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Effects on Transmethylation by High-Dose 6-Mercaptopurine and Methotrexate Infusions During Consolidation Treatment of Acute Lymphoblastic Leukemia

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ABSTRACT. 6-mercaptopurine (6MP) cytotoxicity is caused by thioguanine and methylthioinosine nucleotides. Thiopurine methylation occurs to a large extent *in vivo* and *in vitro*. In this reaction, S-adenosyl-L-methionine (AdoMet), produced from methionine and ATP, is converted into S-adenosyl-L-homocysteine (AdoHcy) which, in turn, is hydrolyzed into homocysteine. Remethylation of homocysteine into methionine is inhibited by methotrexate (MTX). In cultured lymphoblasts, AdoMet:AdoHcy ratio and DNA methylation decrease after incubation with 6MP. The aim of the present study was to investigate the influence of high-dose 6MP on the methylation capacity in children with acute lymphoblastic leukemia.

Five patients received 4 courses with high-dose intravenous MTX (5 $g \cdot m^{-2}$ in 24 hr) immediately followed by high-dose 6MP (1300 $mg \cdot m^{-2}$ in 24 hr). Five control patients received high-dose MTX and oral 6MP (25 $mg \cdot m^{-2}$ daily for 8 weeks). Leucovorin rescue was started at 36 hr in both groups.

In the intravenous 6MP group, 6-methylmercaptopurine, its riboside, and 6-methylmercapto-8-hydroxypurine were detectable in plasma in concentrations of 0.3–2.6 μM (6MP steady state levels: 11.6 μM). In red blood cells, mean methylthioinosine nucleotide levels were one third of those of ATP (13.1 nmol/10^8). AdoHcy levels (10 pmol/10^8) remained constant in both groups and AdoMet was not detectable (<20 pmol/10^8). In both groups, plasma homocysteine increased and methionine decreased following administration of MTX. The delay in the recovery of methionine in the intravenous 6MP group after MTX infusion is probably the result of an increased demand on methyl groups during 6MP infusion. BIOCHEM PHARMACOL 51;9:1165–1171, 1996.

KEY WORDS. 6-mercaptopurine; methotrexate; acute lymphoblastic leukemia; methylation

6MP§ is used in the treatment of ALL. It has no intrinsic cytotoxic activity, but is converted into active metabolites intracellularly (Scheme 1). 6MP is first converted with 5-phosphoribosyl-1-pyrophosphate as cosubstrate into tIMP which, itself, can be converted either into tGMP or MetIMP. Both pathways result in cytotoxicity *in vitro*, either by incorporation of thioguanine nucleotides into DNA and RNA [1, 2] or by inhibition of purine *de novo* synthesis by MetIMP [2, 3]. 6MP is methylated into MeMP and 6MP-riboside into MeMPR (Scheme 1). The thiopurine methylation reactions are catalyzed by thiopurine methyltransferase (EC 2.1.1.67) (TPMT). The TPMT activity is controlled by a genetic polymorphism and the activity in RBC correlates with that in lymphoblasts, lymphocytes, platelets, liver, and kidney [4–7]. Population studies showed that the frequency distribution is trimodal with 88.6% of the subjects displaying high activity, 11.1% intermediate activity, and 1 out of 300 subjects having undetectable TPMT activity [8]. Thiopurine methylation requires AdoMet as methyldonor [9]. AdoMet is the universal methyldonor for many methyltransferase reactions of small molecules, DNA, RNA, proteins, and phospholipids, and plays an important role in the regulation of the action of these compounds [10].

Scheme 2 shows the transmethylation pathway with its principal metabolites, AdoMet, AdoHcy, homocysteine, and methionine. By donation of the methylgroup, AdoMet is converted into AdoHcy, which is hydrolyzed into homocysteine and adenosine. Homocysteine can be degraded into cysteine in the transsulfuration pathway or remethylated into methionine. Two enzymes catalyze the remethylation of homocysteine. Betaine-homocysteine methyl-

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§ *Abbreviations:* AdoHcy, S-adenosyl-L-homocysteine; AdoMet, S-adenosyl-L-methionine; ALL, acute lymphoblastic leukemia; 6MP, 6-mercaptopurine; MeMP(R), 6-methylmercaptopurine (riboside); MeM8OHP, 6-methylmercapto-8-hydroxypurine; MetIMP (MetIDP, MetITP), methylthioinosine mono- (di-, tri-) phosphate; MTX, methotrexate; RBC, red blood cells; tGMP (tGDP, tGTP), thioguaninosine mono- (di-, tri-) phosphate; tIMP, thioinosine monophosphate.

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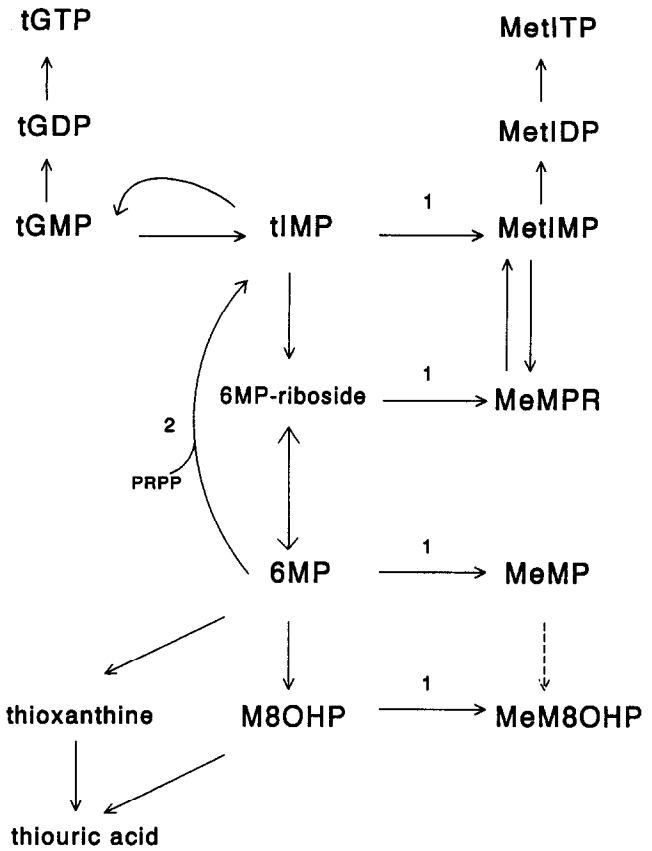


SCHEME 1. The metabolism of 6MP. 1, thiopurinemethyltransferase; 2, hypoxanthineguanine phosphoribosyltransferase (EC 2.4.2.8); M8OHP, 6-mercapto-8-hydroxypurine; PRPP, 5-phosphoribosyl-1-pyrophosphate.

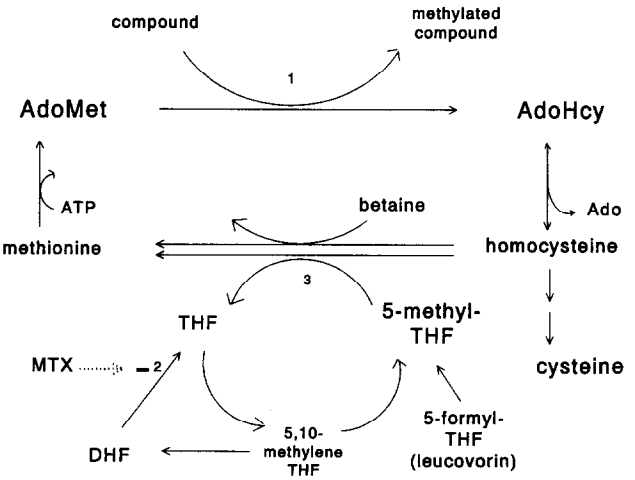
transferase (EC 2.1.1.5) uses betaine as methyldonor and is active only in the liver [11]. 5-Methyltetrahydrofolate homocysteine methyltransferase (EC 2.1.1.13) is active in many, if not all, tissues and uses 5-methyltetrahydrofolate as methyldonor [11]. Next to its role in protein synthesis, the essential aminoacid methionine can be activated as methyldonor by formation of AdoMet, using ATP as cosubstrate [11].

Recently, we reported the effect of 6MP on the principal transmethylation metabolites *it vitro* [12]. Incubation of Molt-F4 human malignant ALL lymphoblasts with 6MP resulted in a decrease in AdoMet and the AdoMet/AdoHcy ratio, in an increase in AdoHcy and methionine in these cells, and a decreased release of homocysteine into the culture medium [12]. The decreased AdoMet/AdoHcy ratio could, theoretically, be caused by a depletion of the AdoMet pool due to its consumption for thiopurine methylation. However, similar effects on AdoMet, AdoHcy, methionine, and homocysteine were demonstrated after incubation of these cells with MeMPR, which already contains a methylgroup. Thus, the effects of 6MP and MeMPR on these transmethylation metabolites were ascribed to the reduced conversion of methionine into AdoMet, due to ATPdepletion produced by MetIMP [12]. More recently, we observed that incubation of Molt-F4 lymphoblasts with 6MP or MeMPR resulted in a decrease in the incorporation of [^{14}C] from [^{14}C]-methionine into total DNA [13], suggesting that thiopurines can influence DNA methylation *in vitro*.

It is well known that quantitative large amounts of 6MP are methylated *in vivo*. In RBC of ALL patients treated daily with oral 6MP (75 mg · m^{-2}), the median methylthioinosine nucleotide levels were 4.36 nmol/8*10^8 RBC (range 0.25–22.5), compared to the much lower median thioguanine nucleotides levels of 335 pmol/8*10^8 RBC (range 144–1263) [14]. Methylthioinosine nucleotide levels were even higher in RBC [15] and mononuclear cells [16], compared to those of thioguanine, when high doses of 6MP were administered intravenously. Influences of 6MP on the transmethylation metabolites *in vivo* have not been investigated up to now.

In the present study, we investigated influences of high-dose 6MP infusions on methylation capacity *in vivo*. In the consolidation treatment of the Dutch Childhood Leukemia Study Group (DCLSG-ALL-8 protocol) ALL patients are randomized for high-dose intravenous 6MP or low-dose oral 6MP. We investigated the oral 6MP group as the control group for the intravenous 6MP group, because both groups also received methotrexate (MTX) infusions. This inhibits dihydrofolate reductase (EC 1.5.1.3), causing a depletion of active tetrahydrofolate compounds (Scheme 2). Thus, it indirectly inhibits folate-dependent reactions, such as remethylation of homocysteine into methionine, purine *de novo* synthesis, and thymidylate synthesis [17].

The amounts of 6MP methylated *in vivo* were investigated by measurement of the methylated metabolites of 6MP in RBC, plasma, and urine. The influence of the infusions on the transmethylation metabolites was investigated by measurement of methionine and homocysteine in plasma and AdoMet, AdoHcy, and purine nucleotides intracellularly. Lymphoblasts were not available for investi-



SCHEME 2. The transmethylation pathway. 1, thiopurinemethyltransferase and other AdoMet-dependent methyltransferases; 2, dihydrofolate reductase; 3, 5-methyl-tetrahydrofolate homocysteine methyltransferase; DHF/THF, di/tetra hydrofolate.

gation, because the patients were in complete remission. We used RBC, because TPMT activity in RBC correlates with that in lymphoblasts and other tissues [4–7]. RBC can synthesize 5-phosphoribosyl-1-pyrophosphate [18], an essential cofactor for the conversion of 6MP into tIMP, but do not manifest an active purine *de novo* synthesis, as ALL lymphoblasts do [19].

METHODS

Ten children with ALL were treated in our center according to the DCLSG-ALL-8 protocol with a consolidation therapy of high-dose MTX (5 g · m^{-2} in 24 hr, 4 courses in 8 weeks). Leucovorin rescue (Scheme 2) was started at 36 hr after the MTX infusion was begun and continued every 6 hr until MTX plasma levels were less than 0.25 μM. Five of the 10 patients were randomized for oral 6MP (25 mg · m^{-2} daily for 8 weeks, starting 7 days before the first MTX course) and the other 5 received 6MP intravenously (1300 mg · m^{-2} in 24 hr, from 24 until 48 hr after MTX infusion, 4 courses in 8 weeks). Patient or parental approval was obtained according to the ethical guidelines of our hospital.

Blood was sampled during the 4 courses of all patients in the oral and intravenous group, before and 24, 28, 48, 52 and 72 hr after the MTX infusion was begun. Plasma and RBC were isolated as described [20]. RBC transfusions, an essential part of the supportive treatment during the intensive consolidation treatment, were given between the courses, but never during a course. Dithiothreitol was added to plasma, RBC, and urine to prevent oxidation of the thiol groups [20]. Urine was collected during the 24 hr of the 6MP infusion as well as during the next 24 hr, either as a pooled 24-hr sample or as fractionated samples of approximately 6 hr. In children with nappies, we collected one urine sample at the end of the infusion. The methylated metabolites of 6MP and the purine nucleotides were extracted and measured with HPLC using ultraviolet absorbance. Detection limits for the methylated metabolites in plasma were 20–50 nM and for MetIMP 11 pmol per 100 μL injection [20]. A previously indeterminate metabolite of 6MP [20] was identified as MeM8OHP. It was measured as the other methylthiopurine metabolites and eluted at 30 min [20]. Total homocysteine was measured with HPLC after reduction with sodium borohydride and derivatization with monobromobimane [21]. Methionine was measured as described [12]. AdoMet and AdoHcy were extracted from 200 μL RBC (2–3.10^{12} RBC/L 0.9% (w/v) saline with 0.1% (w/v) dithiotreitol) and 200 μL water with 40 μL 55% trichloroacetic acid and kept on ice for 10 min. After centrifugation, 300 μL of the supernatants were washed 3 times with 2 volumes of peroxide-free diethylether to remove the trichloroacetic acid. The samples were flushed with nitrogen to a final volume of 150 μL. AdoMet and AdoHcy were measured with HPLC [12] at 248, 254, and 260 nm.

RESULTS

Methylated Metabolites of 6MPin Plasma, RBC, and Urine

Figure 1 shows the mean levels of the methylated metabolites of 6MP in plasma of the intravenous 6MP group. Steady state levels of 6MP and MeMP were 11.6 (sem 1.6) and 0.6 (sem 0.1) μM, respectively. MeM8OHP reached levels of 2.6 (sem 0.5) μM during the 6MP infusion. Upon its termination, MeMP and MeM8OHP were rapidly cleared from plasma. MeMPR increased slowly during the 6MP infusion and reached levels of 0.3 (sem 0.05) μM 24 hr after its termination. Methylthioxanthine and methylthiouric acid were not detectable in plasma of these patients.

Figure 2 shows the levels of MetIMP, MetIDP, and MetITP in RBC of patients in the intravenous 6MP group. These levels increased during and after the infusion. At 72 hr, the mean levels of MetIMP were 3.1 (sem 0.6), of MetIDP 0.8 (sem 0.3), and of MetITP 1.4 (sem 0.4) nmol/10^8 RBC. At the start of the next course, the mean

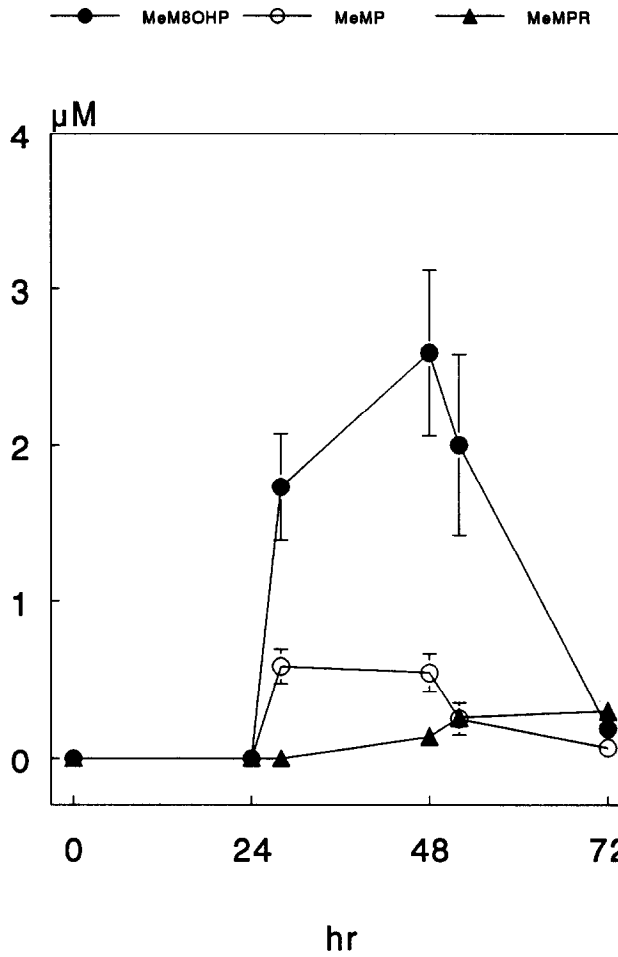


FIG. 1. Methylated metabolites of 6MP in plasma of the intravenous 6MP group Patients were treated with an MTX infusion from 0–24 hr and a 6MP infusion from 24–48 hr. The means of all courses and all patients are indicated by the markers, the error bars indicate the SEM.

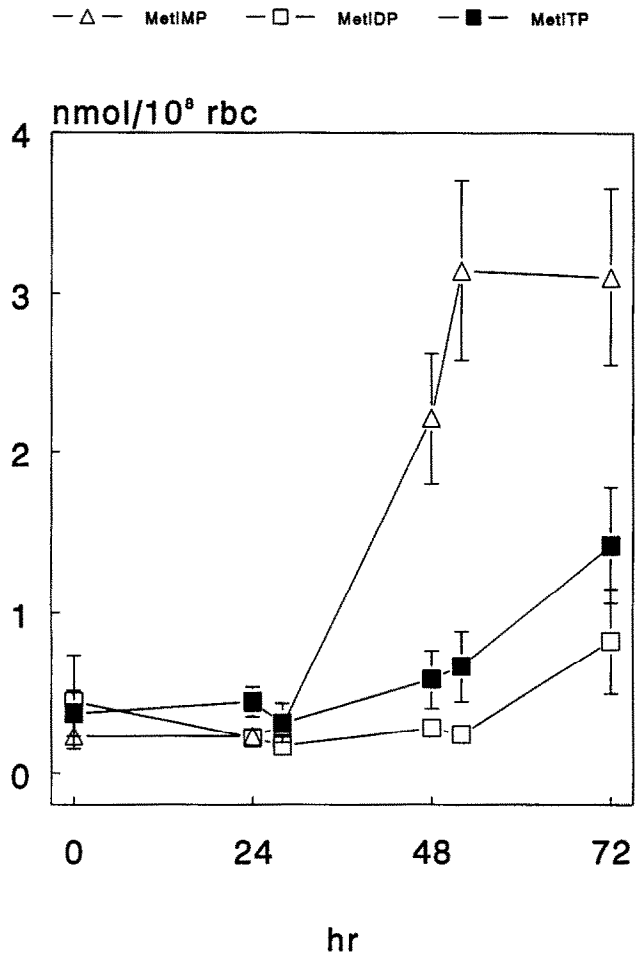


FIG. 2. Methylated metabolites of 6MP in RBC of the intravenous 6MP group. Patients were treated with an MTX infusion from 0–24 hr and a 6MP infusion from 24–48 hr. The means of all courses and all patients are indicated by the markers, the error bars indicate the SEM.

MetIMP levels were decreased to less than one tenth of the level at 72 hr of the previous course, whereas those of MetIDP were one half and of MetITP one third. Four children each received one RBC transfusion between the courses. After this, the methylthioinosine nucleotide levels at the start of the next course were in the same range or higher, compared to those of the previous course. Interindividual variation coefficients of 6MP and the methylated metabolites in plasma and RBC were between 30 and 50%.

In urine 6MP, thioxanthine, and thiouric acid were the main metabolites. MeMP, MeM8OHP, methylthioxanthine, and methylthiouric acid were not detectable in urine. MeMPR accounted for less than 2% of all excreted metabolites of 6MP in urine during the 6MP infusion.

Urine of the oral group was not collected. In plasma and RBC samples of the oral 6MP group at 0, 24, 28, 48, 52, and 72 hr, none of the metabolites of 6MP was detectable. Oral 6MP (25 mg · m^{-2}) was given at 7 a.m., whereas blood was sampled at 1 or 5 p.m..

Purine Nucleotides in RBC

Adenine and guanine nucleotide levels in RBC of the oral and intravenous 6MP group were in the normal range [22] and remained constant during the MTX and 6MP infusions of all courses. ATP levels were 13.1 (sem 0.9) nmol/10^8 RBC.

AdoMet and AdoHcy in RBC

AdoMet levels were analyzed in both groups, but were not detectable in any of the samples. The detection limit of AdoMet in RBC is 20 pmol/10^8 RBC. AdoHcy levels remained constant in both groups. Mean AdoHcy levels were 10.8 (sem 1.3) pmol/10^8 RBC in the intravenous and 9.1 (sem 1.0) pmol/10^8 RBC in the oral group.

Methionine and Homocysteine in Plasma (Fig. 3)

In both groups, homocysteine levels increased by 130–250% upon administration of MTX and returned to initial values after the start of leucovorin administration. Until 48 hr, there were no significant differences in homocysteine levels between the groups but, at 52 and 72 hr, they were significantly lower in the intravenous group (*t*-test, two-tailed, $P = 0.03$ and 0.002, respectively, df 34, all courses). Methionine levels decreased by 35–50% from 0 to 24 hr during all courses in the oral group and during the third and fourth course in the intravenous group. This decrease in methionine was not present during the first or second course in the intravenous group. After termination of the MTX infusion, methionine levels immediately returned to initial levels in the oral group. In the intravenous group, methionine levels began to increase 4 hr after termination of the 6MP infusion and reached levels 137% above the initial levels at 72 hr. At 48 and 52 hr, methionine levels were significantly higher in the oral group (*t*-test, two-tailed, $P = 0.05$ and 0.05, respectively, df 33, all courses). At 0, 24, 28, and 72 hr, there were no significant differences. Methionine in unwashed RBC of 10 random samples of the oral and intravenous 6MP group was in the range of 60–200 pmol/10^8 RBC.

DISCUSSION

The importance of a balanced transmethylation pathway and its role in carcinogenesis and malignancy has been stressed by several studies and reviewed by Laird and Jaenisch [23]. The methylation pattern of DNA of human malignant cells is often characterized by widespread hypomethylation, regional hypermethylation, and elevated expression levels of DNA methyltransferase activity. In animals, the administration of AdoMet was able to prevent or even reverse the development of tumors, whereas methyl-deficient diets were able to induce tumors, which is consistent with the widespread hypomethylation in tumor DNA [23]. Recently, it was shown that DNA methyltransferase

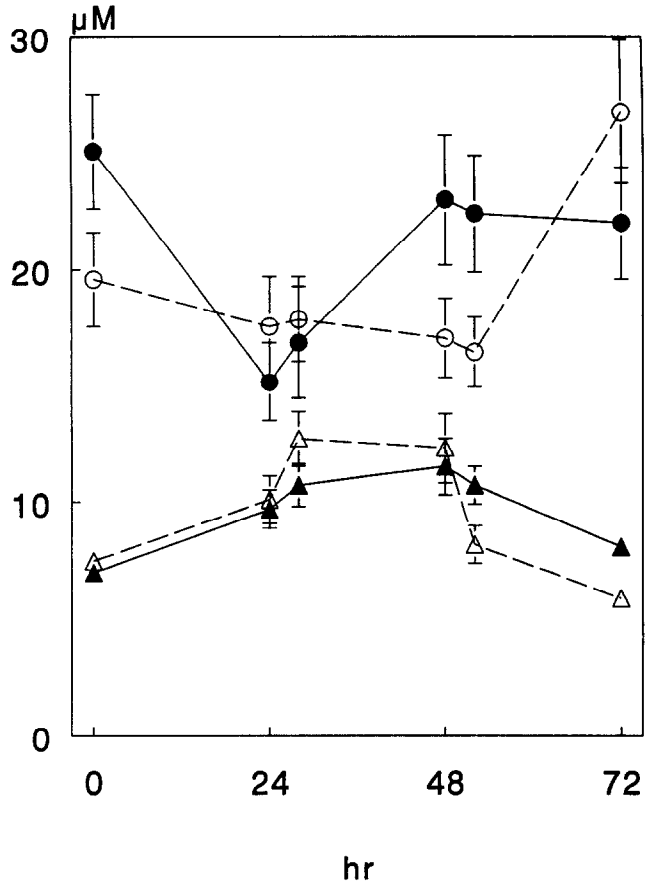


FIG. 3. Homocysteine (triangles) and methionine (circles) in plasma of the oral (solid lines) and intravenous (dotted lines) 6MP group. Patients in both groups were treated with a MTX infusion from 0–24 hr. Patients in the oral group received 6MP daily p.o., whereas patients in the intravenous 6MP group received 6MP as an infusion from 24–48 hr. The means of all courses and all patients are indicated by the markers, the error bars indicate the SEM.

activity contributes substantially to the development of intestinal adenomas [24]. However, it remains unclear whether or not changes in the methylation status (i.e. methionine metabolites, DNA methyltransferase activity, or DNA methylation) play a causal role in carcinogenesis or are a property of the malignant cell. Even without this knowledge, the methylation status might be a target for chemotherapeutic agents. In line with this view, we recently demonstrated that incubation of Molt F4 lymphoblasts with 6MP resulted in changes in the concentrations of transmethylation metabolites [12] and in DNA methylation [13].

The present study is the first to investigate the influence of high-dose 6MP on transmethylation metabolites *in vivo*. Large amounts of methylated thiopurines were detectable in plasma and RBC after high-dose intravenous 6MP, with interpatient variabilities of 30–50% (Fig. 1, 2). TPMT activities in peripheral mononuclear cells ranged from 4.6–16.1 IU/10^9 cells in the present group, demonstrating intermediate and high activity [25]. The genetic polymorphism of TPMT may be partially responsible for the interpatient variability. We could not demonstrate a correlation between TPMT activity and (methyl)thiopurines, because a high percentage of TPMT assays in mononuclear cells fail due to the lack of cells in leukopenic patients as well as other enzymes involved in thiopurine metabolism (Scheme 1) [25]. Measurement of TPMT in RBC would not be informative because RBC are transfused during the induction and consolidation treatment. The presence of methylated metabolites of 6MP in urine was less pronounced, compared to that in plasma and RBC. The absence of MeMP and MeM8OHP in urine might be the result of oxidation [26] or glucuronidation [27] of these compounds or of desulfuration of the drug [27]. Methylthioxanthine and methylthiouric acid were not detectable in plasma or urine, which is in concordance with the results obtained from patients treated with 6MP and MeMP [28] and with *in vitro* data demonstrating that thioxanthine and thiouric acid are inhibitors of TPMT [29].

In the oral group, 6MP and its metabolites were not detectable in plasma nor RBC. Blood sampling was timed after the start of MTX, as in the intravenous group, and not after 6MP intake. Detection of mono-, di- and triphosphate nucleotides in RBC requires higher intracellular concentrations of methylthioinosine nucleotides compared to measurement of MeMP after hydrolysis of the nucleotides. The administered dose (25 $mg \cdot m^{-2}$ daily for 8 weeks) was one third of the dose administered daily during 1.5–2-year maintenance therapy of ALL [14], too low for detection of methylthioinosine nucleotides in the oral group.

The high levels of methylthioinosine nucleotides in RBC in the intravenous group indicate that the transmethylation rate and, thus, the rate of AdoMet synthesis must have been high in RBC. AdoMet levels were below the detection limit in all RBC samples, but the increasing levels of methylthioinosine nucleotides in RBC after termination of the infusion indicate that sufficient AdoMet was available for this methylation reaction. In RBC of healthy controls, mean AdoMet levels were 3.5–5.2 μmol/L packed fresh RBC (30–32) ($\pm 10*10^{12}$ RBC/1 packed cells). Roughly, these levels are just above our detection limit (20 pmol/10^8 RBC). AdoHcy levels were approximately 10 pmol/10^8 RBC and remained constant during the MTX and 6MP infusions. In one study, mean AdoHcy levels were one third of those of AdoMet in fresh RBC of healthy subjects [31] whereas, in our study, AdoMet levels were lower than AdoHcy levels. We used stored RBC from patients treated for leukemia, which may have influenced the difference. We are not aware of a study having been conducted on AdoMet and AdoHcy levels in RBC under similar conditions.

Changes in homocysteine and methionine levels in plasma were observed in both groups. The increase in homocysteine and the decrease in methionine in plasma after the start of MTX administration could be ascribed to the decreased remethylation of homocysteine, itself the result

of the decreased availability of tetrahydrofolate derivatives due to inhibition of dihydrofolate reductase by MTX (Scheme 2). These changes have also been demonstrated by other [33–35]. A decrease in methionine levels was not detectable during the first two courses in the intravenous group, probably because we did not measure between 0 and 24 hr when the nadir was reached [35].

After termination of the MTX infusion, methionine and homocysteine levels were different for the two groups. Upon leucovorin administration, homocysteine levels decreased in both groups, but decreased to significantly lower levels at 52 and 72 hr in the intravenous 6MP group. Methionine levels increased immediately to initial levels after termination of the MTX infusion in the oral group, but remained low in the intravenous group during the 6MP infusion, after which they rebounded above initial levels. Because leucovorin, which increases methylation of homocysteine into methionine, was administered 12 hr after the start of the 6MP infusion, the pure effect of high-dose 6MP on the transmethylation metabolites may be masked. The delay in the recovery of methionine after termination of the MTX infusion in the intravenous group may be the result of the high consumption of methionine for methylation of thiopurines.

Thus, our study in children with ALL in remission shows an effect of high-dose 6MP in plasma (i.e. a delay in the recovery of the decrease in methionine due to MTX). This study does not show evidence for an effect of high-dose 6MP in RBC. This does not exclude the possibility that high-dose 6MP influences the transmethylation metabolites in the lymphoblasts. However, as all patients were in complete remission during the consolidation treatment, lymphoblasts are not available. It would be worthwhile to investigate the effects of high-dose 6MP alone on the transmethylation pathway in the lymphoblasts immediately after diagnosis, which will probably be done in a window phase of the next DCLSG study (ALL-9).

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