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Mechanisms of *NT5C2*-mediated thiopurine resistance in acute lymphoblastic leukemia

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Abstract

Relapse remains a formidable challenge for acute lymphoblastic leukemia (ALL). Recently, recurrent mutations in *NT5C2* were identified as a common genomic lesion unique in relapsed ALL and were linked to acquired thiopurine resistance. However, molecular mechanisms by which *NT5C2* regulates thiopurine cytotoxicity were incompletely understood. To this end, we sought to comprehensively characterize the biochemical and cellular effects of *NT5C2* mutations. Compared to wildtype *NT5C2*, mutant proteins showed elevated 5'-nucleotidase activity with a stark preference of thiopurine metabolites over endogenous purine nucleotides, suggesting neomorphic effects specific to thiopurine metabolism. Expression of mutant *NT5C2* mutations also

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The authors have declared that no conflict of interest exists.

significantly reduced thiopurine uptake *in vitro* with concomitant increase in efflux of MP metabolites, plausibly via indirect effects on drug transporter pathways. Finally, intracellular metabolomic profiling revealed significant shifts in nucleotide homeostasis induced by mutant *NT5C2* at baseline; MP treatment also resulted in global changes in metabolomic profiles with completely divergent effects in cells with mutant vs wildtype *NT5C2*. Collectively, our data indicated that *NT5C2* mutations alter thiopurine metabolism and cellular disposition, but also influenced endogenous nucleotide homeostasis and thiopurine-induced metabolomic response. These complex mechanisms contributed to *NT5C2*-mediated drug resistance in ALL and pointed to potential opportunities for therapeutic targeting in relapsed ALL.

Introduction

In spite of risk-adapted combination chemotherapy, a substantial proportion of children with acute lymphoblastic leukemia (ALL) still experience relapsed disease(1–3). In fact, relapsed ALL is a leading cause of cancer-related death in children with a dismal survival rate of ~30%, even with aggressive salvage therapies including allogeneic stem cell transplantation(4–6). Recent large-scale genomic studies elegantly described clonal evolution of ALL from diagnosis to relapse(7–9) and identified recurrent genomic alterations specifically enriched in relapsed ALL (e.g., *IKZF1*(7), *TP53*(10), *CREBBP*(11), *SETD2*(12), *NT5C2*(13, 14), *PRPS1*(15)), some of which directly confer resistance to commonly used anti-leukemic agents. For example, genetic alterations in *CREBBP* and *IKZF1* are linked to glucocorticoid resistance(11, 16, 17), and mutations in *NT5C2* and *PRPS1* negatively influence thiopurine efficacy(13–15). These findings pointed to potential therapeutic targets in relapsed ALL and novel treatment strategies that may improve the outcome of this group of patients.

Somatic mutations in the *NT5C2* gene are arguably one of the most frequent genomic aberrations in relapsed ALL (~20–30% of cases) and in fact exclusively occur at relapse(18). As a 5'-nucleotidase, *NT5C2* is important for cellular nucleotide homeostasis by regulating the level of inosine monophosphate (IMP)(19). Results from us and others indicated that *NT5C2* mutations identified in relapsed ALL resulted in increased 5'-nucleotidase activity(13, 14, 20) and specifically reduced cytotoxic effects of thiopurines (e.g., 6-mercaptopurine [MP] and 6-thioguanine [TG]) in ALL cells. While thiopurines are indispensable for the cure of ALL in children and adults, prolonged daily exposure to MP during maintenance therapy may also impose a strong selection pressure for the outgrowth of *NT5C2*-mutated leukemia clones. In fact, patients carrying *NT5C2* mutations almost always experience early relapse and in some cases even during thiopurine therapy(13). Better understanding of the molecular and biochemical mechanisms by which *NT5C2* mutations alter thiopurine efficacy is much needed for developing therapeutic strategies to overcome resistance to this important class of anti-leukemic drugs.

In this study, we comprehensively examined the effects of *NT5C2* mutations on its enzymatic properties, and thiopurine metabolism, and drug disposition, and also explored the metabolomic basis of *NT5C2*-mediated thiopurine resistance in ALL.

Materials and Methods

NT5C2 recombinant protein production and purification

C-terminal His₆-tagged full-length cDNA for wildtype *NT5C2* was cloned into the pET30a expression vector using NdeI and HindIII restriction sites. Point mutations, p.Arg39Gln, p.Arg238Trp, and p.Arg367Gln, (hereafter referred to as p.R39Q, p.R238W, and p.R367Q) were introduced by using the QuicChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). BL21 DE3 competent *E.coli* (New England Biolabs.) was transfected with various pET30a expression vectors, and *NT5C2* expression was induced by 0.5mM isopropyl β-D-1-thiogalactopyranoside for 15 hours at 18°C. Cell pellets were collected at 4,000rpm for 30min at 4°C, and were re-suspended in ice-cold lysis buffer (50 mM HEPES, pH 7.8, 500 mM NaCl, 10% glycerol, 1 mM TCEP, 1mM AEBSF, with 1x EDTA-free protease inhibitors (Roche)). Bacteria were lysed by sonication, and lysates were harvested by centrifugation at 14,000rpm for 40min. The supernatant was loaded to Ni-NTA beads (Qiagen), and the beads were washed with lysis buffer containing 40mM imidazole. NT5C2 proteins were eluted with lysis buffer containing 200mM imidazole, and then further purified by gel filtration (HiPrep 16/60 Sephacryl S300 HR column from GE; buffer condition: 50 mM sodium phosphate, pH 7.4, 100 mM NaCl, 10% glycerol and 0.5 mM TCEP). Protein expression and purity was assessed by SDA-PAGE and Coomassie blue staining (Supplementary Figure 1). Formation of the NT5C2 tetramer (R39Q, R238W, and R367Q) was confirmed by gel filtration (Supplementary Figure 2).

5'-nucleotidase assay

5'-nucleotidase activity was measured by quantifying the release of phosphate from IMP (Sigma), GMP (Santa Cruz Biotechnology), TIMP (Toronto Research Chemicals) or TGMP (Jena Bioscience), using the SensoLyte® Malachite Green Assay kit (AnaSpec) in assay buffer (MgCl₂ 2 mM, NaCl 120 mM, KCl 5 mM, Glucose 10 mM, HEPES 20 mM). NT5C2 protein (300ng) was incubated with 40 μM IMP, GMP, TIMP or TGMP at 37°C for 30min. For determination of enzymatic kinetic parameters such as V_{max} and K_m values, reaction condition was optimized to achieve catalytic saturation for mutant or wildtype NT5C2 with each substrate, based on which to ensure robust measurement and specificity. Thus, NT5C2 protein (1 μg, 1 μg, 200 ng and 500 ng for IMP, TIMP, GMP, and TGMP, respectively) was incubated with increasing concentrations of substrates: 41.0–2625 μM (IMP), 268.9–3062.5 μM (GMP) and 27.3–1750 (TIMP or TGMP). The reaction was performed at 37°C for 10min (IMP) or 5min (GMP, TIMP and TGMP). Experiments were assessed in more than duplicates.

NT5C2 overexpression and MP cytotoxicity *in vitro*

N-terminal Flag-tagged cDNA for wildtype and mutant *NT5C2* (R39Q, R238W, and R367Q) was cloned into the lentiviral vector pLenti using SalI and XbaI restriction sites. Viral particles were prepared by using the calcium chloride method in HEK293T cells. Supernatant was harvested 48 hr later and used to transduce human ALL cell lines Nalm6 and REH. The cells were grown in RPMI1640 media supplemented with 10% FBS, 10 mM HEPES and 1mg/ml Geneticin under 5% CO₂ at 37 °C. After geneticin selection, NT5C2 overexpression was confirmed by immunoblotting with GAPDH as the loading control (anti-

NT5C2 antibody [Abcam; 1:1,000 dilution]; anti-Flag antibody [Cell Signaling Technology, 1:3,000 dilution]).

ALL cells overexpressing wildtype or mutant *NT5C2* were treated with thiopurines (MP or TG) at increasing concentrations for 72 hr incubation, and cell viability was then assessed using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

HEK293T or Nalm6 and REH cells were obtained from the American Type Culture Collection or the German Collection of Microorganisms and Cell Cultures in 2012, respectively. Cells were regularly tested for mycoplasma contamination by using MycoAlert Mycoplasma Detection Kit (Lonza # LT07–118). All cell line-based experiments were completed within two weeks after thawing.

MP influx and efflux assay *in vitro*

Drug influx and efflux experiments were performed according to previous published methods (21–23) with slight modification. To characterize combined function of influx and efflux transporter activities (i.e., net accumulation), 1.0×10^6 cells were incubated at 37°C in DMEM containing 10% dialyzed FBS and l-glutamine (i.e., complete medium) with 10 μ M of [14 C] MP (51 mCi/mmol. MC-1447, Moravek Biochemicals) for 2, 6, or 24 hours. After drug treatment, cells were collected by centrifugation and washed twice with ice-cold PBS, and were lysed with 150 μ l 0.5N NaOH. Lysates were neutralized by the equivalent amount of 0.5N HCl, and then radioactivity was measured by liquid scintillation counting. For analyses of drug influx, 1.0×10^6 cells were seeded in glucose-free, pyruvate-free DMEM containing 10% dialyzed FBS and 5 mM sodium azide (=energy depleted medium) to disrupt ATP-dependent efflux transporter activity, then cells were treated with 10 μ M of [14 C] MP for 2 and 6 hours. Intracellular radioactivity was quantified as described above. For drug efflux assay, cells were first loaded with 10 μ M of [14 C] MP in energy depleted medium for 2 hours. The cells were then washed twice with ice-cold PBS and incubated for additional 10, 30, 60 and 120 min in completed medium without the radiolabeled drug. Cells and medium were separately collected to measure radioactivity in each compartment. Radioactivity levels were normalized against intracellular protein (determined by BSA assay) for all experiments. Each experiment was performed in triplicates.

Targeted metabolomic analysis

To evaluate the impact of *NT5C2* mutations and MP treatment on endogenous metabolite homeostasis, Nalm6 and REH cell lines overexpressing wildtype or R238W *NT5C2* were incubated for 48 hours in the presence or absence of 10 μ M MP at 0.5×10^6 cells/ml. After incubation, cells were harvested by centrifugation, washed twice with ice-cold PBS, and flashed frozen in liquid nitrogen to quench cellular metabolism. Cell samples were kept at –80 °C until analysis. Cell samples for each condition were prepared in quintuplicate

Endogenous metabolites in cell samples were extracted as described previously with modifications(24). In brief, 1 mL of 80% methanol (v/v) (cooled to –80 °C) was added to each cell sample. The mixture was vortex-mixed, sonicated, and centrifuged (14,000 rpm, 4°C, 10 min). The supernatant was transferred to a new 2-mL microcentrifuge tube, and 0.8

mL 80% methanol (v/v) (cooled to -80°C) was added to the precipitate followed by vortex-mixing and centrifugation. Supernatant from two extractions was combined and dried in a CentriVap® Refrigerated Centrifugal Concentrator (Kansas City, MO) at 10°C . The residual was reconstituted in acetonitrile-water (50/50, v/v), and the supernatant was subjected to LC-MS/MS-based targeted metabolomic analyses that were based on 3 different chromatographic separation mechanisms to quantitate ~250 targeted metabolites.

Chromatographic separation of most organic acids and amino acids was achieved based on reversed-phase liquid chromatography on a Synergi Polar-RP column (80A, 2.0 mm x 150 mm, 4 μm) under a gradient elution consisted of mobile phase A (0.03% formic acid in water) and mobile phase B (0.03% formic acid in acetonitrile), at a flow rate of 0.25 mL/min. Separation of highly polar metabolites was achieved based on hydrophilic interaction liquid chromatography (HILIC) on an Atlantis HILIC Silica column (2.1 mm x 150 mm, 3 μm) under a gradient elution consisted of mobile phase A (10 mM ammonium formate, pH 3.0 and 0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile), at a flow rate of 0.25 mL/min. Chromatographic separation of oligonucleotides, sugar, and sugar phosphates was achieved based on ion-pair liquid chromatography on an Atlantis T3 column (2.1 mm x 100 mm, 3.0 μm) under a gradient elution consisted of mobile phase A (100 mM hexafluoroisopropanol, and 8.6 mM triethylammonium acetate in water, final pH, 8.3) and mobile phase B (10% acetonitrile in mobile phase A), at a flow rate of 0.5 mL/min. Column eluents were monitored under both positive and negative ionization modes using the multiple reaction monitoring (MRM) on an AB Sciex QTRAP 6500 mass spectrometer. Calibration standards of individual metabolites were prepared in the appropriate mobile phase at the concentration range of 0.001 to 10 μM . Quality control samples were prepared in the appropriate mobile phase at the concentrations of 100 nM. Since concentrations of individual metabolites in cell samples varied significantly, both original samples and 50-fold diluted samples were processed and subjected to LC-MS/MS analyses. The concentrations of individual metabolites in a cell sample were normalized to cellular protein amount (expressed as $\mu\text{mol}/\text{mg}$ protein).

Statistics

5'-nucleotidase activity with a fixed substrate concentration, MP influx and efflux was compared between wildtype and mutants using the student's t-test. Enzymatic parameters (i.e., V_{max} and K_m) were estimated using Hill equation with degree of cooperativity fixed as two. Analysis of the metabolomics data was performed using the MetaboAnalyst web-based statistical package (<http://www.metaboanalyst.ca/>). To create a normal or Gaussian distribution, data normalization of metabolite concentrations was performed by log-transformation and auto-scaling (mean-centered and divided by the standard deviation of each variable). Principal component analysis (PCA) was used to acquire clustering information and global metabolic differences among 4 different groups (i.e., wildtype control, R238W control, wildtype MP-treated, and R238W MP-treated). Student's t-test was performed for the comparison of metabolite changes between two groups. One-way analysis of variance (ANOVA) with Fisher's LSD as post-hoc analysis was performed for the comparison of metabolite changes among 4 groups. For individual metabolites in two groups

comparison, a false discovery rate (FDR) adjusted p-value < 0.05 was considered as significant difference.

Results

While there is a growing number of *NT5C2* mutations identified in relapsed ALL, we elected to focus on p.R39Q, p.R238W, and p.R367Q in mechanistic experiments in this study because they are the most common and representative of distinctive mutation hotspots. To investigate molecular mechanisms of thiopurine resistance, we first established a series of ALL cell lines (Nalm6 and REH) with stable overexpression of wildtype or mutant *NT5C2*. In both Nalm6 and REH, ectopic expression of mutant *NT5C2* consistently resulted in significant resistance to thiopurines, with the effects stronger for TG than MP with minimal variation amongst 3 mutations (Supplementary Figure 3). In contrast, cells over-expressing wildtype *NT5C2* remained highly sensitive to thiopurines indistinguishable from control cells transduced with the empty vector (Supplementary Figure 3). These results pointed to the possibility that mutant *NT5C2* is not merely a hyperactive form of the wildtype protein but instead might have acquired novel catalytic functions specifically related to thiopurine metabolism.

We first evaluated the enzymatic properties of mutant *NT5C2* against 4 purine-based monophosphate nucleotides (Figure 1): IMP and GMP (endogenous purine metabolites) and TIMP and TGMP (thiopurine metabolites). Wildtype *NT5C2* exhibited 5'-nucleotidase activity against all 4 nucleotides, but IMP and TIMP were clearly the preferred substrates with 4.6- and 1.4-fold higher production of phosphate compared to GMP and TGMP, respectively. All leukemia-derived *NT5C2* mutations resulted in substantial increase in 5'-nucleotidase activity against all 4 substrates, with the p.R367Q mutant consistently having the highest activity. Similarly, mutant *NT5C2* showed significant substrate preference of IMP over GMP (24.2 ± 1.6 vs. 6.5 ± 0.21 , 21.2 ± 1.3 vs. 3.8 ± 0.09 , and 26.7 ± 0.24 vs. 10.4 ± 0.42 pmol/min/300 ng protein for p.R39Q, p.R238W and p.R367Q with IMP vs. GMP as the substrate, respectively). However, this inosine over guanosine bias in substrate specificity was reversed with thiopurine metabolites: leukemia-derived mutant *NT5C2*s (except p.R238W) were substantially more efficient in dephosphorylating TGMP than TIMP (27.9 ± 0.38 vs. 34.4 ± 0.61 , 21.7 ± 0.37 vs. 16.9 ± 1.6 , and 27.8 ± 2.2 vs. 41.5 ± 0.23 pmol/min/300 ng protein for p.R39Q, p.R238W and p.R367Q with TIMP vs. TGMP as the substrate, respectively). Interestingly, we observed increased 5'-nucleotidase activity of mutant *NT5C2*s for mTIMP (Supplementary Figure 4A), and we also discovered gain of function of other relapse-related mutants against thiopurine metabolites (Supplementary 4B and 4C). These results suggest that *NT5C2* mutations confer gain of function specifically against thiopurine-related monophosphate nucleosides.

We also quantitatively characterized enzymatic kinetics parameters for wildtype and each of the mutant *NT5C2*s with 4 different substrates (IMP, GMP, TIMP or TGMP, Figure 2, Table 1). Measuring k_{cat} , a measurement of the efficiency at which substrate molecules are converted for each enzyme site, we observed that the mutant *NT5C2*s consistently had increased activity over wildtype proteins regardless of the substrate tested. The “mutant to wildtype” ratio of k_{cat} was significantly greater when thiopurine metabolites (TIMP and

TGMP, ratio ranging 3.6–11.8) were used as substrates than endogenous purine nucleotides (IMP and GMP, ratio ranging 2.0–3.6). These results again indicated that ALL relapse-associated *NT5C2* mutations likely confer not only an increase of endogenous 5'-nucleotidase activity, but more importantly, novel effects specific to thiopurine metabolites.

As described previously, ALL cell lines expressing mutant *NT5C2* showed a significant reduction in overall intracellular levels of thiopurine metabolites than cells expressing the wildtype gene(13), pointing to potential influence on thiopurine disposition (in addition to drug metabolism). Using [¹⁴C] MP, we traced both the influx and efflux of MP metabolites in isogenic Nalm6 and REH cells expressing wildtype or the mutant *NT5C2* (Figure 3A–F). First, we measured intracellular accumulation of [¹⁴C] labeled MP metabolites, i.e., the net result of drug influx and efflux (Figure 3A and 3D). In both cell lines, significant accumulation of radioactive metabolites was observed as early as 2 hours and increased rapidly over time when wildtype *NT5C2* was expressed, whereas MP metabolite levels remained largely unchanged in cells expressing mutant *NT5C2*. At 24 hrs, MP metabolite levels in Nalm6 cells harboring p.R39Q, p.R238W and p.R367Q *NT5C2* were $34.0 \pm 2.2\%$, $22.9 \pm 1.7\%$, and $58.8 \pm 5.9\%$ of that in cell expressing the wild-type protein, respectively (Figure 3A). Similarly, mutant REH cells showed less net accumulation than cells with wildtype *NT5C2*, and the difference in total metabolites level was most significant after 24 hour 6-MP treatment (59.7%, 72.1% and 64.0% reduction compared with wildtype cells for p.R39Q, p.R238W and p.R367Q, respectively. Figure 3D).

To determine if lower net accumulation was the result of perturbation of the influx or efflux functions, we first employed ATP-depleted medium to block efflux by ABC transporters (Figure 3B and 3E) and assess influx activity specifically. In both Nalm6 and REH cells, intracellular MP metabolites were significantly lower in mutant cells compared to wildtype and the differences grew wider with increasing time: 48.2%, 62.4% and 40.4% reduction of MP influx for p.R39Q, p.R238W and p.R367Q in Nalm6 cells; 39.8%, 50.8% and 36.3% reduction for in REH cells, respectively). Conversely, we quantified MP efflux activity by first pre-loading cells with radio-labeled MP and then measuring the appearance of MP metabolites in extracellular medium (Figure 3C and 3F). In Nalm6 cells, appreciable MP metabolite efflux was observed as early as 10 min and ~80% of the intracellular MP metabolites was excreted within 30 min. During the initial rapid efflux phase, mutant cells exhibited 3.3–4.7-fold greater efflux than control cells (Figure 3C). The same trend was also observed in REH cells (2.3-, 3.0-, and 2.1-fold efflux activity in p.R39Q, p.R238W and p.R367Q, respectively Figure 3F). Taken together, these results implied that alterations in the drug transporter pathway also contribute to *NT5C2*-mediated thiopurine resistance in addition to direct effects on thiopurine metabolism.

Given that *NT5C2* is a key enzyme in cellular nucleotide metabolism, we also performed metabolomic profiling to comprehensively identify the effects of *NT5C2* mutations on endogenous metabolite homeostasis. Analyzing 243 cellular metabolites involved in nucleotide, amino acid, and carbohydrate biosynthesis and degradation, we compared Nalm6 and REH cells expressing R238W mutant or wildtype *NT5C2* at baseline and also after MP treatment (Figure 4A–D). Principal component analysis identified 3 distinctive clusters from these 4 experimental groups in each cell line: wildtype and mutant *NT5C2* cells before MP

treatment grouped together with identifiable but modest differences in metabolite profiles (Figure 4A, 4B and 4C, and Supplementary Table 1); in contrast, MP treatment globally altered cellular metabolite profiles but had completely divergent effects in NT5C2 mutant vs wildtype cells (as represented by two distinctive clusters, Figure 4A, 4B and 4D and Supplementary Table 1), suggesting a metabolomic basis of NT5C2-mediated thiopurine resistance. At baseline, an example of differences between R238W and wildtype NT5C2 cells were intermediate metabolites in the purine salvage synthesis pathway (e.g., guanosine, $P = 0.002$ or 0.007 in Nalm6 or REH, respectively, Figure 4C, and Supplementary Table 1), with a partially significant increase in the purine nucleoside plausibly due to the increased 5'-nucleotidase activity (Supplementary Figure 5). Interestingly, there was also a significant increase in S-adenosylmethionine in mutant Nalm6 cells compared to wildtype at baseline (fold change 2.3, $P = 0.04$, Figure 4C and Supplementary Table 1), and the higher level of this methyl donor in turn would lead to increased conversion of MP to its inactive methylated MP, plausibly contributing to MP resistance. Exposure to MP dramatically disturbed nucleotide homeostasis (both *de novo* and salvage pathways of purine and pyrimidine biosynthesis), consistent with the known effects of MP as a purine analog. We also observed significant differences of individual metabolites and disturbed pathways between wildtype and R238W after MP treatment (Figure 4D and Supplementary Tables 1), which might contribute to negative alteration of MP sensitivity in mutant cells.

Discussion

Originally introduced in 1950's, thiopurines remain as the mainstay of ALL therapy in children and adults with a narrow therapeutic index and large inter-patient variability in response and toxicities(25–28). Thiopurines are prodrugs and require multiple enzymatic reactions to be converted to active metabolites (e.g., TGTP)(29). The mechanisms of action are thought to involve DNA-incorporation of thioguanine and subsequent DNA damage repair-triggered apoptosis(29–31). Genetic variations have been identified in a number of genes regulating different aspects of thiopurine metabolism and hence cytotoxic effects, e.g., germline polymorphisms in *TPMT* and *NUDT15*(27, 32–36), and somatic mutations in *NT5C2* and *PRPS1*(13–15). In fact, thiopurines are among the very few anti-leukemic agents for which drug sensitivity is strongly influenced by a small number of genetic factors with definitive and clinically actionable effects. In particular, *NT5C2* mutations are seen in ~20% of relapsed ALL and directly linked to thiopurine drug resistance and can emerge prior to clinical relapse(18). Because *NT5C2* mutations do not cause cross-resistance to other anti-leukemic agents, early detection of these mutations might warrant treatment intensification with non-antimetabolite based therapy to prevent the outgrowth of the MP-resistant leukemia clone and subsequent relapse. One may also postulate that regimens that rotate distinctive classes of chemotherapeutics during maintenance might be advantageous over prolonged exposure to anti-metabolite therapy alone to minimize the risk of developing thiopurine resistance(37). On the other hand, *NT5C2* mutations might also represent a meaningful therapeutic target for relapsed ALL given its specificity and prevalence in this group of patients. The gain-of-activity nature of the mutations also makes them attractive for developing small molecular inhibitors(20, 38, 39). The selective effects of *NT5C2* mutations on thiopurine metabolites (TIMP and TGMP) are important because inhibitors may be

developed to specifically block *NT5C2*-mediated thiopurine inactivation while sparing its activity against endogenous nucleotides (IMP and GMP). It was recently reported that there are several classes of *NT5C2* mutations and each class of mutants drives resistance to thiopurine in a different mechanism; majority of mutant *NT5C2* proteins (e.g., R39Q, R238W and R367Q) disrupts self-inactivation regulatory mechanisms and then increases nucleotidase activity. Other mutations such as K359Q maintain a constitutively-activated conformation and become independent of the allosteric regulation by ATP(20, 39). However, these mutations all lead to increased *NT5C2* activity and therefore similar downstream effects on thiopurine metabolism and other cellular process. Although it is well established that thiopurines can interfere with *de novo* purine synthesis(40), there is a paucity of data of the global metabolomic effects of thiopurines. Our targeted metabolomic profiling results indicated that MP treatment broadly alter nucleotide homeostasis (not only purine but pyrimidine nucleotides also) as well as amino acid biosynthesis which was not previously recognized. More importantly, we observed dramatic differences in the metabolomic response to MP in cells expressing wildtype vs mutant *NT5C2*, pointing to potentially novel mechanisms of thiopurine resistance. Interestingly, even at baseline, expression of mutant *NT5C2* resulted in significant shift in endogenous purine metabolism as described by other group(38). However, our metabolic profiles exhibited no depletion of purine nucleotides as opposed to the previous study (Supplementary Figure 5), which might be due to differences in studied types of cell lines or *NT5C2* mutants. Future studies are warranted to determine whether this confers vulnerability to other anti-metabolite agents and thus a potential therapeutic opportunity. It is also needed to elucidate the exact mechanisms how mutant *NT5C2* proteins influence transporter function, with which to explore transporter-related treatment approaches.

In this study, our results indicated that relapsed ALL-related *NT5C2* mutations selectively influence thiopurine metabolism, indirectly alter drug influx and efflux, and also globally affect metabolomic response to thiopurine therapy (Figure 5)(41, 42). These multiple mechanisms might be integrated to confer leukemic cells carrying *NT5C2* mutations with thiopurine resistance, and as a consequence, they may be subjected to weaker selection pressure for clonal evolution during maintenance therapy. Our results might point to potential opportunities for therapeutic targeting in relapsed ALL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviation list

ALL	acute lymphoblastic leukemia
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GMP	guanosine monophosphate
IMP	inosine monophosphate
MP	6-mercaptopurine
rMP	mercaptopurine ribose
rTG	thioguanosine ribose
TG	6-thioguanine
TGDP	thioguanosine diphosphates
TGMP	thioguanosine monophosphate
TGTP	thioguanosine triphosphates
TIMP	thioinosine monophosphate

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Figure 1. Mutant NT5C2 showed neomorphic effects with elevated 5'-nucleotidase activity preferentially against thiopurine metabolites.

Purified NT5C2s were incubated with endogenous nucleotides (IMP and GMP) or thiopurine metabolites (TIMP and TGMP) as substrates and 5'-nucleotidase activity was measured by quantifying the release of free phosphate using the Malechite Green assay. Abbreviations: GMP, guanosine monophosphate; IMP, inosine monophosphate; TGTP, thioguanosine monophosphate; TIMP, thioinosine monophosphate; Bars represent the mean values; the error bars represent the standard deviation from triplicate. Each experiment was repeated at least two times. * $P < 0.05$ (student's t-test)

Figure 2. Enzyme kinetics of mutant and wildtype *NT5C2* with endogenous or thiopurine 5'-nucleotides as substrates.

Initial reaction velocity (i.e., 5'-nucleotidase activity over reaction time) was measured at increasing substrate concentrations. The error bars represent the standard deviation from triplicate.

Figure 3. Effects mutant *NT5C2* on MP influx and efflux *in vitro*.

Isogenic ALL cell lines (Nalm6, top, and REH, bottom) ectopically expressing mutant or wildtype *NT5C2* were incubated with [14 C]-labeled MP to measure drug disposition, and radioactivity represented the sum of intact drug and MP metabolites. MP net accumulation (A, D) indicated intracellular drug concentration at the steady state, whereas influx activity (B, E) and efflux activity (C, F) reflect drug uptake and excretion, respectively. The bars represent the mean values; the error bars represent the standard deviation of triplicates. * $P < 0.05$ (student's t-test)

Figure 4. *NT5C2* mutation and global metabolomic profile in ALL.

Nalm6 and RHE cells expressing wildtype or mutant *NT5C2* were treated with PBS or MP (10 μ M) for 48 hours and cell lysates were subjected to metabolomic profiling (243 metabolites) using LC-MS. Principal component analysis identified 3 distinctive clusters (**A**): wildtype and mutant *NT5C2* cells before MP treatment grouped together with identifiable but modest differences in metabolite profiles (green and blue dots); in contrast, MP treatment globally altered cellular metabolite levels and had completely divergent effects on *NT5C2* mutant vs wildtype cells (red and purple dots). (**B**) Heatmaps from unsupervised clustering analysis identified metabolomic differences between ALL cells with wildtype vs mutant *NT5C2* at baseline (highlighted by red rectangle) and after MP treatment (highlighted by green rectangle). Top twenty five compounds with $P < 0.05$ in t-test are shown in **C** (at baseline) and **D** (after 6MP treatment).

Figure 5. NT5C2 mutations and proposed resistant mechanisms.

Relapsed ALL-related *NT5C2* mutations selectively influence thiopurine metabolism, indirectly alter drug influx and efflux, and also globally affect metabolomic response to thiopurine therapy. These multiple mechanisms are associated with thiopurine resistance and leukemic cells with *NT5C2* mutations might be less subject to selection pressure for clonal evolution during maintenance therapy. MP, 6-mercaptopurine; rMP, mercaptopurine ribose; rTG, thioguanosine ribose; TG, 6-thioguanine; TGDP, thioguanosine diphosphates; TGMP, thioguanosine monophosphate; TGTP, thioguanosine triphosphates; TIMP, thioinosine monophosphate.

Table 1.
5'-nucleotidase enzyme activity and kinetics parameters of mutant vs wildtype NT5C2.

$V_{\max}$ and K_m were estimated using Hill equation (R^2 represents the fitness of the model). Mutant proteins changed cooperativity of proteins for the substrates, and mutant proteins exhibited significant increase of k_{cat} compared with wt, especially with thiopurine metabolites as the substrates.

Substrate	Kinetic parameters	WT	R39Q	R238W	R367Q
IMP	$V_{\max}$ (pmol/min/ μg)	246 ± 15	512 ± 21	506 ± 28	522 ± 14
	K_m (μM)	599 ± 74	267 ± 27	380 ± 48	136 ± 10
	R^2	0.9072	0.9241	0.8895	0.9466
	k_{cat} (s^{-1})	0.27 ± 0.02	0.57 ± 0.02	0.56 ± 0.03	0.58 ± 0.02
	k_{cat} ratio (vs WT)	1	2.1	2.1	2.1
TIMP	$V_{\max}$ (pmol/min/ μg)	38.9 ± 1.5	269 ± 17	220.6 ± 7.4	460.6
	K_m (μM)	82.3 ± 9.2	106 ± 19	55.9 ± 6.0	111 ± 11
	R^2	0.9374	0.858	0.9121	0.9424
	k_{cat} (s^{-1})	0.04 ± 0.00	0.30 ± 0.02	0.24 ± 0.01	0.51 ± 0.00
	k_{cat} ratio (vs WT)	1	6.9	5.7	11.8
GMP	$V_{\max}$ (pmol/min/ μg)	412 ± 27	1478 ± 48	821 ± 24	1197 ± 58
	K_m (μM)	867 ± 100	1040 ± 54	1711 ± 62	893 ± 74
	R^2	0.8985	0.9858	0.9956	0.9589
	k_{cat} (s^{-1})	0.46 ± 0.03	1.6 ± 0.05	0.91 ± 0.03	1.3 ± 0.06
	k_{cat} ratio (vs WT)	1	3.6	2.0	2.9
TGMP	$V_{\max}$ (pmol/min/ μg)	353 ± 42	1252 ± 78	1364 ± 107	1848 ± 91
	K_m (μM)	624 ± 125	843 ± 76	607 ± 81	528 ± 47
	R^2	0.8902	0.9797	0.9504	0.9763
	k_{cat} (s^{-1})	0.39 ± 0.05	1.4 ± 0.09	1.5 ± 0.12	2.1 ± 0.10
	k_{cat} ratio (vs WT)	1	3.6	3.9	5.2