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ORIGINAL ARTICLE

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Leucocyte versus erythrocyte thioguanine nucleotide concentrations in children taking thiopurines for acute lymphoblastic leukaemia

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Abstract *Purpose*: The aim of this study was to compare leucocyte and erythrocyte thioguanine nucleotide (TGN) cytotoxic metabolite concentrations in children with lymphoblastic leukaemia taking mercaptopurine (MP) or thioguanine (TG) as part of their long-term remission maintenance chemotherapy. *Methods*: Ten consecutive children treated on the MRC ALL97 protocol were studied. Six were randomized to TG and four to MP. Leucocyte and erythrocyte thiopurine nucleotide metabolites were measured after the children had been titrated to the standard thiopurine protocol dose, or higher. *Results*: Children taking TG accumulated significantly higher erythrocyte TGN concentrations than those taking MP (median difference 1171 pmol/8×10^8 erythrocytes, 95% CI 766 to 2169, $P < 0.02$), but there was no significant difference in the concentration range of leucocyte TGNs generated from TG or MP. In those children taking TG, median TGN concentrations were 5142 pmol/8×10^8 leucocytes and 1472 pmol/8×10^8 erythrocytes (3.5-fold difference, median difference 3390 pmol/8×10^8 cells, 95% CI 1559 to 7695, $P = 0.005$), compared to 5422 pmol/8×10^8 leucocytes and 261 pmol/8×10^8 erythrocytes (20-fold difference, median difference 5054 pmol/8×10^8 cells, 95% CI 2281 to 6328, $P = 0.03$) in those taking MP. *Conclusions*: Despite the accumulation of significantly higher erythrocyte TGN concentrations for TG compared with MP, the accumulation of leucocyte TGNs in children taking TG was similar to the range of leucocyte TGNs in children taking MP. Therefore, when correlating intracellular TGNs to clinical effect, the range of erythrocyte TGN metabolites will be higher for those children taking TG than in those taking MP.

Keywords Thiopurines · Thioguanine nucleotides · Childhood lymphoblastic leukaemia · Erythrocytes · Leucocytes

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Introduction

Mercaptopurine (MP) has been the backbone of 'maintenance' chemotherapy in childhood lymphoblastic leukaemia over three decades, and the most successful protocols for 'standard risk' disease include a prolonged period of daily MP coupled with weekly methotrexate. In current randomized trials, in both Europe and the United States, MP is being compared with the closely related drug thioguanine (TG) [6, 12, 15, 18]. The cytotoxic effects of MP and TG are mediated, in part, by intracellular TG nucleotide (TGN) metabolites. In an early trial protocol for children with lymphoblastic leukaemia, the concentration of MP-derived TGNs correlated with relapse-free survival independently of other prognostic factors [14]. In vitro data have suggested that lymphoblasts are more sensitive to TG than to MP [1].

Thiopurine metabolism is complex and thiol methylation competes with nucleotide metabolite formation (Fig. 1). TG forms TGNs directly, but the formation of MP-derived TGNs is influenced by methylmercaptopurine nucleotide formation catalysed by thiopurine methyltransferase (TPMT). MP, MP nucleotide and TG are good substrates for TPMT; TGNs are poor substrates [4]. TPMT activity is regulated by a common genetic polymorphism [11, 22]. Patients with low TPMT activity form high concentrations of erythrocyte TGNs from MP and experience profound neutropenia. Conversely, children with very high TPMT activities do not

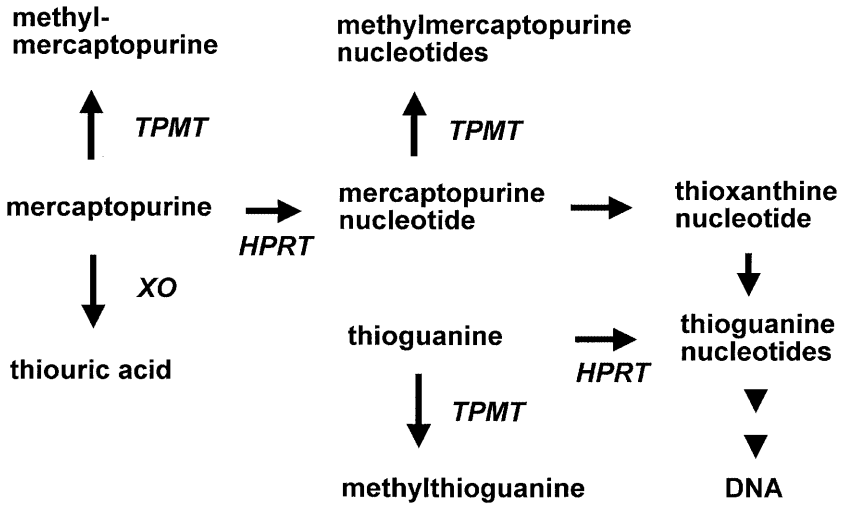

Fig. 1. Thiopurine metabolism. Mercaptopurine is metabolized along three competing routes; oxidation, methylation and nucleotide metabolite formation. Nucleotide metabolite formation is catalysed by the enzyme hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) and *S*-methylation by TPMT. Oxidation of mercaptopurine is catalysed by xanthine oxidase (*XO*), although the liver has very high xanthine oxidase activities, this enzyme activity is lacking in all blood cells. TG is not a direct substrate for xanthine oxidase. TG forms TG nucleotides directly

respond to standard thiopurine dosages [10, 11, 16]. The genetic polymorphism that controls erythrocyte TPMT activity also controls the enzyme activity in all other cells and tissues, including lymphoblasts [17, 22].

Traditionally, the easily accessible and plentiful erythrocyte has been used as a surrogate marker for thiopurine metabolism in other tissues. There is little information available on leucocyte thiopurine metabolism. This cell population may serve as a more appropriate marker tissue in leukaemic children. Measurement of thiopurine metabolites in renal transplant patients receiving the MP prodrug azathioprine have shown that neutrophil TGN concentrations were over 30-fold higher than erythrocyte concentrations [2]. Leucocyte DNA TG is derived from TGNs, and in children with lymphoblastic leukaemia on MP therapy the incorporation of TG base ranges from 95 to 710 fmol TG base per microgram of leucocytes [21]. In Crohn's disease patients receiving azathioprine, leucocyte DNA TG correlates directly with erythrocyte TGNs [3].

The aim of this study was to compare the accumulation of erythrocyte and leucocyte thiopurine nucleotide metabolites in children with lymphoblastic leukaemia on either MP or TG maintenance therapy. The total leucocyte metabolite concentrations were measured, rather than individual cell population concentrations, due to the low leucocyte count in this group of patients.

Patients and methods

Consecutive children with lymphoblastic leukaemia, in remission, attending The Royal London Hospital and treated on the UK Medical Research Council lymphoblastic leukaemia trial ALL97 [18], were studied. The local research ethics committee approved the protocol and fully informed written consent was obtained from the parents. The 2-year programme of maintenance therapy included daily oral thiopurine, weekly oral methotrexate, monthly intravenous vincristine and randomized steroid (prednisolone or dexamethasone) with intrathecal methotrexate every 12 weeks. All the children were studied at the same point in the maintenance cycle. The study blood sample was withdrawn in the late morning prior to the monthly vincristine sample on the day that the child was attending for intrathecal methotrexate, which was given in the afternoon. The intrathecal methotrexate dosage was 12.5 mg for those children aged over 3 years and 10 mg for those children aged 2 to 3 years. The weekly oral methotrexate (20 mg/m^2) was taken 3 days later. Each child was randomized to MP or TG (standard protocol dose of 75 and 40 mg/m^2, respectively). The thiopurine dosage was titrated to toxicity, escalated by 25% every 4 weeks if the neutrophil count was above 1.0×10^9/l and the platelet count was above 100×10^9/l and reduced if the cell counts fell below those thresholds. Children were studied when they had been receiving MP or TG at the standard protocol dose, or above, for at least 7 days and when their peripheral neutrophil counts exceeded 1.0×10^9/l and platelet counts exceeded 100×10^9/l.

Venous blood samples (20 ml in EDTA) were taken prior to the daily thiopurine dose and processed within 2 h. Whole blood was layered onto an equal volume of Polymorphprep (Nycomed Pharma) and the leucocytes prepared according to the manufacturer's instructions. Contaminating erythrocytes were removed from the harvested leucocytes by lysis in 5 ml ammonium chloride (154 m*M*) containing sodium bicarbonate (10 m*M*) and EDTA (0.1 m*M*) for 5 min at room temperature. Phosphate-buffered saline (25 ml) was added and the leucocytes pelleted at 400 *g* and 22°C for 10 min. These steps were repeated, and the washed leucocytes resuspended in 600 µl Hanks' balanced salt solution. Prior to drug metabolite analysis, the leucocytes were lysed by freeze/thawing three times. The erythrocytes, sedimented beneath the Polymorphprep, were washed and prepared for metabolite assay as described previously [8].

Intracellular drug metabolite concentrations were measured by a high-performance liquid chromatographic assay [8], using an isocratic gradient that enabled the separation and detection of both TGNs and methylmercaptopurine nucleotides in the same clinical sample. The lower limits of sensitivity for both TGNs and methylmercaptopurine nucleotides were 30 pmol/8×10^8 erythrocytes and 30 pmol/5×10^6 leucocytes. The interassay coefficients of variation for the thiopurine quality control samples at 300 pmol and 3000 pmol/8×10^8 erythrocytes were 5% and 3% respectively. Leucocyte interassay calibration data, run over four assays, for quality controls at 100 and 360 pmol TG and 1000 and 3600 pmol methylmercaptopurine predicted 99, 362, 984 and 3686 pmol with coefficients of variation ranging from 4% to 11%. The TG extraction efficiency was 56% (coefficient of variation 10%) at leucocyte concentrations ranging from 5×10^6 to 1 x10^8 cells. The leucocyte samples from the children taking TG and those taking MP were assayed on the same day. Leucocyte calibration curves containing 3×10^7 leucocytes, and spiked with 0 to 600 pmol TG

and 0 to 6000 pmol methylmercaptopurine, were run in parallel with the patient samples. The curves were linear with correlation coefficients > 99% and described by the equations $y = 17 + 7.3x$ and $y = -45 + 4.8x$, where y is the peak height in microvolts and x is the concentration in picomoles, for TG and methylmercaptopurine, respectively.

Erythrocyte TPMT activity was measured as previously described [9]. Statistical comparisons were made by the Mann-Whitney test and correlations were assessed using Spearman's rank correlation coefficient (r_S).

Results

Ten children, median age 6.2 years (range 2.8 to 10.5 years), were studied. Six were randomized to receive TG (three boys, three girls) and four MP (two boys, two girls). At the time of study, the median dose of TG was 43 mg/m^2 (range 36 to 58 mg/m^2) and MP 75 mg/m^2 (range 63 to 77 mg/m^2), and the duration of thiopurine treatment at this dosage varied from 1 to 6 weeks, median 2 weeks. All children had high (homozygous wild-type) TPMT activity [11], median activity 16.2 U TPMT/ml packed erythrocytes (range 12.7 to 20.3). The median leucocyte count in the sample preparations was 3.6×10^7 (range 9.3×10^6 to 1.4×10^8). The metabolite concentrations measured are summarized in Table 1. The concentration range of erythrocyte TGNs produced from TG were significantly higher than from MP (median difference 1171 pmol/8×10^8 erythrocytes, 95% confidence interval (CI) 766 to 2169 pmol, $P < 0.02$), but there was no significant difference in the concentration range of leucocyte TGNs generated from the two thiopurines.

In both groups of children, cell for cell, the accumulation of TGNs in leucocytes was higher than in erythrocytes, but in the children taking TG the leucocyte/erythrocyte ratio was less. The median leucocyte/erythrocyte TGN ratio was 2.8 for those children taking TG in contrast to 14 for those taking MP (median difference 10.3, 95% CI 6.3 to 27.4, $P < 0.02$). For those children taking TG, the median TGN concentrations were 5142 pmol/8×10^8 leucocytes and 1472 pmol/8×10^8 erythrocytes (3.5-fold difference, median difference 3390 pmol/8×10^8 cells, 95% CI 1559 to 7695, $P = 0.005$), compared to 5422 pmol/8×10^8 leucocytes and 261 pmol/8×10^8 erythrocytes (20-fold difference, median difference 5054 pmol/8×10^8 cells, 95% CI 2281 to 6328, $P = 0.03$) for those taking MP.

In this small group of children there was no direct relationship between leucocyte and erythrocyte TGNs for the group as a whole ($n = 10$, $r_S = 0.52$) nor for the TG subgroup ($n = 6$, $r_S = 0.77$) or the MP subgroup ($n = 4$, $r_S = 0.2$). For those children taking MP the erythrocyte methylmercaptopurine nucleotides were not correlated with the leucocyte concentrations ($n = 4$, $r_S = 0.4$), and the abnormally high erythrocyte methylmercaptopurine nucleotides [13] measured in children 8 and 10 were not reflected in the leucocytes. All children continue, at the time of writing, in complete remission.

Discussion

These results highlight important differences in thiopurine metabolism between erythrocytes and leucocytes. Children taking TG accumulated significantly higher concentrations of erythrocyte TGN than those taking MP, confirming the findings of previous studies [5, 7]. This is probably a direct reflection of the differences in the metabolic fates of the two thiopurines coupled with the ability of the erythrocytes to salvage, and so accumulate, circulating thiopurines throughout their prolonged life [19]. Mature erythrocytes are incapable of de novo purine synthesis but are endowed with a highly efficient salvage mechanisms for using preformed purines. TGNs can be formed from hepatic and extrahepatic thiopurine metabolites taken up by erythrocytes throughout their life [19]. This mechanism could also explain the elevated erythrocyte methylmercaptopurine nucleotide concentrations observed in this (children 8 and 10) and other studies [13]. The measurement of methylmercaptopurine nucleotides in the leucocytes indicates that these cells have functional TPMT activity

Table 1. Thiopurine nucleotide concentrations in leucocytes and erythrocytes during TG and mercaptopurine therapy (*TG* thioguanine, *MP* mercaptopurine, *TGN* thioguanine nucleotides, *MeMPs* methylmercaptopurine nucleotides, *WBCs* leucocytes, *RBCs* erythrocytes)

Child	Drug	Dose (mg/m^2)	WBCs (pmol/8×10^8)		RBCs (pmol/8×10^8)		WBC/RBC ratio	
			TGNs	MeMPs	TGNs	MeMPs	TGNs	MeMPs
1	TG	58	3602	0	1285	0	2.8	
2	TG	42	9921	0	1721	0	5.8	
3	TG	47	6682	0	2459	0	2.7	
4	TG	38	3280	0	1395	0	2.4	
5	TG	43	8740	0	1548	0	5.6	
6	TG	36	2969	0	1045	0	2.8	
Median		43	5142	0	1472	0	2.8	
7	MP	73	6545	4291	217	3,810	30.2	1.1
8	MP	77	2800	5128	232	21,700	12.1	0.2
9	MP	63	6000	3486	519	2,842	11.6	1.2
10	MP	83	4844	2028	290	11,296	16.7	0.2
Median		75	5422	3889	261	7,553	14.0	0.7

[20]. This is contrary to the findings of Bergan et al. [2] who reported erythrocyte methylmercaptopurine nucleotides but failed to detect these methylated metabolites in neutrophils isolated from renal transplant recipients receiving azathioprine. This may be partly have been due to the fact that these patients were studied at the start of azathioprine therapy.

Incorporation of TGN-derived TG bases into leucocyte DNA [21] is generally considered to be central to thiopurine-mediated myelotoxicity, and the results of this study indicate that both thiopurines produce similar ranges of leucocyte TGN concentrations. This observation is clinically important. The leucocyte cell line is one step nearer the target lymphoblast than the surrogate erythrocyte, but using current techniques it is not practicable to run a routine therapeutic drug monitoring programme for leucocyte metabolites in these myelosuppressed children. This study, and other clinical studies [5, 12], have now shown that the concentrations of erythrocyte TGNs generated from TG therapy are far higher than the concentration range of TGNs generated from MP. Therefore, when correlating intracellular TGNs with clinical effect, the range of erythrocyte TGN metabolites will be higher in those children taking TG than in those taking MP. The results of this study indicate that this does not mean that, in children taking MP, the leucocyte is exposed to lower TGN concentrations.

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