Cancer Chemother Pharmacol (1998) 42: 266–272

ORIGINAL ARTICLE

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Pharmacokinetics and metabolism of thiopurines in children with acute lymphoblastic leukemia receiving 6-thioguanine versus 6-mercaptopurine

Received: 23 June 1997 / Accepted: 4 February 1998

Abstract Mercaptopurine (6MP) has been the standard drug for maintenance therapy of acute lymphoblastic leukemia. In a multicenter study we investigated whether thioguanine (6TG), which is converted more directly to the cytotoxic thioguanine nucleotides (TGN), offers a therapeutic advantage over 6MP. In this study (CO-ALL-92), 6TG was randomized versus 6MP in maintenance therapy, whereby the doses of both drugs had to be adjusted to a white blood cell (WBC) count of between 2 and 3/nl. In 19 children the plasma levels of both drugs and/or the accumulation of their metabolites in red blood cells (RBC) were measured during intensive treatment in two consecutive chemotherapy blocks, and in 54 children the metabolites in RBC were measured every 3 months during daily treatment in maintenance therapy. There was a marked interindividual difference in the plasma kinetics of the two drugs; after identical doses of 100 mg/m^2 an about 4-fold higher peak concentration of the parent drug was reached with 6MP. The main metabolites of 6TG were thioguanine nucleotides (TGN), whereas during 6MP treatment, methylated thioinosine nucleotides (TIN) predominated in erythrocytes. In patients receiving 6TG during maintenance therapy (22 patients) the concentration of methylated TGN reached about 40% of that of unmethylated TGN; after 6MP administration (32 patients) the methylated TIN were concentrated about 26-fold higher in RBC than were TGN. In contrast to 6TG, for 6MP the pattern of metabolites shifted toward the methylated ones with increasing dose. The median TGN concentration was about 7-fold higher in the TG branch, although the median dose was only about 70% of that of 6MP. The WBC values were equivalent in the two treatment groups. Our results suggest that the cytotoxic effect of 6MP is not based solely on the formation of TGN.

Key words Acute lymphoblastic leukemia · 6-Thioguanine · 6-Mercaptopurine · Pharmacokinetics · Metabolism

This investigation was supported by the Fördergemeinschaft Kinder-Krebs-Zentrum Hamburg eV

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Introduction

The antimetabolites 6-thioguanine (6TG) and 6-mercaptopurine (6MP), analogues of guanine and hypoxanthine, have been used in the treatment of acute leukemia for more than four decades. Only for historical, not for scientific, reasons has 6MP become the drug of choice for acute lymphoblastic leukemia, whereas 6TG is used primarily in acute myelogenous leukemia [12].

6TG and 6MP are prodrugs and have to be converted into their active metabolites. As the first step the corresponding ribonucleotides thioguanosine and thioinosine monophosphate (TGMP and TIMP) are formed. TIMP is converted via two additional enzymatic steps into TGMP, which is finally incorporated into DNA and RNA in its (deoxy-)triphosphate form. In addition, TIMP as well as other metabolites and the parent drugs 6MP and 6TG serve as substrates for the enzyme thiopurine methyltransferase (TPMT). Catabolism of both drugs to the cytostatically inactive thiouric acid (TUA) is catalyzed by xanthine oxidase; first, 6TG has to be deaminated by the enzyme guanase.

It is commonly assumed that cell damage caused mainly by the incorporation of the thioguanine nucleotides (TGN) into DNA is of decisive importance for the cytotoxic effect of 6MP as well as 6TG [26, 37, 39, 44]. On the other hand, methylthioinosine monophosphate (MeTIMP) [5, 8, 11, 17] and, to a lesser extent, methylthioguanosine monophosphate (MeTGMP) [3] are inhibitors of purine de novo synthesis (PDNS) in vitro.

Controversial, however, is whether this also applies to the situation in vivo.

In our cooperative study for treatment of acute lymphoblastic leukemia in childhood, COALL-92, we investigated whether 6TG, which is converted more directly to the cytotoxic TGN, would offer a therapeutic advantage over 6MP. Patients were randomized to receive either 6MP or 6TG in maintenance treatment in this study. The metabolites of both drugs were measured in red blood cells (RBC) during daily application of the drugs. In addition, in some patients, plasma pharmacokinetics and RBC metabolites were measured during intensive treatment with short-term courses of the drugs at higher doses. Preliminary results have been reported elsewhere [1, 19, 20].

Patients and methods

Treatment protocol

Study COALL-92 is a multicenter treatment study for children with acute lymphoblastic leukemia whose age ranges from 1 to 18 years. Prior to maintenance therapy the patients are randomized to receive either daily 6MP p.o. and weekly methotrexate (MTX) p.o. or daily 6TG p.o. and weekly MTX p.o. The drug doses should be adjusted to maintain a WBC count of between 2 and 3/nl, the starting dose for 6MP and 6TG being 50 mg (329 and 299 μmol)/m^2. (the conversion factor from milligrams to micromoles is 6.6 for 6MP and 6.0 for 6TG). The starting dose of MTX was 20 mg/m^2, giving a desired ratio of 2.5 between 6MP/6TG and MTX; the minimal dose was 10 mg/m^2. In our study, 7 day courses of either 6MP or 6TG at 100 mg/m^2 are part of the intensive phase of the protocol in which various drug combinations are given (Fig. 1).

Pharmacology studies involved 54 children in maintenance therapy and 19 in intensive therapy, all of whom were treated at the Children's University Hospital in Hamburg. Measurements during maintenance treatment were performed at least 3 weeks after the beginning of therapy and every 3 months thereafter, provided that the patient had an unchanged daily drug dose for at least 1 week before sampling and that the dose given daily in the preceding week differed only slightly, if at all. In all, 32 patients from the 6MP arm and 22 from the 6TG branch fulfilled this condition; children in the 6TG group often had treatment interruptions due to thrombocytopenia. The thiopurines were taken in the evening and blood sampling was done the next morning. The doses of 6MP and 6TG during the intensive phase were fixed at 100 mg/m^2 for 7 days in combination therapy with other cytostatic drugs (Fig. 1) and were given 2–3 h after breakfast. According to the protocol, in low-risk patients the first treatment block contained 6MP and the second, 6TG, but in five children the sequence was changed. Plasma concentrations of the parent drugs were measured every full hour during the first 5 h, and measurements of metabolites in RBC were done hourly between h 1 and 5 and, additionally, at 24 h after the first drug intake in two consecutive blocks.

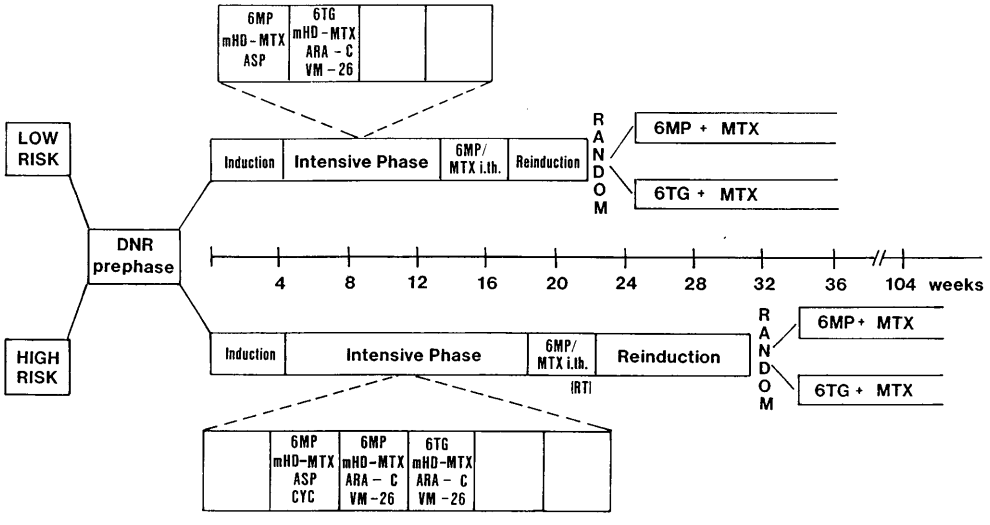

Fig. 1 Simplified therapeutic scheme of study COALL-92 for treatment of children with acute lymphoblastic leukemia. During the intensive phase the drugs are given as follows. *Low risk* – 6-mercaptopurine (*6MP*): days 29–35; 6-thioguanine (*6TG*): days 43–49 (the first dose of both drugs is given 5 h before mHD-MTX); medium-high-dose methotrexate (*mHD-MTX*): days 29, 43, 57, 78; cytosine arabinoside (*ARA-C*) and teniposide (*VM-26*): day 45; asparaginase (*ASP*): days 31, 33, 59, 61, 80, 82. *High risk* – 6MP: days 43–49 and 57–63; 6TG: days 71–77 (the first dose of both drugs is given 5 h before mHD-MTX); mHD-MTX: days 29, 30, 43, 44, 57, 71; ARA-C and VM-26: days 59 + 73; cyclophosphamide (*CYC*): 29 + 43; ASP: days 32, 34, 46, 48, 87, 89, 108, 110. Radiotherapy (*RT*) is carried out in selected patients. Blocks in the intensive phase without pharmacokinetics studies are shown as *blanks*

Analytical methods

Chemicals

The thiopurines and dithiothreitol (DTT) were obtained from Sigma (Munich, Germany); Hanks' balanced salt solution (HBSS), from Life Technologies (Paisley, UK); and all other chemical from Merck (Darmstadt, Germany). TUA was prepared by incubation of TX with xanthine oxidase, and water was purified in a Milli-Q System (Millipore, Eschborn, Germany).

Preparation of standards

The thiopurines were dissolved in 0.1 N NaOH, further diluted with water and refrigerated at −20 °C. The final concentrations of the aqueous high-performance liquid chromatography (HPLC) standards prepared from these stock solutions were 1.20 μ*M* (6TG), 1.31 μ*M* (6MP), 5.52 μ*M* (6MeTG), 6.02 μ*M* (6MeMP), and 5 m*M* (DTT) in 0.5 *M* $HClO_4$.

Table 1 Drug dose and concentration of thiopurine metabolites detected in erythrocytes of children during maintenance therapy after oral administration of 6-mercaptopurine (90 samples in 32 patients) and 6-thioguanine (61 samples in 22 patients). The median doses were calculated from the individual daily doses given in the week before blood sampling, corresponding to 54.3 (6MP) and 41.8 (6TG) mg/m^2, respectively

Drug	Dose [μmol/m^2] (median, range)	E-TGN [pmol/10^9 RBC] (median, range)	E-MeTIN [pmol/10^9 RBC] (median, range)	E-MeTGN [pmol/10^9 RBC] (median, range)
6MP (n = 90)	357 (105–670)	366 (115–1,263)	9,563 (834–67,456)	Detectable in only a few samples
6TG (n = 61)	250 (74–616)	2538 (511–5,584)	–	1,056 (293–2,481)

Preparation of samples

First, 5 ml of whole blood was collected in heparinized tubes and centrifuged immediately. The RBC were washed twice in HBSS, resuspended in 2 ml of medium, and counted in a Celldyn Counter. Plasma and RBC were stored at −20 °C until analysis.

The extraction of thiopurines from plasma and RBC was performed according to Boulieu et al. [7] and Erdmann et al. [14], with some modifications. In brief, 1 ml of plasma was mixed with 25 μl of DTT (0.2 *M*) and then deproteinized and extracted with 50 μl of perchloric acid. After 10 min the supernatant was centrifuged, filtered (Acrodisc GHP 0.45 μ, Gelman), and chromatographically analyzed. For measurement of the drug metabolites in RBC an aliquot of the suspension was diluted with HBSS to 1 ml containing 0.5×10^6 RBC/μl and, after the addition of DTT, was heated for 15 min to 37 °C. After deproteinization and extraction as described above the supernatant was then heated for 2 h at 100 °C in Eppendorf Safe Lock tubes to achieve hydrolysis of the nucleotides. By this procedure, MeMP is totally transformed into a not yet identified substance ("MeMP") [29]. Hydrolysis of MeTGN was shortened to 45 min. After this time the released MeTG is reduced to about 65%, after 2 h it is nearly completely broken down to guanine. Because of the instability of the methylated thiopurines the aqueous standard solutions were treated likewise before HPLC by the addtion of an equal volume of perchloric acid and heating as described above.

Chromatography

The HPLC system (TSP, Darmstadt) consisted of a pump (P 200), an automatic autosampler (AS 300), and a variable UV/VIS detector (UV 2000) set at 342 nm (6TG derivates, TX, TUA), 322 nm (6MP derivates), 315 nm (MeTG derivates), and 290 nm (MeMP derivates). "MeMP" was measured at 310 nm. All separations were performed at room temperature on a reversed-phase column (Nucleosil 120, C18, particle size 3 μm, 125 × 4.6 mm with a 20-mm guard cartridge; GAT Analysentechnik, Bremen). The flow rate was 1.1 ml/min and the injection volume, 50 μl. The mobile phase consisted of sodium dihydrogen phosphate (0.01 *M*) adjusted to pH 2.7 with phosphoric acid. The extracts from the RBC were analyzed with 3% (6TG and "MeMP" in a single run) or 7.5% (MeTG) acetonitrile, and plasma extracts were chromatographed without it. For the identification of different (un-)methylated metabolites, RBC and plasma extracts were additionally analyzed with acetonitrile contents varying from 0 to 10%.

Results

Metabolites detected in RBC during maintenance therapy and blood counts

The MTX dose was 20 ± 12.7 mg/m^2 in the 6MP arm and 22.3 ± 8.3 mg/m^2 in the 6TG branch; in the analyzed blood samples the median WBC count was 3.6 ± 1.3 and 3.5 ± 1.9/nl, respectively. The daily thiopurine doses given before blood sampling and the concentrations of drug metabolites detected in RBC are shown in Table 1. The median molar thiopurine dose was 30% lower and the median E-TGN concentration about 7-fold higher in the 6TG branch than in the 6MP branch. The predominant erythrocytic metabolites found after 6MP administration were MeTIN, concentrated about 26-fold higher than the TGN and nearly 4-fold higher than the TGN after 6TG dosing. Methylated TGN could be detected only in a few samples in the 6MP arm, whereas in patients receiving 6TG their concentrations reached about 40% of that of the TGN.

In the 6TG group the concentration of the methylated as well as of the unmethylated TGN in RBC increased in parallel with the dose (Fig. 2). In the 6MP group the dose dependency of the E-TGN concentration seemed to be slight. The E-MeTIN concentration, however, rose considerably (Fig. 3). Thus, in contrast to the 6TG group, the pattern of metabolites shifted toward the methylated ones with increasing dose. In some cases the concentration of MeTIN was up to 160-fold higher than that of the TGN as demonstrated in Fig. 4, which shows the ratio between the methylated and

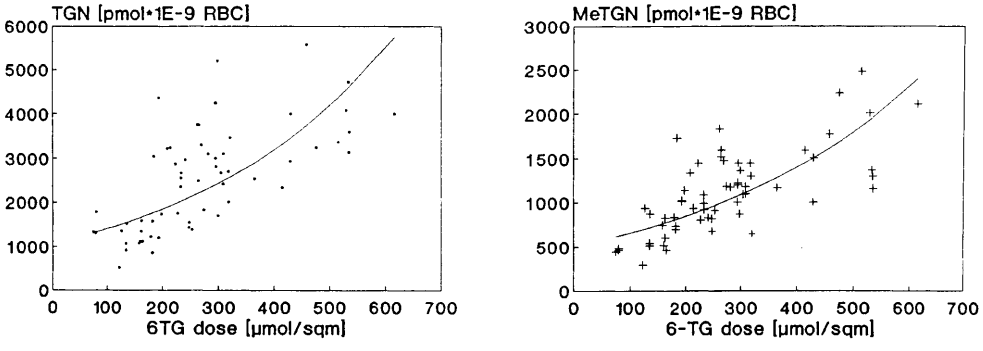

Fig. 2 Concentrations of 6-thioguanine (*left*) and 6-methylthioguanine (*right*) nucleotides measured in erythrocytes of 22 children (61 samples) in maintenance therapy after 6-thioguanine dosing (299 μmol corresponds to 50 mg 6TG). Curves were created using Havard Graphics; correlation coefficients and *P* values were not calculated

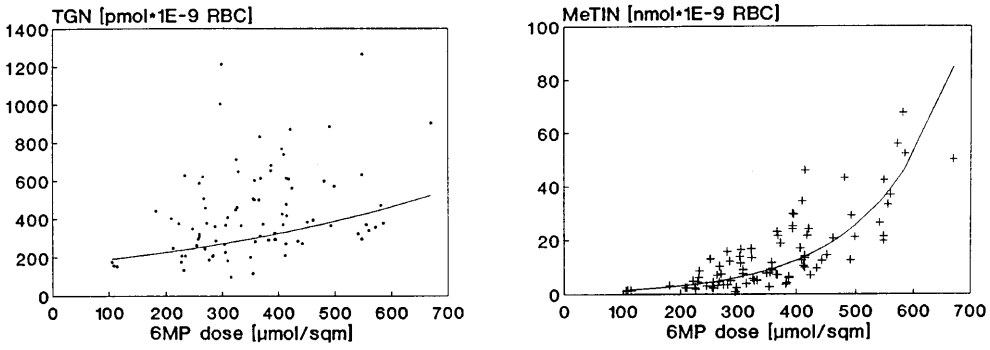

Fig. 3 Concentrations of 6-thioguanine (*left*) and 6-methylthioinosine (*right*) nucleotides measured in erythrocytes of 32 children (90 samples) in maintenance therapy after 6-mercaptopurine dosing (329 µmol corresponds to 50 mg 6MP)

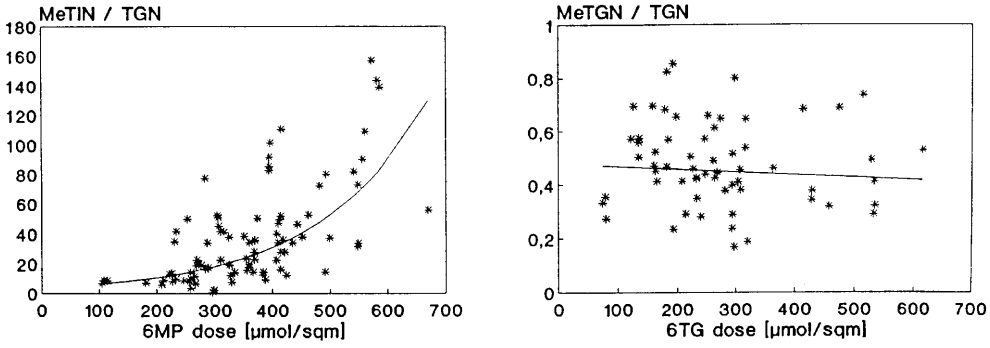

Fig. 4 Ratio between methylated and unmethylated thiopurine metabolites detected in erythrocytes of children in maintenance therapy after 6-mercaptopurine (*left*; 90 samples in 32 patients) and 6-thioguanine (*right*; 61 samples in 22 patients) administration (329 µmol 6MP or 299 µmol 6TG corresponds to 50 mg)

unmethylated metabolites during 6MP and 6TG treatment.

Without hydrolysis, methylated or unmethylated thiopurine bases or the corresponding ribosides (MeTG riboside was not examined) could not be detected in erythrocytes. Thus, we conclude that all measured bases derived from nucleotides.

RBC metabolites detected during the intensive phase

After treatment with 6TG at 100 mg/m^2, TGN in RBC were measurable as early as at 5 h post treatment. Five children who had been pretreated with 6MP 2–3 weeks earlier had markedly higher values than five others who had not been pretreated, (200–1,700 versus 45–360 pmol/10^9 RBC). Due to the small number of samples, however, these results must be regarded as preliminary. After treatment with 6MP at 100 mg/m^2, TGN and MeTIN were not detectable in all patients, even after 24 h. The mean MeTIN concentrations recorded at this time point were nearly 20 times higher than the TGN concentrations (Table 2). After 7 days of treatment with 6MP, MeTIN and TGN levels continued to persist (1,175–8,313 and 37–94 pmol/10^9 RBC, respectively) for 2–3 weeks.

Table 2 Thioguanine and methylthioinosine nucleotide concentration measured in erythrocytes of patients in intensive therapy after the first single 100-mg/m^2 dose of mercaptopurine (*ndet* Not detected).

Patient	5 h after intake		24 h after intake	
	E-TGN [pmol/10^9 RBC]	E-MeTIN [pmol/10^9 RBC]	E-TGN [pmol/10^9 RBC]	E-MeTIN [pmol/10^9 RBC]
A.D.	21	694	85	793
B.A.	ndet	ndet		
E.I.	46	633		
K.M.	28	ndet	124	2,329
M.J.	29	726		
P.J.	56	269	85	301
S.P.	39	ndet	100	ndet
S.I.	ndet	698	ndet	540
S.J.	75	299		
W.V.			28	ndet
C.J.	29	ndet	81	4,259
K.K.	21	379	26	318
M.K.	34	870		
T.D.	ndet	381		
Mean[a]	38	550	75	1,424

[a] Only patients with detectable metabolites were included

Table 3 Pharmacokinetic data recorded for patients in intensive therapy receiving the first dose of mercaptopurine and thioguanine. The mean doses correspond to 102 (6MP) and 101 mg/m^2 (6TG)

Patient	6MP			6TG		
	Dose [μmol/m^2]	Time to peak [h]	Peak conc. [pmol/ml]	Dose [μmol/m^2]	Time to peak [h]	Peak conc. [pmol/ml]
A.D.	648	2	888	581	4	196
B.A.	632	1	2,231			
D.R.	614	2	1,056	626	4	194
E.I.	738	1	1,460			
H.B.				592	2	493
K.M.	671	1	726			
M.J.	651	4	889			
M.S.	684	3	1,387			
P.J.	681	2	1,075			
S.P.	661	2	2,166	687	2	221
S.I.	710	3	1,864	603	2	981
S.H.				591	1	64
S.J.	714	2	752	607	1	227
W.J.				594	4	144
Mean	673	2.2	1,318	610	2.5	315

Pharmacokinetics

On oral administration of either 6MP or 6TG, large interindividual differences could be demonstrated with regard to peak concentrations and times to peak concentration (Table 3) as well as the course of elimination of the drugs (data not shown). Peak concentrations were reached after 1–4 h (mean 2.5 h) and were about 4-fold higher after treatment with 6MP as compared with 6TG (1,318 versus 315 pmol/ml). At certain time points after treatment with the two drugs, thioxanthine and thiouric acid (6MP arm) and thiouric acid and thioguanosine (6TG arm) could be identified in addition to the parent drugs. These compounds have not yet been quantified; 6MP, however, seems to be oxidized to a much greater extent to TUA. For the three patients investigated, the height of the TUA peak recorded at 3 h after drug uptake was 40- to 60-fold higher in the 6MP branch than in the 6TG arm (data not shown).

Discussion

The cytotoxic effect of 6MP and 6TG was first demonstrated in the early 1950s; in a review on thiopurines, Gertrude Elion stated: "Thioguanine was more active but also more toxic. It was also more difficult to synthesize and, since its mechanism of action appeared to be similar to that of 6MP, its metabolic fate and clinical activity was explored somewhat later" [12]. These may have been the reasons why 6TG never came to be used in maintenance treatment of acute lymphoblastic leukemia, although in contrast to 6MP, it is more directly converted to the cytotoxic TGN and can be degraded by xanthine oxidase only after deamination. Furthermore, in vitro experiments with leukemic cells have demonstrated that 6TG exerts its cytotoxicity after shorter periods of exposure and at lower concentrations [2, 40]. In our randomized comparison of the two drugs we found considerable differences in pharmacokinetics, metabolism, and accumulation of RBC metabolites.

Peak plasma concentrations were considerably lower after a single dose of 6TG than after 6MP dosing. Previous investigations on the pharmacokinetics of either 6TG [9, 33, 35] or 6MP [13, 21, 25, 37] also showed a lower peak concentration after 6TG administration. The plasma concentrations and the bioavailability of such intensively metabolized drugs are of little consequence; however, crucial is the formation of the active metabolites in the target cells. Since leukemic cells are not available for measurement once remission has been achieved, determination of the concentrations of the metabolites in RBC has to serve as a substitute and could allow certain conclusions to be drawn regarding the treatment intensity and even the clinical outcome. TGN levels measured in RBC (E-TGN) during 6MP treatment correlated with neutropenia or leukocytopenia [30, 42]. Moreover, children with TGN levels below the group median had a significantly higher relapse rate [28, 32]. In addition, the concentration of methylated thioinosine nucleotides in RBC (E-MeTIN) seems to be of prognostic relevance [6].

As we and other investigators [33, 35] have shown, TGN are demonstrable in RBC within a few hours of a single dose of 6TG. After 6MP administration the TGN concentrations measured in RBC are low relative to the concentration of MeTIN; in our study, at 24 h after a single 6MP dose, E-MeTIN concentrations reached values similar in magnitude to those recorded for E-TGN after 6TG treatment. It has yet to be shown in a larger number of patients whether pretreatment with 6MP enhances TGN formation after 6TG treatment.

The E-TGN concentrations measured in our patients during daily treatment with 6MP are in accordance with those reported from other studies [22, 28, 34, 36]. Bostrom and Erdmann [6], however, found markedly

lower concentrations. In their study, as in our investigation, E-MeTGN were not detectable in all patients.

During 6MP treatment we found E-MeTIN concentrations that far exceeded the E-TGN concentrations. This has also been described by other investigators [6, 29, 41]. On the other hand, Keuzenkamp et al. [22] used a 6MP dose of 25 mg/m^2 and found no methylated metabolite. The reason for this finding as well as for the lower concentrations measured by Lennard et al. [29] is the dose dependency of E-MeTIN, which we could demonstrate in our patients. Intravenous administration of 6MP resulted in even higher E-MeTIN levels, whereas E-TGN levels were no higher than those detected after the lower oral dose [22]. An increase in the 6MP dose beyond a certain limit resulted only in increased MeTIN levels but not in higher TGN concentrations in some patients in our study as well (data not shown).

The formation of TGN from 6MP is dependent on the availability of TIMP and on the activity and interplay of the three enzymes that catalyze its further metabolism. The best studied of these enzymes is thiopurine methyltransferase (TPMT), which is subject to genetic polymorphism ([46]; reviewed by Krynetski et al. [24]). Its activity is negatively correlated with the E-TGN levels detected during 6MP treatment [32]; patients without measurable TPMT activity developed exceedingly high TGN levels [15, 34]. In contrast to previous investigations [10], Krynetski et al. [23] have demonstrated that 6MP and 6TG and their nucleotides are substrates for TPMT with similar kinetic properties.

Inosine monophosphate dehydrogenase is the rate-limiting enzyme for the interconversion of IMP to GMP [45]; it can be inhibited by TIMP [11, 18] as well as by TGMP [4, 16, 38]. After incubation of leukemic cells with 6MP the intracellular concentration of TIMP is markedly higher than that of TGN [23, 43, 47]; an increase in the 6MP concentration in the incubation medium enhances TIMP and MeTIMP levels without increasing TGN levels [43]. During short-term incubation, RBC are capable of converting 6MP to TIMP but not to TGN [27]. The formation of TIMP itself, therefore, does not seem to be the limiting factor for TGN formation. The metabolic pattern observed after a single dose of 6MP in our investigation suggests that TIMP, either directly or after dephosphorylation, is methylated more quickly than it is converted to TXMP.

In contrast to the numerous investigations regarding the formation of E-TGN after 6MP treatment, to date, only limited data have been published on E-TGN formation after 6TG dosing. Lilleymark et al. [35] investigated TGN accumulation after single-dose 6TG therapy, as did Lennard et al. [33] after daily treatment of relapsed patients with 6TG. The latter group also found higher TGN levels after 6TG dosing as compared with 6MP treatment, even though their concentrations were lower than ours despite the use of comparable doses. Methylated metabolites were not measured in either study.

As we have shown, the more direct and more rapid conversion of 6TG leads to much higher erythrocytic levels of TGN versus 6MP, which is predominantly metabolized to its methylated nucleotide. If TGN were the principal cytostatic metabolites of the thiopurines, this would lead to a distinct superiority of 6TG in maintenance therapy. In our randomized study, however, the event-free survival in the two arms was equal after a median observation period of 27 months [1, 19, 20]. A median molar 6TG dose amounting to 70% of that of 6MP was sufficient to achieve an equivalent WBCU. Considering that the TGN concentrations were 7 times higher in the 6TG arm, the cytotoxic effect on the WBC should have been more pronounced in comparison with that seen in the 6MP arm. On the other hand, during 6MP treatment the concentration of MeTIN was 26-fold higher than that of TGN. Inhibition of purine de novo synthesis by MeTIMP could be another major mechanism of action, as could the inhibition of inosinate dehydrogenase [12], a key enzyme in the de novo synthesis of guanine nucleotides. Further studies have to elucidate which mechanism is the most important one for leukemic cells. More than 20 years ago, Zimmermann et al. [48] concluded: ''In spite of the relatively lengthy history on long-term clinical use of this drug its precise mechanism of cytotoxicity has thus far eluded definition although many loci of action are known.'' From our studies it seems likely that the cytotoxicity of 6MP is only partly mediated through TGN.

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