methyl-TIMP) and unaffected 6-TGN concentration. The lack of effect of MTX on 6-TGN concentration might be explained by the many metabolic steps between PRPP (the site of action of MTX) and 6-TGN. In contrast, TIMP and MMPRP is closer to PRPP in the metabolic map of thiopurines. However, the rate-limiting steps in the conversion of 6-MP to its different metabolites remain to be identified.

The tendency of TPMT activity to decrease during the study period was unexpected. In a study by H.L. Wold (unpublished data) blood was sampled from 10 Wistar rats weekly for 7 weeks. RBC TPMT activity was stable throughout this period (mean intraindividual CV 11.9%). In addition, in three pilot studies including 26 rats given saline, 6-MP 2–20 mg/kg, MTX 80 mg/kg or 6-MP 20 mg/kg plus MTX 20, 40 or 60 mg/kg, no change in TPMT activity was noted (unpublished data). Since there was no difference in RBC TPMT activity in the rats given 6-MP plus MTX compared with those given 6-MP alone in the present study, the observed decrease in RBC TPMT activity can not be explained by the MTX treatment.

Since MMPRP itself is cytotoxic, the results from the present in vivo study in rats indicate an increase in the total amount of cytotoxic 6-MP metabolites (MMPRP and 6-TGN) in RBCs in the presence of MTX. However, the importance of MMPRP levels and hence the significance of the present observations for the clinical outcome in patients on 6-MP therapy is not known.

Acknowledgements We thank Roy A. Lysaa, Department of Pharmacology, and the staff at the Department for Animal Research, University of Tromsø, for technical assistance.

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