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Erythrocyte-incorporated 6-mercaptopurine metabolite levels are not affected by recent drug administration during maintenance therapy for childhood acute lymphoblastic leukemia

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ABSTRACT

Background: Childhood acute lymphoblastic leukemia (ALL) maintenance therapy with oral 6-mercaptopurine (6-MP) and methotrexate prevents leukemic relapse by acting against residual lymphoblasts. Non-adherence to oral maintenance therapy significantly increases ALL relapse risk. The levels of intracellular 6-MP metabolites erythrocyte incorporated (ery-) thioguanine nucleotides (ery-TGN) and methylated 6-MP metabolites (ery-MeMP) can be used to evaluate adherence to 6-MP, however, little is known on their short-term pharmacokinetics. The aim of this study was to assess the changes between trough levels and the levels at 2-hours after 6-MP intake when the 6-MP levels in plasma is expected to have peaked.
Materials and methods: Ten ALL patients with stable 6-MP dose were prospectively included. Two blood samples were collected for ery-TGN and ery-MeMP analysis (before 6-MP intake and 2 h after).
Results: The median trough ery-TGN was 173 (range 126–299) nmol/mmol hemoglobin (HGB), and 2 h after 6-MP intake the median level was 175 (range 120–293) nmol/mmol HGB. For ery-MeMP, the median trough and 2 h-concentrations were 9 239 (range 1 278–19 645) nmol/mmol HGB and 9 216 (range 1 215–19 519) nmol/mmol HGB, respectively. The median absolute percentual change for both ery-TGN and ery-MeMP were not statistically different from 0, $p = 0.28$ and $p = 0.06$, respectively.
Conclusions: Intracellular 6-MP metabolites, ery-MeMP and ery-TGN, remain stable 2 h after oral 6-MP intake in patients with a stable 6-MP dose. This data support that blood samples may be used to assess patient adherence irrespective of the timing of 6-MP intake.

1. Introduction

Combination therapy with oral 6-mercaptopurine (6-MP) and methotrexate (MTX) is the long-standing backbone of maintenance therapy (MT) for childhood acute lymphoblastic leukemia (ALL). The aim of MT is to prevent leukemic relapse by prolonged antileukemic activity against residual lymphoblasts [1,2].

6-MP is a prodrug with large inter- and intra-individual variability and a short half-life of 1.8 (range 0.6–2.5) hours [3]. The cytotoxic effect of 6-MP is primarily mediated by thioguanine nucleotides (TGN) incorporated into DNA of leukocytes (DNA-TGN) [4]. 6-MP and its intermediary metabolites are methylated intracellularly by thiopurine S-methyl transferase (TPMT), creating both inactive and active methylated 6-MP metabolites (MeMP) [2].

DNA-TG, as the common downstream metabolite of MT, has been associated with relapse risk for patients with a detectable measurable

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residual disease (MRD) at the end of induction therapy [5,6]. Direct association of erythrocyte (ery-) TGN and ery-MeMP levels with relapse risk has not been confirmed in contemporary studies, but their levels are associated with DNA-TG [5]. Poor treatment adherence has been associated with a 2.5–3.9-fold increased risk of relapse [7]. The levels of intracellular metabolites with a rapid turnover like ery-TGN and ery-MeMP can be used to evaluate adherence to MT [8,9]. Low or highly variable levels of ery-TGN and ery-MeMP has been associated with non-adherence to MT [8–11].

The current European ALLTogether1 protocol (EudraCT: 2018–001795–38) reports back to physicians upon low ery-TGN or ery-MeMP levels to address possible non-adherence. The levels of ery-TGN and ery-MeMP has been investigated on the long timescale of MT revealing large inter- and intraindividual variability [12,13]. However, changes in metabolite levels in a short time period and the significance of measuring trough levels are not well described. We therefore compared measurements of ery-TGN and ery-MeMP trough levels with repeated measurements two hours after oral 6-MP administration.

2. Materials and methods

2.1. Study patients and treatment risk stratification

Patients with newly diagnosed B-cell precursor (BCP) or T-cell acute lymphoblastic leukemia (T-ALL) aged 1–18 years at diagnosis and treated according to intermediate risk-low (IR-low) or intermediate risk-high (IR-high) treatment arm in the ALLTogether1 protocol (EudraCT: 2018–001795–38) were included into the study. This included all *BCR::ABL*-negative patients who had detectable < 5% MRD at the end of induction (day 29), except the patients with: (i) minimal residual disease (MRD) > 0.01% after Consolidation 1 (day 78) for NCI high risk (age ≥10 years and/or leukocytes ≥50 $\times 10^9$/L and/or T-ALL at diagnosis) or MRD ≥ 0.05% for NCI low risk patients, respectively; (ii) remaining testicular disease or mediastinal mass ≥ 1/3 of initial volume after Consolidation 1; (iii) all patients with translocation t(17;19)/*TCF3::HLF* and (iv) patients ≥ 16 years and *KMT2A*-rearrangement.

Eligible patients were those who reached MT phase and were taking stable (+/- 20%) 6-MP dose for at least four weeks. According to the ALLTogether1 protocol recommendations, the drug could be taken at any time of the day, but on a stable schedule. Patients were excluded if they had red blood cell transfusion within 6 weeks.

The study was approved by the Regional Biomedical Research Ethics Committee of Vilnius, Lithuania (2021/4–1332–808), and written informed consent was obtained from all participants and their legal guardians.

2.2. Maintenance therapy in ALLTogether protocol

All patients received standard MT with a backbone of daily oral 6-MP, initial dose of 75 mg/m^2/day (for *TPMT* wild type and heterozygous patients), and weekly oral MTX, initial dose of 20 mg/m^2/week. In patients with homozygous *NUDT15* or *TPMT*-deficiency, the starting 6-MP dose is reduced to 5 mg/m^2. The 6-MP and MTX dose is adjusted to keep the absolute neutrophil count (ANC) level between 0.75 and 1.5 × 10^9/L and platelets above 75 × 10^9/L. MT is continued until the end of the therapy (two years from end of induction therapy).

Every four weeks throughout MT, patients receive pulses of oral Dexamethasone (Dexa) 6.0 mg/m^2/day for 5 days and Vincristine (VCR) 1.5 mg/m^2 (maximum single dose 2.0 mg) intravenously on day 1 of the pulse. Pulses are given regardless of blood counts.

Intrathecal therapy with MTX is given on the first day of VCR/Dexa pulse every 3rd cycle (every 12 weeks) three times for IR-low group or throughout MT phase up to a total of six doses for patients stratified to IR-high [1].

Table 1
Patient characteristics.

Patient number	Diagnosis	Treatment risk group	Sex	BSA (m^2)	Age at sampling (years)	6-MP dose (mg/m^2)
1	BCP	IR-High	M	1.1	9.3	68
2	BCP	IR-High	F	1.74	17.2	72
3	BCP	IR-Low	F	1.14	7.9	44
4	ALAL	IR-High	F	1.72	14.6	58
5	BCP	IR-Low	F	0.75	5.1	33
6	BCP	IR-Low	F	1.68	13.1	30
7	BCP	IR-High	M	0.61	3.2	41
8	BCP	IR-High	M	2.22	17.7	68
9	BCP	IR-Low	M	0.78	5.2	32
10	BCP	IR-High	F	1.16	7.8	43

ALAL – acute leukemia of ambiguous lineage, BCP – B-cell precursor acute lymphoblastic leukemia, BSA – body surface area, F – female, IR – intermediate risk, M – male.

2.3. Blood sampling

Blood samples were collected during MT at inpatient or outpatient visit for VCR/Dexa pulse therapy. The patients who were taking 6-MP on the evening schedule and came in for their VCR/Dexa pulse for an outpatient visit, were asked to change to morning schedule for three days before the visit. Hospitalized patients continued with the evening 6-MP schedule, and the blood sample was collected in the evening.

The blood samples were collected from a central venous line to 8 mL tubes with EDTA just before 6-MP administration (trough level) and two hours after 6-MP administration. Whole blood samples were immediately transported to the hospital laboratory, aliquoted to eight separate cryo-tubes of 1 mL each and stored at − 80°C. All samples were shipped on dry ice to the Pediatric Oncology Laboratory, Copenhagen University Hospital Rigshospitalet, Denmark for analysis.

2.4. 6-MP metabolite analysis

After thawing, the samples were deproteinized by precipitating with perchloric acid. The samples were then centrifuged at 3000 g for 15 min at 4 °C, and supernatant was incubated for 45 min at 100 °C to hydrolyze acidic thiopurine nucleotides into their bases. Ery-TGN and ery-MeMP were quantified by ultra-performance liquid chromatography with ultraviolet detection and assessed using internal standards [14]. Intra- and inter-day precision for both analytes were < 10% relative standard deviation (RSD). Ery-TGN and ery-MeMP metabolite results were normalized to samples' measured hemoglobin (HGB) levels and are presented as nmol/mmol HGB. All samples were analyzed in one run. Every sample was run twice, and mean values were used for further analysis.

2.5. Statistical analysis

Descriptive statistics such as frequency and median (range) were used to describe patients' demographic and clinical data. Wilcoxon signed-rank test was used to check the null hypothesis that the median of absolute percentual change of ery-TGN and ery-MeMP was not statistically significantly different from 0. A two-tailed p value < 0.05 was considered to be statistically significant. Coefficient of variation (CV) within each run's duplicate analysis and between the pre- and 2 h after 6-MP dose for every sample was calculated. Statistical analysis was performed using IBM SPSS version 28.

3. Results

Ten consecutive patients (four male, six female) with a median age of 8.6 (range 3.2–17.7) years at the sampling time were included in the

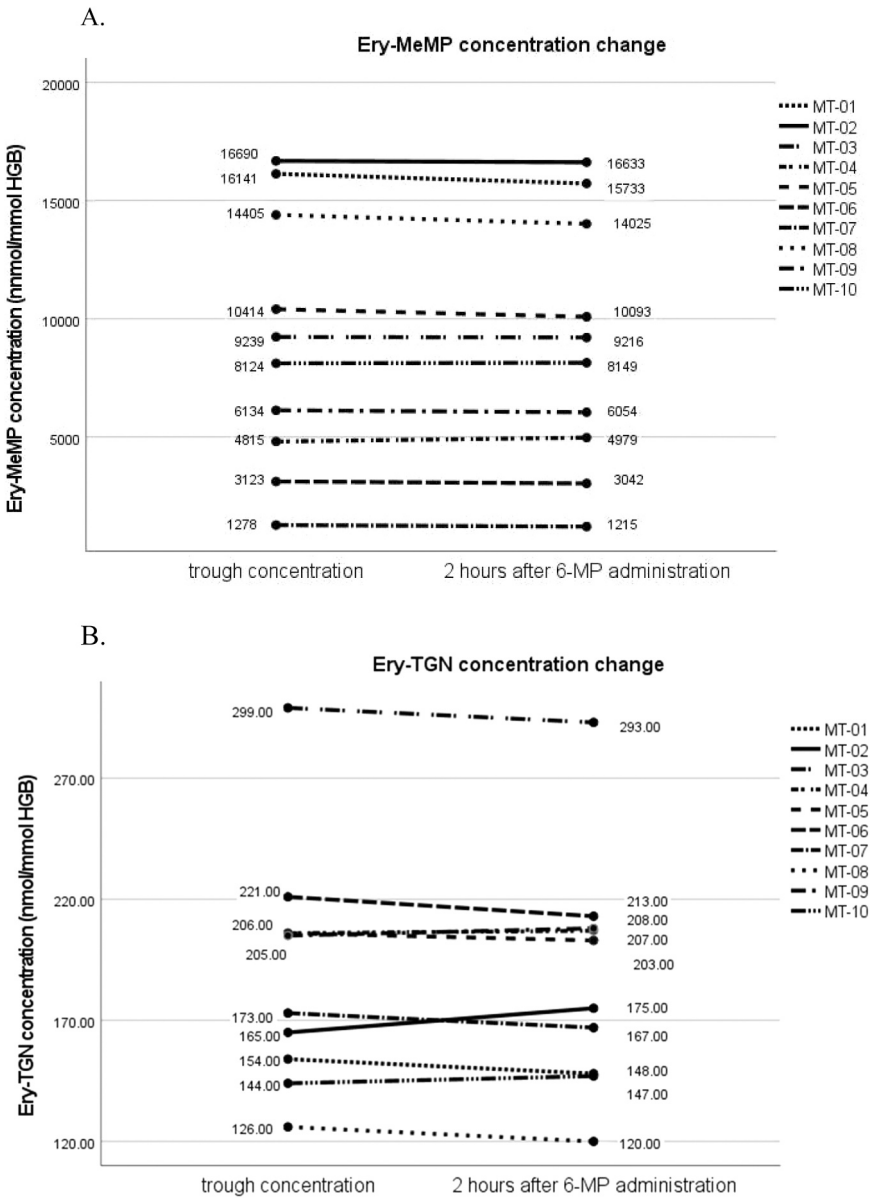

Fig. 1. Ery-MeMP (**A**) and ery-TGN (**B**) concentrations at trough levels and two hours after 6-MP administration.

study. Stable daily 6-MP dose varied from 30 mg/m^2 to 72 mg/m^2 (median 44 mg/m^2) (Table 1). Prior to inclusion into the study, all patients were taking 6-MP in the evening. All patients had *TPMT* and *NUDT15* wild type genotypes.

The median (min–max) trough ery-TGN was 173 (range 126–299) nmol/mmol HGB, and 2 h after 6-MP administration 175 (120−293) nmol/mmol HGB. For ery-MeMP, the median trough concentration and 2 h-concentration were 9 239 (range 1 278–19 645) nmol/mmol HGB and 9 216 (range 1 215–19 519) nmol/mmol HGB, respectively.

The absolute difference between the two time points varied from 1 to 10 (median 6) nmol/mmol HGB for ery-TGN and from 24 to 408 (median 81) nmol/mmol HGB for ery-MeMP, respectively. The median of absolute percentual change between the two time points was 2.9% (range 0.4–6.2%) for ery-TGN and 2.6% (range 0.3–5.0%) for ery-MeMP (Fig. 1). CV ranged 0.000–0.094 in duplicate analyses of ery-TGN in samples before 6-MP intake and 0.000–0.053 in samples 2 h after 6-MP intake. Whereas CV between the two time points ranged 0.002 – 0.042. For ery-MeMP, CV ranged 0.001 – 0.028 in duplicate samples before and 0.011 – 0.027 for the samples 2 h after 6-MP intake, whereas CV between the two time-points ranged from 0.002 to 0.036, respectively. The median absolute percentual change for both ery-TGN and ery-MeMP were not statistically different from 0, $p = 0.280$ and $p = 0.059$, respectively. Thus, ery-TGN and ery-MeMP levels were not affected by recent administration of 6-MP. There was no correlation of ery-MeMP or ery-TGN levels difference between the two time-points neither with patients age, sex, height, weight, body surface area or 6-MP dose ($p > 0.05$).

4. Discussion

We found no differences in intracellular levels of the 6-MP metabolites ery-MeMP and ery-TGN between trough levels and 2 h after administration, when the maximum 6-MP level in plasma is expected. Not surprisingly, large inter-individual variations in 6-MP metabolites were observed.

Different levels of intracellular metabolites are determined by variations in individual 6-MP pharmacokinetics, which subsequently lead to differences in efficacy and toxicity among the patients[15] [2]. Ery-MeMP and ery-TGN levels can elucidate the balance between intracellular pathways in individual patients, representing

pharmacogenetic variance in e.g., *TPMT*. However, currently the most applicable clinical use of intracellular 6-MP metabolites is their ability to reveal potential non-adherence to MT.

Adherence to treatment is crucial during the entire 2-year ALL treatment including the prolonged MT phase which requires daily self-controlled oral 6-MP intake. Low intensity of 6-MP exposure increases relapse risk 2.5–3.9-fold [8,16–18]. In children with ALL, non-adherence rates (defined as taking <95% of doses) has been assessed as high as 40–50%, explaining almost half of relapses [19,20]. Differences in adherence may partly explain differences in relapse rates between different ethnic groups, as well as in adolescents and young adults compared to smaller children [7,11,16–18].

WBC or neutrophil counts are generally used for 6-MP dose adjustment as surrogate markers for MT efficacy and toxicity in contemporary ALL treatment protocols and have been shown to be associated with relapse risk [21]. However, due to variations caused by endogenous (age, gender, circadian rhythm) and exogenous (e.g., infections, concomitant medications) factors, blood counts are not reliable treatment adherence markers. Increments of 6-MP dose induce hepatotoxicity and the raise in liver transaminases is associated with increased ery-MeMP [22]. Thus, in cases where no increment in transaminases occur upon a 6-MP dose increment, non-adherence should be considered [9,23]. However, hepatotoxicity may be induced by other chemotherapy or infectious agents, and is therefore not a reliable adherence measure.

Currently, electronic Medication Event Monitoring System (MEMS) which captures opening of the bottle, is considered the gold standard for measuring medication adherence. However, opening of the bottle does not necessarily mean drug ingestion [9]. In this context, measuring the levels of 6-MP metabolites could be a feasible alternative. Measuring of 6-MP metabolites can be a consistent direct method for adherence assessment and could serve as more specific parameter than neutrophil count or liver transaminases. Compared to DNA-TG, which concentration has been shown to be associated with relapse risk, however, fluctuates with neutrophil counts, Ery-TGN and ery-MeMP, due to their stability and shorter half-life, would be more useful for monitoring treatment adherence.

This study demonstrated the independence of ery-TGN and ery-MeMP levels from recent 6-MP ingestion, hence the timing of 6-MP intake does not need to be considered when taking blood samples for ery-TGN or ery-MeMP analysis. However, larger prospective studies with more samples within 24 h would be useful to characterize 6-MP metabolites pharmacokinetics more precisely.

5. Conclusions

Intracellular 6-MP metabolites, ery-MeMP and ery-TGN, remain stable 2 h after the intake of oral 6-MP in patients with a stable 6-MP dose. This finding is of clinical importance, as it underlines that blood samples may be used to assess patient adherence irrespective of the timing of 6-MP intake.

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CRediT authorship contribution statement

Linea Natalie Toksvang and **Kjeld Schmiegelow**: Conceptualization, Methodology, Supervision, Writing – review & editing; **Goda Elizabeta Vaitkeviciene**: Investigation, Data Curation, Formal analysis, Writing – original draft, Supervision, Writing – review & editing; **Lauryna Aukstikalne**: Investigation, Data Curation, Formal analysis, Writing – original draft; **Ignas Klejus**: Investigation, Data curation; **Maria Thastrup**: responsible for metabolite analyses; **Tadas Zvirblis**: statistical analysis. All authors reviewed and agreed with the last version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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