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Differential effects of thiopurine methyltransferase (*TPMT*) and multidrug resistance-associated protein gene 4 (*MRP4*) on mercaptopurine toxicity

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Abstract

Purpose—Mercaptopurine plays a pivotal role in treatment of acute lymphoblastic leukemia (ALL) and autoimmune diseases, and inter-individual variability in mercaptopurine tolerance can influence treatment outcome. Thiopurine methyltransferase (TPMT) and multi-drug resistant Protein 4 4(MRP4) have both been associated with mercaptopurine toxicity in clinical studies, but their relative contributions remain unclear.

Methods—We studied the metabolism of and tolerance to mercaptopurine in murine knockout models of *Tpmt*, *Mrp4*, and both genes simultaneously.

Results—Upon mercaptopurine treatment, *Tpmt*^{−/−} *Mrp4*^{−/−} mice had the highest concentration of bone marrow thioguanine nucleotides (8.5 pmol/5 × 10⁶ cells, P = 7.8 × 10^{−4} compared with 2.7 pmol/5 × 10⁶ cells in wild-types), followed by those with *Mrp4* or *Tpmt* deficiency alone (6.1 and 4.3 pmol/5 × 10⁶ cells, respectively). *Mrp4*-deficient mice accumulated higher concentrations of methylmercaptopurine metabolites compared with wild-type (76.5 vs 23.2 pmol/5 × 10⁶ cells, P = 0.027). Mice exposed to a clinically relevant mercaptopurine dosing regimen displayed differences in toxicity and survival among the genotypes. The double knock-out of both genes experienced greater toxicity and shorter survival compared to the single knockout of either *Tpmt* (P = 1.7 × 10^{−6}) or *Mrp4* (P = 7.4 × 10^{−10}).

Conclusions—We showed that both *Tpmt* and *Mrp4* influence mercaptopurine disposition and toxicity.

Keywords

Mercaptopurine; Thiopurine methyltransferase; *Mrp4*; ABCC4; adverse effects

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Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Introduction

Mercaptopurine is widely used as an anticancer agent and immunosuppressant in the treatment of leukemia and autoimmune diseases. Dose-limiting toxicities of mercaptopurine, including hematopoietic, hepatic and gastrointestinal (GI) toxicities, were associated with treatment interruptions[1] and inferior outcomes in some studies[2–5]. Genetic variation in both *TPMT* and *MRP4* have been shown to alter the metabolism and disposition of mercaptopurine. To investigate the impact of deficiency of these two genes on the disposition and toxicity of mercaptopurine *in vivo*, we developed murine models with combinations of *Tpmt* and *Mrp4* deficiency.

The relationship between mercaptopurine toxicity and thiopurine methyltransferase (TPMT) has been extensively characterized.[6,1] Erythrocyte TPMT activity is inversely related to the concentration of the active metabolites, thioguanine nucleotides (TGN), after mercaptopurine administration.[7] Heterozygotes who carry one copy of a non-functional *TPMT* allele have intermediate TPMT activity and moderate toxicity to thiopurines, and the rare homozygous deficient individuals may develop severe thiopurine-induced myelosuppression.[6] Therefore, *TPMT*-guided dosage individualization of thiopurines is recommended.[8,6]

We have previously established a *Tpmt*-deficient murine model that recapitulates clinical features of the human *TPMT* no-function variants.[9] Recently, using genome-wide approaches, we confirmed that TPMT activity is a monogenic trait determined by *TPMT* genetic polymorphisms.[10] Based on the clinical genotype of *TPMT* (presence or absence of *2, *3A and *3C variant alleles), we were able to predict TPMT activity with >90% sensitivity and specificity; however, the tolerability of mercaptopurine could not be fully predicted using *TPMT* genetic tests alone.[10] Other studies [11–14] also reported inter-individual variations in mercaptopurine toxicity that were not fully explained by TPMT phenotypes and genotypes.

MRP4 (Multi-resistant Protein 4), also designated ABCC4 (ATP-Binding Cassette, Sub-Family C, Member 4), is an efflux transporter of nucleoside monophosphate analogs, including all major thiopurine metabolites.[15,16] Overexpression of MRP4 in cells conferred resistance to mercaptopurine and thioguanine.[17,15,18] A murine model with *Mrp4* deficiency confirmed the protective effect of MRP4 against mercaptopurine hematopoietic toxicity by reducing intracellular TGN accumulation.[19,20] Polymorphisms in *MRP4* have been related to increased thiopurine toxicity in patients with ALL[21] and patients with inflammatory bowel disease (IBD).[22] However, the magnitude of the effect of *MRP4* deficiency (or heterozygosity) on thiopurine toxicity relative to *TPMT* deficiency is not well defined.

Methods

Animals

The generation of *Tpmt* knockout mice and *Mrp4* knockout mice were previously described.[9,23] Both strains were backcrossed into 129X1/SvJ for five generations. Wild-type,

Tpmt^{-/-} *Mrp4*^{+/+} (*Tpmt*^{KO}), *Tpmt*^{+/+} *Mrp4*^{-/-} (*Mrp4*^{KO}), *Tpmt*^{-/-} *Mrp4*^{-/-} (double knockout, DKO) and *Tpmt*^{+/-} *Mrp4*^{+/-} (double heterozygotes, DHET) were used in this study. Mice of all genotypes are indistinguishable by appearance, reproductive capacity, and histology of heart, lungs, liver, kidney, adrenal glands, thymus, spleen, lymph nodes, stomach, small and large intestine, pancreas, adrenals, reproductive organs, brain and bone marrow. All animal experiments were approved by the Institutional Animal Care and Use Committee of St. Jude Children's Research Hospital.

Chemicals

Mercaptopurine monohydrate was obtained from Acros Organics (Morris Plains, NJ). Thioguanine nucleotides (TGN), mercaptopurine riboside, methylmercaptopurine, methylmercaptopurine riboside and tetracycline were purchased from Sigma (St. Louis, MO). All chemicals and reagents were of high-performance liquid chromatography grade. A folic acid-deficient purified diet for mice was obtained from TestDiet (Richmond, IN). Methotrexate and sulfamethoxazole/trimethoprim oral suspension were purchased from Alparma (Fort Lee, NJ).

Animal treatment and sample collection

Mice of both genders, aged 8 to 12 weeks, were used in the study. To mimic the antifolate therapy given to children with leukemia, mice were placed on a folate-deficient diet (containing less than 0.05 ppm residual folic acid). Tetracycline (1 g in 1 L drinking water, 7 days a week) and sulfamethoxazole/trimethoprim (600 mg sulfamethoxazole and 120 mg trimethoprim in 1 L drinking water, 3 days a week) were used to prevent infections.

To compare pharmacokinetics of mercaptopurine in different genotypes, 4 males and 4 females of each genotype were given a single intraperitoneal injection of mercaptopurine at 80 mg/kg. Four hours after administration, bone marrow cells were collected and the intracellular level of thiopurine nucleotides was measured by high-pressure liquid chromatography as previously described.[24,25]

To evaluate the chronic toxicity of mercaptopurine, 10 male and 10 female mice (or 5 males and 5 females as indicated) of each genotype were given mercaptopurine (10 mg/L or 2.5 mg/kg/day, given the estimated water intake) continuously in their drinking water for 6 months. Methotrexate was also administered i.p. at 1 mg/kg/wk to mimic the exposure conditions of combination drug therapy.[26] Mice were sacrificed when they completed the 6-month treatment or became moribund. Blood was collected into tubes with anticoagulant, and all organs were dissected and fixed in 10% formalin, embedded in paraffin, sectioned (5 μ m) and stained with hematoxylin and eosin (H&E) for screening of any pathologic changes associated with mercaptopurine treatment.

Statistical analysis

Differences in pharmacokinetic data between two genotypes were compared using the Wilcoxon rank-sum test. The Kaplan-Meier method and the log-rank test were used to compare the differences in survival. $P < 0.05$ was considered statistically significant.

Results

Mercaptopurine pharmacokinetics differed by genotypes

To assess TPMT and MRP4 impact on bone marrow accumulation of mercaptopurine derived nucleotides, mice received a single intraperitoneal injection of mercaptopurine (80 mg/kg), and metabolite levels were determined in bone marrow cells from *Tpmt*^{KO}, *Mrp4*^{KO} and DKO mice. The median TGN levels in mice wild-type for both genes (2.7 pmol/5 × 10⁶ cells) were lower than *Tpmt*^{KO} mice (4.3 pmol/5 × 10⁶ cells, *P* = 0.021), *Mrp4*^{KO} (6.1 pmol/5 × 10⁶ cells, *P* = 0.011) and DKO mice (8.5 pmol/5 × 10⁶ cells, *P* = 7.8 × 10⁻⁴; (by an average of 37%, 56% and 68% respectively), Figure 1A). As expected, the methylated metabolites were not detected in *Tpmt*^{KO} and DKO mice. *Mrp4*^{KO} mice had over 3-fold higher median MMPN levels (76.5 pmol/5 × 10⁶ cells) than *Mrp4* wild-type (23.2 pmol/5 × 10⁶ cells, *P* = 0.027; Figure 1B), indicating greater intracellular accumulation of methylated mercaptopurine metabolites in mice lacking *Mrp4*. Moreover, DKO, *Mrp4*^{KO} and DHET mice had higher intracellular concentrations of mercaptopurine (median = 12.5, 7.5 and 4.8 pmol/5 × 10⁶ cells; Figure 1C) compared with wild-type mice (median = 0.6 pmol/5 × 10⁶ cells; *P* = 0.004, 0.047 and 0.048, respectively). The DHETs also tended to have slightly higher median TGN (3.3 pmol/5 × 10⁶ cells) and MMPN levels (35.3 pmol/5 × 10⁶ cells) than wild-types, although the differences were not statistically significant.

Tpmt provided superior protection against chronic oral mercaptopurine toxicity than *Mrp4*

To mimic clinical regimens used to treat ALL, we next assessed the effect of chronic orally administered mercaptopurine, when combined with a single weekly dose of methotrexate. We provided wild-type, *Tpmt*^{KO}, *Mrp4*^{KO}, DKO and DHET mice with mercaptopurine in their drinking water (equivalent to 2.5 mg/kg/d) and weekly methotrexate injections (1 mg/kg/wk i.p.). All of the wild-type and 80% of the DHET mice survived for the 6-month treatment (Figure 2). The median duration of survival of the DKO mice was only 17 days, followed by that of *Tpmt*^{KO} (56 days; *P* = 7.4 × 10⁻¹⁰), which was shorter than that of *Mrp4*^{KO} mice (111 days; *P* = 1.7 × 10⁻⁶), suggesting a greater protective effect of *Tpmt* compared to *Mrp4* against chronic orally administered mercaptopurine. In accordance with this, *Tpmt*^{KO} mice had an average of 23% lower leukocyte counts (*P* = 0.009), 86% lower neutrophils (*P* = 4.1 × 10⁻⁴), 77% lower platelets (*P* = 4.0 × 10⁻⁵) and 75% lower erythrocyte counts (*P* = 8.7 × 10⁻⁵) than *Mrp4*^{KO} at the end of treatment (Figure 3). The survival did not differ by sex (Figure S1).

After chronic mercaptopurine, we performed necropsies on all mice regardless of whether they succumbed to toxicity or completed the 6-month treatment. Severe bone marrow hypocellularity was observed in DKO mice after only 18 days of treatment (Figure 4A), while the *Tpmt*^{KO} or *Mrp4*^{KO} mice exhibited myelosuppression after 6 weeks and 9 weeks, respectively (Figure 4B–C). Wild-type mice had only mild to moderate myelosuppression at the end of 6-months of treatment (Figure 4D). Moreover, 4/4 (100%) DKO, 1/9 (11%) *Tpmt*^{KO} and 1/9 (11%) *Mrp4*^{KO} mice had spontaneous CNS hemorrhage (related to severe thrombocytopenia) (Figure S2).

GI toxicity that resembled mercaptopurine-induced GI toxicity in patients was also observed (Figure S3). Gastric epithelial hyperplasia, hyperkeratosis and/or ulceration were found in 4 of 9 *Tpmt*^{KO} mice that received at least 30 days of treatment. This was not observed in either *Mrp4*^{KO} (n = 9) or wild-type mice (n = 7) that received more than 30 days of treatment. The short survival (< 20 days) of DKO mice prevented the evaluation of GI toxicity; however, an exception was one DKO mouse that had a gastric submucosal hemorrhage after 18 days of treatment.

Renal tubular degeneration was observed in mice of all genotypes (12/30, 40%) at the time of sacrifice (Figure S4). Hepatocellular lipidosis was common in mice that received more than 2 months of treatment (Figure S5), including 3 of 9 (33%) *Tpmt*^{KO} (average duration 77 days), 7 of 9 (78%) *Mrp4*^{KO} (average duration 100 days) and 3 of 7 (43%) wild-type mice (average duration 6 months).

Discussion

Mercaptopurine toxicity has been associated with genetic factors, including polymorphisms in *TPMT* and *MRP4*. [22,1] Deficiency in *TPMT* results in excessive intracellular accumulation of TGN and severe myelosuppression. [7] Approximately 10% of patients carry one non-functional *TPMT* allele, and those who inherit homozygous deficiency in *TPMT* universally experience severe hematopoietic toxicity with conventional mercaptopurine doses. [1,27] In addition to *TPMT*, polymorphisms in *MRP4* have also been associated with TGN accumulation and hematopoietic toxicity in patients. [22,21] The frequency of damaging *MRP4* variants G2269A, C912A and G559T is higher in the Japanese population compared to people of European ancestry, occurring in 19%, 30% and 14%, respectively [19]. Given the high prevalence of variants in these two genes, it is important to know if *TPMT* and *MRP4* act independently or cooperate in protecting against mercaptopurine toxicity.

In the current study, we established preclinical mouse models that investigated how combinations of *Tpmt* and *Mrp4* deficiency impact the type and extent of mercaptopurine toxicity. As expected, both *Tpmt* and *Mrp4* protect against mercaptopurine-induced toxicities. However, the accumulation of cytotoxic TGN shows that each gene acts independently, with the absence of both *TPMT* and *MRP4* producing the greatest intracellular accumulation of TGN. Accordingly, mice lacking both *Tpmt* and *Mrp4* had significantly shorter durations of survival and markedly lower blood counts, indicative of greater hematopoietic toxicity than mice lacking either *Tpmt* or *Mrp4*. Notably, mice that were compound heterozygotes of *Tpmt* and *Mrp4* were more susceptible to mercaptopurine than wild-type mice, indicating one functional allele of each gene is not sufficient to fully protect from mercaptopurine toxicity, consistent with greater TGN accumulation in bone marrow cells of these mice.

A clinically relevant oral administration schedule of mercaptopurine revealed that mice with *Tpmt* deficiency were more susceptible to toxicity than mice that lacked *Mrp4* (Figure 2–4). This observation is consistent with clinical findings that a homozygous defect of *TPMT* has a more dramatic effect than that of *MRP4* on the hematopoietic toxicity after chronic

thiopurine treatment; patients who are homozygous for any *TPMT* damaging variants (*2, *3A or *3C) only tolerate ~10% of the conventional dose of mercaptopurine, [27] while those who are homozygous for any *MRP4* damaging variants (G2269A, C912A and G559T) tolerated about 75% of the conventional dose.[21] This was surprising given *Mrp4* deficiency resulted in greater accumulation of both cytotoxic TGN and MMPN in bone marrow cells of mice receiving parenteral mercaptopurine (Figure 1). One possible explanation is that mercaptopurine was administered orally and *Mrp4* is known to facilitate the oral absorption of its substrates. For example, previous studies have shown that the *Mrp4* substrate, dasatinib, exhibits poor oral absorption in the absence of *Mrp4*, due to its high expression in the stomach.[28] We postulate that decreased mercaptopurine absorption from the stomach of *Mrp4* knockout mice could account for relatively greater tolerance in *Mrp4*^{KO} mice compared to *TPMT*^{KO} mice.

Mercaptopurine is also commonly associated with GI toxicity which can be severe, preventing patients from normal food intake and may have an adverse impact on the therapy. [26] Studies have reported that polymorphisms in *TPMT* and reduced *TPMT* activity predisposed patients to thiopurine-induced GI toxicity.[11,29] In the current study we observed GI toxicity in mice after at least 30 days of oral exposure to low-dose mercaptopurine, and GI toxicity was more severe in mice with *Tpmt* deficiency compared with *Tpmt*^{+/+} mice (Figure S3).

In summary, we showed that *Tpmt* and *Mrp4* both contribute to protecting from mercaptopurine toxicity, with a greater protective effect from *Tpmt* than *Mrp4* following the low-dose chronic oral exposure that is used to in the clinic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Effect of *Tpmt* and *Mrp4* genotype on mercaptopurine nucleotide accumulation in bone marrow

Mice with wild-type (+/+), heterozygous (+/-) or homozygous (-/-) deficiency of *Tpmt* and/or *Mrp4* genes (n = 8 for each genotype) were given a single administration of mercaptopurine (80 mg/kg). At 4 hours after injection, bone marrow concentration of (A) TGN, (B) MMPN and (C) mercaptopurine (MP) were measured.

Figure 2. Effect of *Tpmt* and *Mrp4* genotype on survival with chronic mercaptopurine treatment Mice with wild-type (+/+), heterozygous (+/-) or homozygous (-/-) deficiency for *Tpmt* and/or *Mrp4* received mercaptopurine from the drinking water (10 mg/L) and weekly methotrexate i.p. at 1 mg/kg. Numbers of mice of each genotype are shown. *log-rank $P < 0.01$.

Figure 3. Hematologic toxicity is impacted by *Tpm1* and *Mrp4*

Mice with wild-type (+/+), heterozygous (+/-) or homozygous (-/-) deficiency for *Tpm1* and *Mrp4* genes were given mercaptopurine (10 mg/L) in the drinking water and weekly injections of methotrexate. Blood was collected at the end of 180-day treatment or at the time of sacrifice due to toxicity, and the median treatment duration (days) of each genotype are shown. Empty circles represent outliers, and asterisks (*) indicate significant difference ($P < 0.05$) compared with wild-types.

Figure 4. Bone marrow suppression of mice with chronic exposure to mercaptopurine
H&E staining of (A) markedly hypocellular marrow from a DKO mouse at day 18; (B) severely hypocellular marrow from a *Tpm1^{-/-}* mouse at week 6; (C) moderately hypocellular marrow from an *Mrp4^{-/-}* mouse at week 9; (D) mildly hypocellular marrow from a wild-type mouse. Magnification: 40x. Mice received mercaptopurine from drinking water (10 mg/L) and weekly methotrexate i.p. at 1 mg/kg.