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Impact of *SLC43A3*/ENBT1 Expression and Function on 6-Mercaptopurine Transport and Cytotoxicity in Human Acute Lymphoblastic Leukemia Cells[S]

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ABSTRACT

6-Mercaptopurine (6-MP) is used extensively in the treatment of acute lymphoblastic leukemia (ALL) and inflammatory bowel diseases. Our laboratory determined previously, using a recombinant HEK293 cell model, that the *SLC43A3*-encoded equilibrative nucleobase transporter 1 (ENBT1) transports 6-MP into cells and significantly impacts the cytotoxicity of 6-MP in that model. To further investigate the clinical relevance of this finding, we now extend this work to an analysis of the impact of *SLC43A3*/ENBT1 expression and function on 6-MP uptake and cytotoxicity in leukemic lymphoblasts, the therapeutic target of 6-MP in ALL. A panel of ALL cell lines was assessed for *SLC43A3*/ENBT1 expression, ENBT1 function, and sensitivity to 6-MP. There was a significant difference in *SLC43A3* expression among the cell lines that positively correlated with the rate of ENBT1-mediated 6-MP uptake. Cells with the lowest expression of *SLC43A3* (SUP-B15: V_{max} = 22± 5 pmol/μl per second) were also significantly less sensitive to 6-MP–induced cytotoxicity than were the highest expressing cells (ALL-1: V_{max} = 69 ± 10 pmol/μl per second). Furthermore, knockdown of ENBT1 using short hairpin RNA interference (shRNAi) in RS4;11 cells caused a significant decrease in ENBT1-mediated 6-MP uptake (V_{max}: RS4;11 = 40 ± 4 pmol/μl per second; RS4;11 shRNAi = 26 ± 3 pmol/μl per Second) and 6-MP cytotoxicity (EC_{50}: RS4;11 = 0.58 ± 0.05 μM; RS4;11 shRNAi =1.44 ± 0.59 μM). This study showed that ENBT1 is a major contributor to 6-MP uptake in leukemia cell lines and may prove to be a biomarker for the therapeutic efficacy of 6-MP in patients with ALL.

SIGNIFICANCE STATEMENT

This study shows that *SLC43A3*-encoded equilibrative nucleobase transporter 1 is responsible for the transport of 6-mercaptopurine (6-MP) into leukemia cells and that its level of expression can impact the cytotoxicity of 6-MP. Further studies are warranted to investigate the therapeutic implications in patient populations.

Introduction

The nucleobase analog 6-mercaptopurine (6-MP), in combination with other drugs such as methotrexate, is a mainstay of maintenance protocols for the treatment of acute lymphoblastic leukemia (ALL) (Pui et al., 2015; Toft et al., 2018), the most common malignancy in children and adolescents. Although the survival rate of pediatric patients with ALL has increased in recent decades, there are still about 20% of patients who do not respond adequately to treatment and experience relapses with far lower survival rates (Chouchana et al., 2015). A complicating factor in the use of 6-MP to treat ALL is the wide variability in its plasma concentration (Lönnerholm et al., 1986), as well as in the levels of active intracellular thionucleotide metabolites, achieved with a standard dose (Lennard, 1992; Estlin, 2001; Schmiegelow et al., 2014). This suggests extensive individual variability in factors that influence 6-MP metabolism and biodistribution. 6-MP must be metabolized by intracellular enzymes to its phosphorylated derivatives in the target cells to exert its cytotoxic effects. Changes in activity of intracellular enzymes such as thiopurine methyltransferase (TPMT) contribute to clinical variability in thioguanine nucleotide levels (Lennard et al., 2015; Abaji and Krajinovic, 2017). However, *TPMT* polymorphisms do not explain all of the

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ABBREVIATIONS: 6-MP, 6-mercaptopurine; ABC, ATP-binding cassette; ALL, acute lymphoblastic leukemia; ALL-1R, resistant ALL-1 cell; ENBT1, equilibrative nucleobase transporter 1; ENT4, equilibrative nucleoside transporter 4; G418, geneticin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HEK293, human embryonic kidney 293; HRP, horseradish perdoxidase; Km, Michaelis constant; MRP, multidrug resistance protein; MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); NMG, N-methyl-D-glucamine; PCR, polymerase chain reaction; shRNA, short hairpin RNA; shRNAi, short hairpin RNA interference; SLC, solute carrier; TBS, Tris-buffered saline; TBS-TM, TBS containing 0.2% v/v Tween-20 and 5% w/v skim milk powder; TPMT, thiopurine methyltransferase; V_{max}, maximum rate of reaction.

therapeutic variability observed, and particularly not the variable plasma concentrations (Chouchana et al., 2015; Patel and Papachristos, 2015), nor do changes in other genes/proteins that have been linked with variations in 6-MP activity, such as *PRPS1, NUDT15, mTORC1, MSH6,* and *NT5C2* (Tzoneva et al., 2013, Li et al., 2015, Liu et al., 2017; Tanaka, 2017; Vo et al., 2017; Zgheib et al., 2017; Dieck et al., 2018; Evensen et al., 2018). Further understanding of factors that influence 6-MP activity is still required to optimize treatment protocols.

Hydrophilic drugs such as 6-MP require specific transporter proteins to enter and exit cells. Variations in transporter expression/activity are well known to impact both the clinical effectiveness of drugs and their off-target toxicities (Nakanishi, 2007; Colas et al., 2016; Wu and Li, 2018). Since 6-MP produces its therapeutic effects via interference with intracellular processes, the very first step in 6-MP action, prior to conversion to its therapeutic cytotoxic form, is its transfer into leukemic cells across the plasma membrane. The ATP-binding cassette (ABC) transporters MRP4 (*ABCC4*) and MRP5 (*ABCC5*) are known to mediate the efflux of 6-MP and metabolites from cells (Wijnholds et al., 2000; Wielinga et al., 2002; Janke et al., 2008; Tanaka et al., 2015; Liu et al., 2017). However, the mechanism by which 6-MP gets into cells, especially lymphoblasts (the target in ALL treatment), remained elusive until the recent identification of equilibrative nucleobase transporter 1 (ENBT1) encoded by the solute carrier (SLC) gene *SLC43A3*. There are two alternative splice variants of *SLC43A3* that we have shown to encode transport proteins that are functionally similar with respect to their ability to transport 6-MP and adenine (Ruel et al., 2019). Furthermore, we have established that ENBT1, heterologously expressed in human embryonic kidney 293 (HEK293) cells, can transport 6-MP at concentrations within the therapeutic range. We also showed, using that model, that changes in ENBT1 activity directly impact the ability of 6-MP to reduce cell viability. Given these findings, we now propose that endogenous ENBT1 expression may be an important variable in 6-MP accumulation by acute lymphoblastic leukemia cells, and variations in ENBT1 activity may modify the therapeutic effectiveness of 6-MP. In the present study, we show for the first time that acute lymphoblastic leukemia cells express *SLC43A3* and exhibit robust ENBT1-mediated 6-MP transport activity. Furthermore, the rate of 6-MP accumulation by these cell lines correlates directly with the level of expression of *SLC43A3*.

Materials and Methods

Materials

[8-^{14}C]-6-MP (50–60 mCi/mmol), and [^{3}H]-water (1 mCi/g) were obtained from Moravek Biochemicals (Brea, CA). Adenine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dipyridamole, geneticin(G418), Dulbecco's modified Eagle's medium, FBS, penicillin-streptomycin, D-glucose, doxycycline, 2-mercaptoethanol, polybrene, and puromycin were purchased from Sigma-Aldrich (St. Louis, MO). RPMI 1640 medium and the ECL Prime western blotting system were purchased from Cytiva Life Sciences (Marlborough, MA). All primers were ordered through Integrated DNA Technologies (Coralville, IA). Agarose, oligo (dT)12–18 primer, Iscove's Modified Dulbecco's Media, HEPES, sodium pyruvate, PowerUp SYBR Green, HALT Protease Inhibitor Cocktail, TRIzol Reagent, and SuperScript III Reverse Transcriptase came from Thermo Fisher Scientific (Waltham, MA). The 100 bp DNA Ladder was supplied by Truin Science (Edmonton, AB). Leu-Fect A was purchased from RJH Biosciences (Edmonton, AB). MOLT-4, NALM-6, SUP-B15, and HEK293 were purchased from ATCC (Manassas, VA). ALL-1, REH, and RS4;11 cells were generously provided by Dr. David Eisenstat (University of Alberta, Edmonton). The antibodies used for immunoblotting were mouse monoclonal IgG1 anti-Myc antibody (Clone 4A6, 05-724, Lot #3013479; EMD Millipore, Canada), rabbit polyclonal anti-*SLC43A3* (HPA030551, Lot #000001575; Sigma-Aldrich, St. Louis, MO), and mouse monoclonal anti-β-actin (C4, sc-47778, Lot #B0719; Santa Cruz Biotechnology Inc., Texas). Secondary antibodies were mouse anti-rabbit IgG horseradish perdoxidase (HRP) (sc-2357, Lot #2517) and m-IgGk BP-HRP (sc-516102, Lot #F1016) from Santa Cruz Biotechnology. The characteristics of the cell lines used in this study are shown in Supplemental Table 1. SMARTvector 2.0 Inducible Lentiviral shRNA particles targeting *SLC43A3* and SMARTvector 2.0 Nontargeting short hairpin RNA (shRNA) Control particles were from GE Healthcare Dharmacon, Inc. (Lafayette, CO). SMARTvector 2.0 Lentiviral shRNA particles bind to cells and deliver their shRNA to the cytoplasm. The SMARTvector 2.0 includes a TurboGFP reporter gene to facilitate assessment and optimization of transduction efficiencies and also contains a puromycin resistance gene for selection and isolation of clonal cell populations.

Cell Culture

ALL-1, MOLT-4, RS4;11, NALM-6, and REH cells were cultured in RPMI 1640 medium with 10% FBS supplemented with D-glucose (4500 mg/L), sodium pyruvate (1 mM), penicillin (100 U/mL), streptomycin (100 μg/mL), and HEPES (10 mM). SUP-B15 cells were cultured in Iscove's Modified Dulbecco's Media but with 20% FBS and 0.05 mM 2-mercaptoethanol. Suspended cells were centrifuged and washed in appropriate assay buffer solutions prior to their use in subsequent assays. HEK293 cells were cultured in Dulbecco's modified Eagle medium with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/mL), and sodium pyruvate (1 mM). G418 was added (300 μg/ml) in the *SLC43A3*-HEK293 (HEK293 cells stably transfected with *SLC43A3*) cell media to maintain selection pressure on the stable transfectants. The HEK293 cells were removed from flasks by exposure to 0.05% trypsin/0.18 mM EDTA for 5 minutes at 37°C, and the suspended cells were washed in the appropriate buffer solution (without G418) immediately prior to use in subsequent assays.

Cell Viability

Cells were seeded into a 24-well plate at a density of 5×10^4 cells per well in culture medium. Following plating, medium containing 6-MP (75 nM–1.28 mM) was added and incubated for 48 hours at 37°C in a humidified 5% CO_2 atmosphere. Cells were then transferred to a 1.5 mL microcentrifuge tube and centrifuged at 3000 rcf for 10 minutes. Following centrifugation, media was removed and replaced with 250 μL of Dulbecco's PBS (137 mM NaCl, 2.7 mM KCl, 6.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, pH 7.4) containing MTT (1 mg/ml) for 90 minutes. Microcentrifuge tubes were centrifuged again at 23,500 rcf for 15 minute, and the Dulbecco's PBS containing MTT was removed. The resultant formazan crystals were solubilized in 450 μl of DMSO, and absorbance was measured at 570 nm in a Bio-Rad xMark Microplate Absorbance Spectrophotometer (Hercules, CA).

Nucleobase Uptake

Cells were suspended in nominally sodium-free buffer (to eliminate potential contribution of sodium-dependent transporters) [N-methyl-D-glucamine (NMG) buffer; 140 mM NMG, 5 mM KCl, 4.2 mM $KHCO_3$, 0.36 mM K_2HPO_4, 0.44 mM KH_2PO_4, 10 mM HEPES, 0.5 mM $MgCl_2$, 1.3 mM $CaCl_2$, pH 7.4] containing 1 μM dipyridamole (to block potential equilibrative nucleoside transporter–mediated uptake) and incubated for 15 minutes at room temperature prior to assay. Cellular uptake was initiated by adding 250 μl cell suspension to 250 μl [^{14}C]6-MP layered over 21:4 silicone:mineral oil (v:v) (200 μl) in 1.5 ml microcentrifuge tubes. The uptake reaction was terminated after

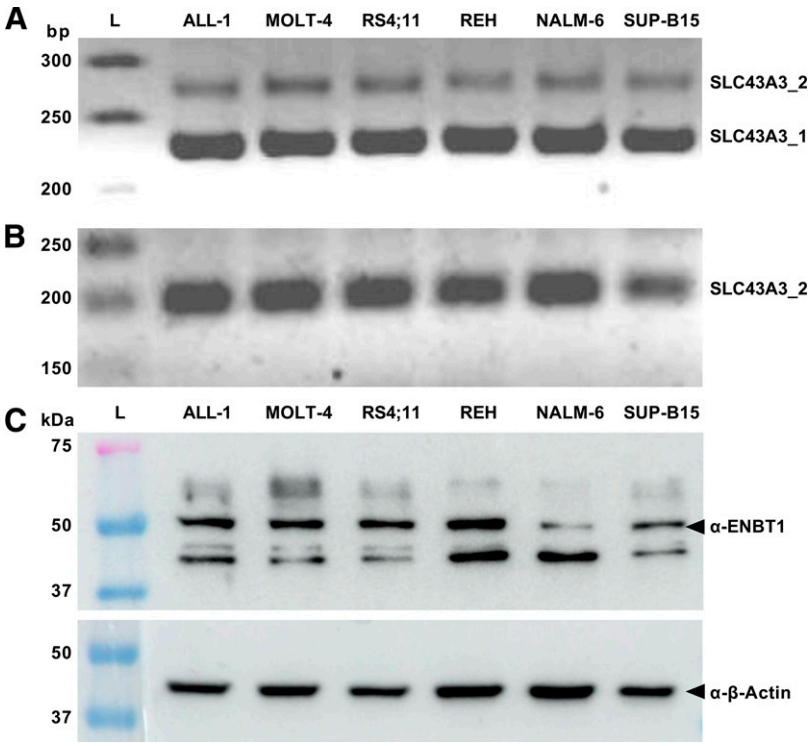

Fig. 1. *SLC43A3* isoform expression and ENBT1 protein levels. cDNA was prepared from mRNA isolated from ALL-1, MOLT-4, REH, RS4;11, NALM-6, and SUP-B15 human acute lymphoblastic leukemia cell lines. Qualitative PCR was used to screen for the two known isoforms of *SLC43A3* (*SLC43A3_1* and *SLC43A3_2)* using primers that flanked the region that differs between the two isoforms [to amplify both isoforms (A)] and a primer set where the forward primer was designed against the region that is unique to *SLC43A3_2* [to specifically amplify isoform 2 (B)] (see Supplemental Table 2 for primer sequences). (C) A representative immunoblot for ENBT1 in these cell lines. Samples were resolved on SDS-PAGE gels, transferred to polyvinyl membranes, and probed with anti-ENTB1 (upper panel) and anti–β-actin (lower panel) antibodies. L, the respective DNA [(A) and (B)] and protein (C) molecular size ladders.

specified times by centrifugation of the cells through the oil layer at ~10,000*g*. The aqueous layer was aspirated, and the tube was washed with ~1 ml of NMG buffer prior to removal of the oil layer. The resulting cell pellet was digested in 1 M NaOH overnight (~16 hours), with aliquots of digested cells assessed for radioactive content using standard liquid scintillation counting techniques in a Beckman Coulter LS6500 scintillation system (Brea, CA). Total uptake was defined as the uptake of [^{14}C]6-MP in the absence of adenine. Nonmediated uptake was defined as the uptake of [^{14}C]6-MP by cells in the presence of 1 mM adenine. Mediated uptake was defined as the difference between the total and nonmediated uptake components. Cell volume (in microliters) was estimated by incubating cells with [^{3}H] water for 3 minutes, centrifuging the cells through the oil layer, sampling 100 μl of the supernatant, and then processing as described above. Total cellular water volume was determined from the ratio of the decays per minute of the cell pellet to the decays per minute of the supernatant, allowing for interexperimental normalization via calculation of picomoles of substrate accumulated per microliter of cell-associated water.

Polymerase Chain Reaction

Cells from confluent 10 cm plates were suspended in 1 ml of TRIzol reagent and homogenized for extraction of RNA according to the manufacturer's protocol (Thermo Fisher Scientific). Total RNA concentration and purity were determined using a Nanodrop 2000 spectrophotometer (Life Technologies Inc.). For qualitative polymerase chain reaction (PCR), 1 μg of total RNA was reverse transcribed to cDNA using Oligo (dT)12–18 primer and SuperScript III Reverse Transcriptase and amplified using recombinant Taq DNA Polymerase (Thermo Fisher Scientific) and primers designed flanking the nucleotide sequence of the 13 additional amino acids in *SLC43A3_2* (forward primer: 5′- CTGTGTGG ACCAGATGC; reverse primer 3′- TAGCCTTCACCTCTGCA), or a primer designed within the nucleotide sequence for the 13 additional amino acids in *SLC43A3_2* (forward primer: 5′-GAGACCTTTTTTC TACTCC) The following conditions were used for amplification: 3 minutes at 95°C, followed by 40 cycles of 30 seconds at 95°C; 30 seconds at 56°C; and 60 seconds at 72°C, followed by extension for 10 minutes at 72°C in a BioRad T-100 Thermocycler. Semiquantitative PCR was conducted using cDNA (~100 ng/well) prepared as described above with the primer sets shown in Supplemental Table 2 using Power Up SYBR

TABLE 1
Summary of experimental parameters measured

			ENBT1-Mediated Transport	6-MP Cytotoxicity[c]		
Cell Line	*SLC43A3* Transcript[a]	ENBT1 Protein[b]	V_{max} (pmol/μL per s)	% Sensitive	EC_{50_1} (μM)	EC_{50_2} (mM)
MOLT-4	1.10 ± 0.11 (7)[d]	0.84 ± 0.34 (13)	87 ± 4 (5)	46 ± 14 (7)	1.46 ± 0.95 (7)	1.72 ± 0.78 (7)
ALL-1	1.01 ± 0.12 (7)	0.74 ± 0.38 (13)	69 ± 10 (5)	44 ± 10 (14)	0.66 ± 0.23 (14)	1.71 ± 1.55 (14)
RS4;11	1.00 ± 0.08 (7)	0.91 ± 0.42 (13)	52 ± 8 (5)	39 ± 8 (6)	0.96 ± 0.39 (6)	3.73 ± 2.21 (6)
REH	0.86 ± 0.07 (7)	1.07 ± 0.47 (13)	65 ± 2 (5)	28 ± 12 (6)	1.21 ± 0.42 (6)	0.72 ± 0.93 (6)
NALM-6	0.55 ± 0.06 (7)	0.48 ± 0.24 (6)	15 ± 2 (5)	37 ± 7 (9)	0.92 ± 0.42 (9)	0.49 ± 0.46 (9)
SUP-B15	0.53 ± 0.10 (7)	0.65 ± 0.37 (12)	22 ± 5 (5)	44 ± 12 (5)	2.06 ± 0.49 (5)	0.25 ± 0.21 (5)

% Sensitive, the proportion of cells that were sensitive to low μM concentrations of 6-MP; EC_{50_1} and EC_{50_2}, the log molar concentrations of 6-MP that reduced the viability of the 6-MP sensitive and resistant cell populations, respectively.
[a]Expression relative to ALL-1 (Fig. 2).
[b]Expression relative to β-actin (see Fig. 1C for representative immunoblot).
[c]Data derived from the biphasic 6-MP cytotoxicity profiles shown in Fig. 4.
[d]Mean plus or minus S.D. from the number of independent experiments shown in parentheses.

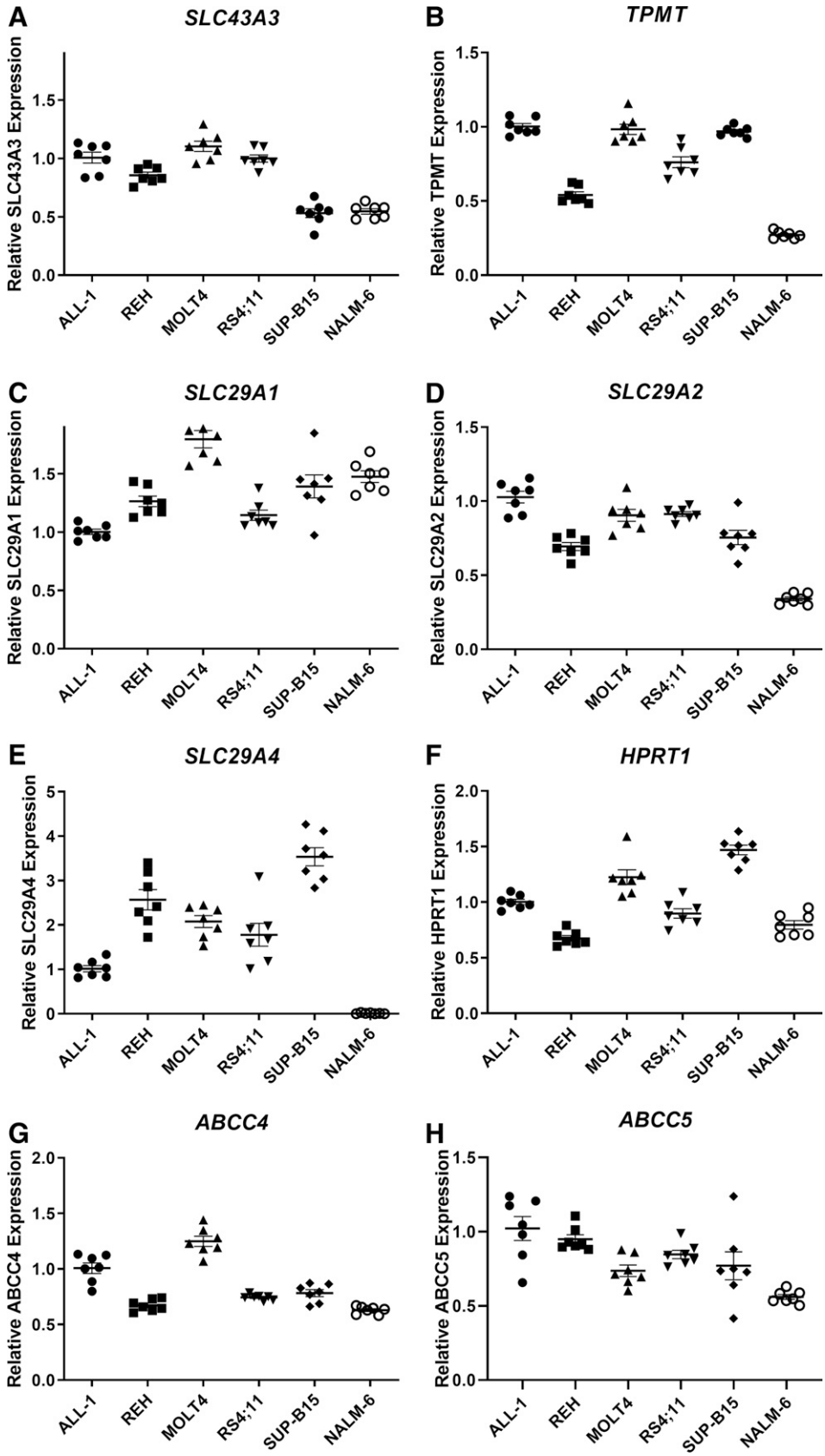

Fig. 2. Transcript levels of various enzymes and transporters potentially associated with 6-MP metabolism. Gene expression was assessed by semiquantitative PCR and is shown relative to the ALL-1 cell line. Expression was normalized to three separate reference genes (*GAPDH, 18S ribosomal N5,* and *β-actin*) and quantified using the delta-delta Ct method. (A–H) Relative expression of *SLC43A3, TPMT, SLC29A1, SLC29A2, SLC29A4,* hypoxanthine phosphoribosyltransferase 1, *ABCC4*, and *ABCC5* as compared with the ALL-1 cell line. Lines represent the mean plus or minus S.D. of six independent samples done in triplicate.

Green fluorescence on a Roche Light Cycler 480 System (Cardiovascular Research Centre, Edmonton, Canada). Primer efficiency and melt curves were assessed prior to their use for gene expression analysis. Semiquantitative PCR conditions were: 2 minutes at 50°C (uracil-DNA glycosylase activation); 2 minutes at 95°C (denaturation), followed by 50 cycles of 15 seconds at 95°C; and 60 seconds at 60°C for

amplification, with a final melt curve analysis. Gene expression was normalized to either glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) alone or the geographic mean of three separate reference genes, *GAPDH*, 18S ribosomal N5, and β-actin, and analyzed relative to expression in the untransfected HEK293 or ALL-1 cells using the $\Delta\Delta C_t$ method.

Immunoblotting

Samples were extracted using radioimmunoprecipitation buffer (150 mM NaCl, 50 mM Tris, 1% NP-40, 0.5% sodium deoxycholate, 1% SDS) containing HALT Protease Inhibitor Cocktail and adjusted to 2% (v/v) β-mercaptoethanol. Samples were resolved using SDS-PAGE on 12.5% (w/v) acrylamide gels and transferred to Immobilon-P PVDF membranes at 280 mA for 1.5 hours. Following the transfer, membranes were rinsed in Tris-buffered saline (TBS; 0.15 M NaCl, 50 mM Tris, pH 7.5) and incubated with TBS-TM (TBS containing 0.2% v/v Tween-20 and 5% w/v skim milk powder) at room temperature for 1 hour to block nonspecific binding. Anti-Myc, rabbit anti-*SLC43A3*, or mouse anti–β-actin at 1:1000, 1:250, and 1:500, respectively, were then incubated for 16 hours at 4°C in TBS-TM (containing 1% skim milk). Membranes were washed several times in TBS-TM before being incubated in mouse anti-rabbit IgG-HRP or m-IgGk BP-HRP at 1:5000 or 1:3000, respectively, for 1 hour at room temperature. After further washing in TBS containing 0.2% (v/v) Tween-20, proteins were detected using ECL Prime western blotting system and visualized on an Amersham Imager 680 (GE Healthcare, Chicago, IL). Image J software was used to conduct the densitometry analyses.

Suppression of *SLC43A3* by Short Hairpin RNA Interference (shRNAi)

HEK293 Cells. The base HEK293 cells and *SLC43A3*-HEK293 cells were transduced with the SMARTvector Inducible Lentiviral shRNA vector containing SLC43A3 shRNAi according to the manufacturer's protocol and stably selected using puromycin. To induce expression of the shRNA, doxycycline was added to the cells at 400 ng/mL for 48 or 72 hours before analysis. shRNA expression efficiency was assessed based on the reporter GFP fluorescence. *SLC43A3* knockdown was confirmed by immunoblots with anti-*SLC43A3* antibodies.

Leukemia Cells. Attempts to transduce ALL-1 and RS4;11 leukemia cells with the SMARTvector Inducible Lentiviral shRNA system, using the same procedure that was used for the HEK293 cells, were unsuccessful. Therefore, transfection of RS4;11 cells with *SLC43A3* shRNAi vector lentiviral particles or nontargeting control shRNAi was done using Leu-Fect A according to manufacturer's protocols. Cells were transfected and incubated at 37°C in a humidified 5% CO_2 atmosphere for 48 hours in culture media, without FBS or penicillin/streptomycin, containing the Leu-Fect A and shRNAi lentiviral particles. Following the 48 hours, culture media containing FBS and penicillin/

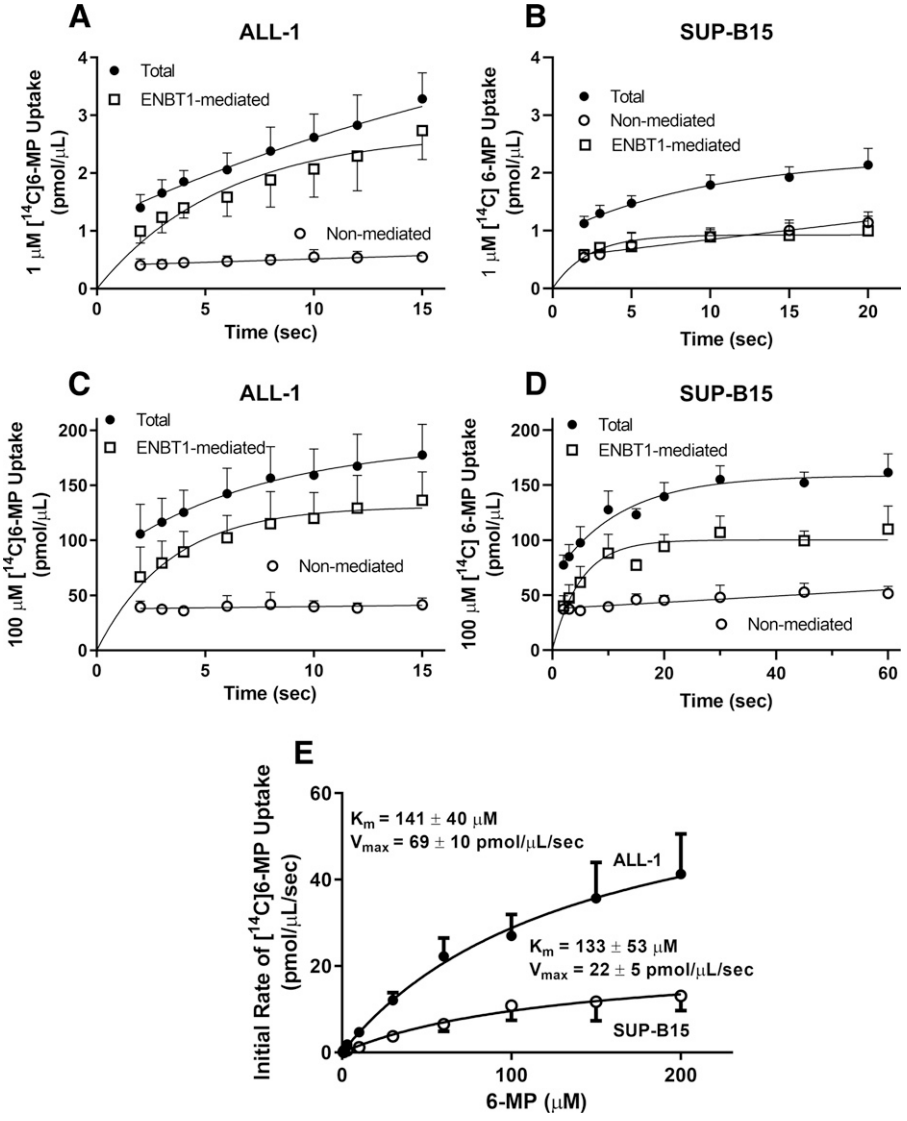

Fig. 3. [^{14}C]6-MP transport by ENBT1. ALL-1 and SUP-B15 cells were incubated at room temperature with 1 μM (A and B) and 100 μM (C and D) [^{14}C]6-MP for the indicated times (abscissa) in the absence (Total uptake) and presence (Nonmediated uptake) of 1 mM adenine. Transport was terminated by centrifugation of the cells through an oil layer. Cell pellets were digested overnight in 1 M NaOH and assessed for their [^{14}C] content via liquid scintillation counting. ENBT1-mediated uptake is defined as the difference between the total uptake and the nonmediated uptake. Data are represented as the mean plus or minus S.D. of five experiments done in duplicate. (E) The kinetics of ENBT1-mediated transport of 6-MP in ALL-1 and SUP-B15 cells were determined by incubating the cells in a range of concentrations of [^{14}C]6-MP for 2 seconds (ALL-1) or 3 seconds (SUP-B15) in the presence and absence of 1 mM adenine, with ENBT1-mediated transport calculated as the adenine-sensitive transport component. Data are shown as the pmol of 6-MP accumulated by the cells per μl of cell volume/s. Data points are the mean plus or minus S.D. of five experiments done in duplicate.

streptomycin was added, and cells were grown for 1 week. Puromycin was then added at 2 μg/mL, and the cells were incubated until no untransfected cells (cultured in parallel) remained. Transfected cells were allowed to continue proliferating in the presence of 1 μg/mL puromycin to a density of approximately 1,000,000 cells per mL. Cells were then sorted on a BD FACSAria III BSL2 cell sorter (BD Biosciences, San Jose, CA) (University of Alberta, Faculty of Medicine & Dentistry Flow Cytometry Facility) for GFP-positive cells and maintained in culture media with 1 μg/mL puromycin. GFP expression was assessed on each cell passage using a Zeiss inverted widefield epifluorescence Axio Observer Z1 microscope (Oberkochen, Germany) at 388 nm, and cells were resorted if expression started to decrease (this was typically done every five passages to ensure sufficient signal and knockdown of target).

Data Analysis and Statistics

Data are expressed as mean plus or minus S.D. from a minimum of five independent experiments done in technical duplicate or triplicate. A sample size of five is the minimum needed to define statistical differences based on the known variability inherent in substrate flux studies. Higher sample sizes ($N > 10$) were used for the more variable types of assays, such as the immunoblot densitometry measurements. Nonlinear curves were fitted to the transport and cytotoxicity data, and statistical analyses, were done using GraphPad Prism 8.01 software. In all cases, if the P value determined from a statistical test was less than 0.05, the difference was considered significant, and the null hypothesis (no difference between data sets) was rejected. Cytotoxicity curves were best represented by a biphasic curve fit (versus one phase). Correlation data were fit using linear regression and statistically tested using the Spearman rank correlation coefficient. Influx data were fit using a one-phase association for time course data, and Michaelis-Menten curves were fit to the concentration-dependent uptake data for determination of Michaelis constant (K_m) and maximum rate of reaction (V_{max}) values. Statistical differences between K_m and V_{max} values were determined using the extra sum-of-squares F test. Significant differences between groups were assessed using a one-way or two-way ANOVA, corrected for multiple comparisons with the Holm-Sidak method.

Results

***SLC43A3* Expression.** All of the cell lines tested expressed both isoforms of *SLC43A3*, with isoform 1 being dominant in all cases (Fig. 1, A and B). Immunoreactivity using an ENBT1-selective antibody was also detected at the molecular mass expected (~54 kDa) for the ENBT1 protein in all cell lines (Fig. 1C). Most of the cell lines had similar levels of expression of *SLC43A3* transcript and ENBT1 protein (Table 1), with the exception of NALM-6 and SUP-B15, which had about a twofold-lower expression relative to the other cell lines tested.

Expression of Other Transporters/Enzymes. The expression of transcripts encoding enzymes involved in 6-MP metabolism (hypoxanthine phosphoribosyltransferase, *TPMT*) and other transporters (i.e., *ABCC4, ABCC5, SLC29A1, SLC29A2, SLC29A4*) that have been implicated in nucleoside/nucleobase flux are shown in Fig. 2. Of all the genes tested, only *SLC29A4,* which encodes for equilibrative nucleoside transporter 4 (ENT4), varied significantly among the cell lines, with the SUP-B15 cells having a fourfold higher expression than the ALL-1 cells and NALM-6 cells having ~50-fold lower expression than the ALL-1 cells.

6-MP Uptake. Initial studies were done to assess the rate of uptake of 6-MP by the ALL-1 and SUP-B15 cell lines using either 1 μM [which reflects its therapeutic plasma concentration (Lönnerholm et al., 1986)] or 100 μM 6-MP. [^{14}C]6-MP uptake was very rapid with a half-time of ENBT1-mediated uptake

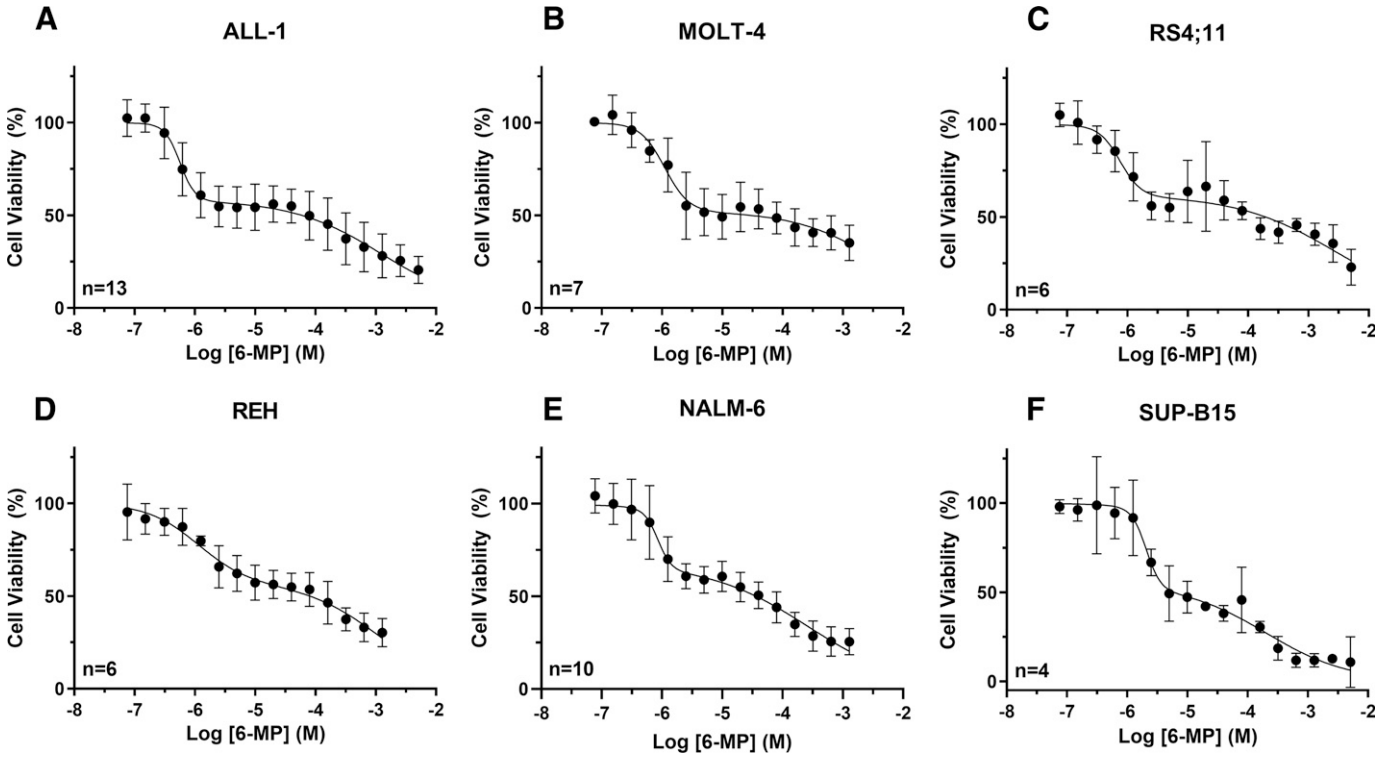

Fig. 4. Cytotoxicity of 6-MP. The leukemia cell lines indicated in (A–F) were plated at a density of 5×10^4 cells per well in 24-well plates and incubated with a range of concentrations of 6-MP for 48 hours at 37°C in a humidified incubator (5% CO2/95% air). Cell viability was assessed using the MTT assay and expressed as a percentage of the cell viability measured at 48 hours in the absence of 6-MP. Data were fitted with biphasic concentration-response curves, and each point represents the mean plus or minus S.D. from the number of experiments (N) indicated on each panel.

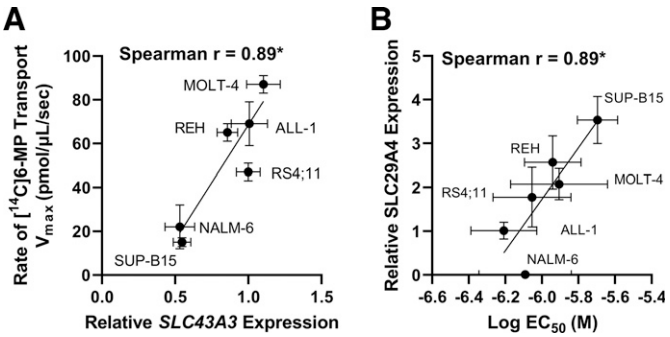

Fig. 5. Correlation analyses. Correlation of the various independent variables measured in this study revealed two significant ($P < 0.05$) correlations among the cell lines, between (A) *SLC43A3* expression and the rate (V_{max}) of ENBT1-mediated 6-MP uptake, and (B) the log EC_{50} for phase 1 (6-MP sensitive component) of the 6-MP cytotoxicity profile and the expression of *SLC29A4*. Data points represent the mean plus or minus S.D. for each independent measure. Data were analyzed using simple linear regression, and correlations assessed using the Spearman rank-order correlation method.

(adenine inhibitable) of ~4.0 seconds and ~2.3 seconds at 1 μM and 100 μM, respectively, for the ALL-1 cells and ~1.6 seconds and ~3.5 seconds, respectively, for the SUP-B15 cells (Fig. 3). Based on these time-course profiles, a 2-second timepoint was chosen to estimate initial rate of influx; this is the shortest incubation time that can be practically achieved using this method.

When initial rates were derived in this manner over a range of [^{14}C]6-MP concentrations, a classic Michaelis-Menten relationship was observed with a V_{max} of 69 ± 10 pmol/μl per second and a K_m of 141 ± 40 μM for ALL-1 cells. In the SUP-B15 cells, we observed a significantly lower V_{max} for ENBT1-mediated 6-MP uptake of 22 ± 5 pmol/μl per second and a K_m of 133 ± 53 μM (Fig. 3E). These K_m values for 6-MP transport by ENBT1 are similar to those determined previously for *SLC43A3*-transfected HEK293 cells (Ruel et al., 2019). Several other leukemia cell lines (RS4:11, REH, NALM-6, MOLT-4) were also examined for their adenine-inhibitable (ENBT1-mediated) uptake of [^{14}C]6-MP, revealing a similar affinity across cell lines. They did, however, vary significantly in the V_{max} of 6-MP transport; these data are summarized in Table 1.

6-MP Cytotoxicity. Each of the cell lines tested displayed a biphasic sensitivity to 6-MP, with ~45% of the cells sensitive to 6-MP with an EC_{50} of ~1 μM (sensitive cell component), and the remainder requiring concentrations greater than 500 μM to affect cell viability (resistant cell component) (Fig. 4). Given that the therapeutic plasma concentrations of 6-MP are in the range of 1 μM, cell lines were compared with respect to the EC_{50} of 6-MP cytotoxicity for the sensitive component. ALL-1 cells were the most sensitive to 6-MP (EC_{50} = 0.66 ± 0.23 μM), and the SUP-B15 cells were the least sensitive (EC_{50} = 2.06 ± 0.49 μM). Cytotoxicity data for all cell lines is compiled in Table 1.

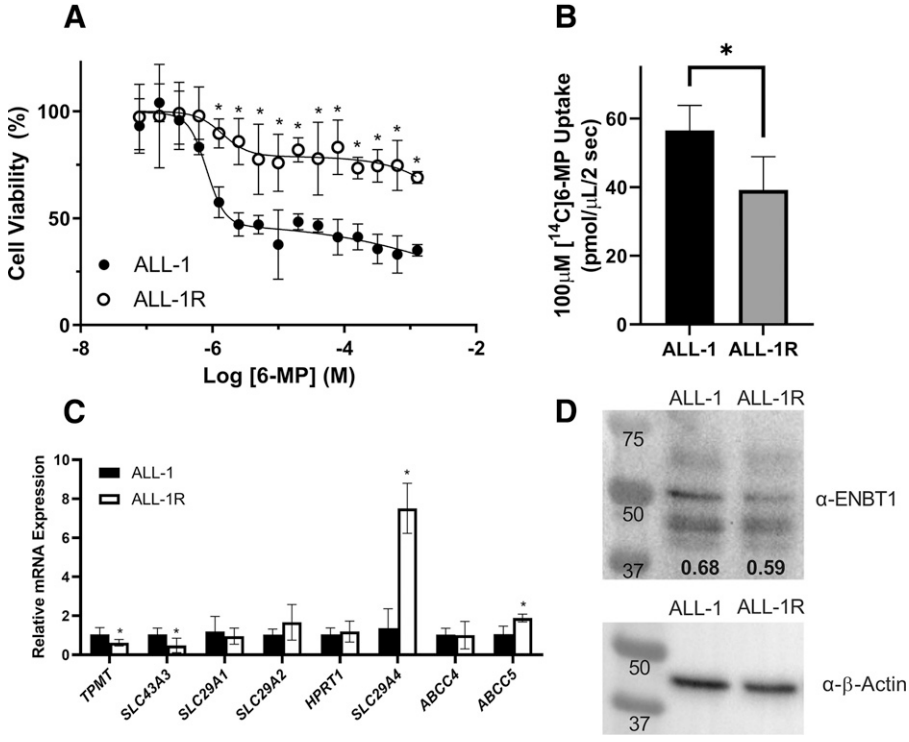

Fig. 6. ALL-1 cells resistant to 6-MP have decreased *SLC43A3* expression. 6-MP resistant ALL-1 cells (ALL-1R) were derived by incubating the cells with 640 μM 6-MP for 48 hours and subculturing the surviving cells for 10 passages. (A) Cells were assessed for their viability via the MTT assay after exposure to a range of concentrations of 6-MP as described in Fig. 4. Data points are expressed as the mean plus or minus S.D. of six (ALL-1) and five (ALL-1R) experiments and fitted to a biphasic concentration-response relationship. *Indicates a significant difference between the ALL-1R and ALL-1 cells using a two-way ANOVA with a Holm-Sidak post hoc test ($P < 0.0001$ for all points). (B) The rate of 6-MP accumulation by the ALL-1 and ALL-1R cells was determined as described in Fig. 3. Bars represent the mean plus or minus S.D. of eight experiments. *Indicates a significant difference in the rate of influx (Student's t test, $P = 0.049$). (C) Transcripts levels were determined as described in Fig. 2, with the exception that *GAPDH* alone was used as the reference gene. Bars represent the mean plus or minus S.D. of 5–7 independent samples done in triplicate. *Indicates a significant difference in gene expression between the ALL-1 and ALL-1R cells based on an unpaired t test (P = 0.032, 0.019, <0.0001, and 0.0037 for *TPMT, SLC43A3, SLC29A4,* and *ABCC5,* respectively). (D) ENBT1 protein levels in ALL-1 and ALL-1R cell lines. Samples were resolved on SDS-PAGE gels, transferred to polyvinyl membranes, and probed with anti-ENBT1 (upper panel) and anti–β-actin (lower panel) antibodies. The ratio of ENBT1 to β-actin determined by densitometry is indicated for each lane below the ENBT1 gel image.

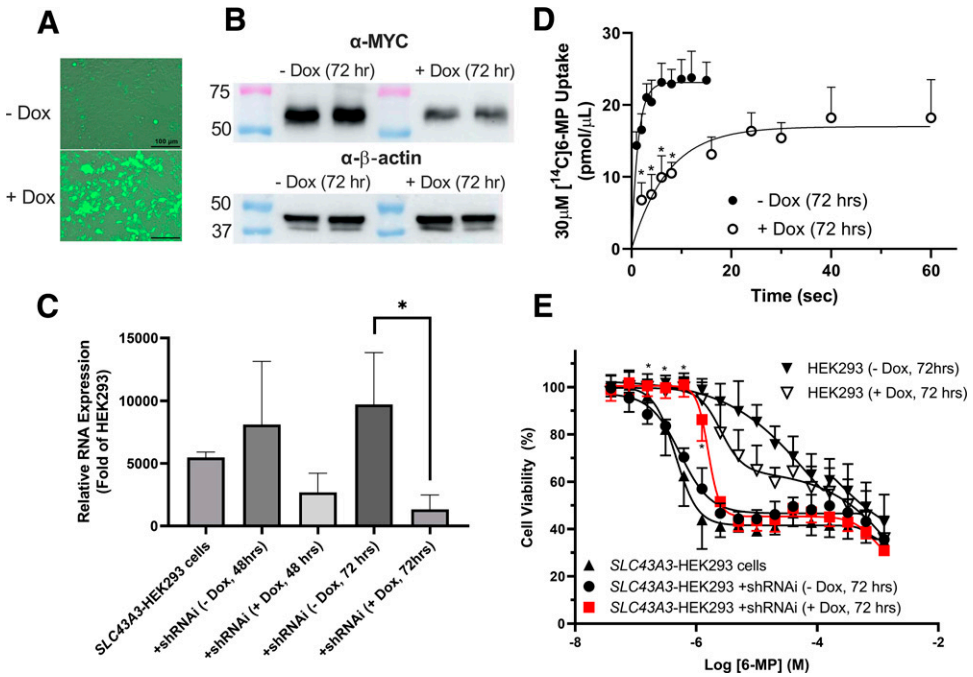

Fig. 7. shRNAi suppression of *SLC43A3* decreases uptake and cytotoxicity of 6-MP in *SLC43A3*-transfected HEK293 cells. Myc-tagged *SLC43A3*-HEK293 cells stably transfected with an inducible shRNAi vector targeting *SLC43A3* were treated with or without 400 ng/μL doxycycline for 48 or 72 hours to induce shRNAi expression (with concomitant GFP). (A) Expression of the GFP indicator without and with 72-hour exposure to doxycycline. Scale bars, 100 μm. (B) MYC-ENBT1 levels before and after exposure to doxycycline for 72 hours. Samples were resolved on SDS-PAGE gels, transferred to polyvinyl membranes, and probed with anti-MYC (upper panel) and anti–β-actin (lower panel) antibodies. (C) *SLC43A3* transcript levels were quantified by semiquantitative PCR using *GAPDH* as the reference gene in shRNAi-transfected *SLC43A3*-HEK293 cells following incubation with and without doxycycline for 48 and 72 hours. Expression levels in the base *SLC43A3*-HEK293 cells are shown for comparison. Bars represent the mean plus or minus S.D. of 4–6 independent samples done in triplicate. *Indicates a significant difference using a one-way ANOVA with a Holm-Sidak post hoc test (P = 0.0027). (D) Time course of the cellular uptake of 30 μM [^{14}C]6-MP by *SLC43A3*-targeting shRNAi-transfected *SLC43A3*-HEK293 cells with and without exposure to doxycycline for 72 hours. Data are expressed as the mean plus or minus S.D. of five experiments done in duplicate. *Indicates a significant difference plus or minus doxycycline (multiple t tests with a Holm-Sidak post hoc test, P = 0.00016, 0.00011, <0.0001, and <0.0001 for 2-, 4-, 6-, and 8-second timepoints, respectively). (E) Effect of 6-MP on cell viability for HEK293 cells, HEK293 cells stably transfected with *SLC43A3* (*SLC43A3*-HEK293), and *SLC43A3*-HEK293 cells stably transfected with *SLC43A3*-targeting shRNAi, with and without incubation of cells with doxycycline for 72 hours. Points represent the mean plus or minus S.D. of 5–7 independent experiments. *Indicates a significant difference plus or minus doxycycline [two-way ANOVA with a Holm-Sidak post hoc test, $P \leq$ 0.0001, <0.0001, 0.0005, and 0.0281 for −5.9, −6.2, −6.5, and −6.8 (log molar 6-MP), respectively].

Correlation of 6-MP Uptake, Cytotoxicity, and Gene Expression. Pairwise comparisons of all the independent measures made in this study revealed two significant correlations. First, the rate of 6-MP uptake (V_{max}) positively correlated with *SLC43A3* mRNA expression (Spearman r = 0.89) (Fig. 5A). There was, however, no significant correlation between 6-MP uptake (V_{max}) by ENBT1 and the cytotoxicity of 6-MP (Spearman r = 0.03) when comparing the full panel of cell lines. Second, there was a significant positive correlation between the phase 1 (6-MP sensitive component) cytotoxicity EC_{50} value and the expression of *SLC29A4* (Spearman r = 0.89) (Fig. 5B). In other words, as *SLC29A4* expression increased, the cytotoxicity of 6-MP decreased.

6-MP Resistant Cells. To further examine the 6-MP–resistant cell population that was apparent from the cytotoxicity profiles shown in Fig. 4, ALL-1 cells were grown in the presence of 640 μM 6-MP for 48 hours (concentration selected to ensure complete elimination of the 6-MP–sensitive cell population; see Fig. 4A) and then subcultured for at least 10 passages. These cells retained their relative insensitivity to 6-MP. The resistant ALL-1 cells (ALL-1R) still showed a biphasic cytotoxicity profile to 6-MP (Fig. 6A) but with only ∼20% of the cell population being relatively sensitive to 6-MP–induced cell death (EC_{50} = 1.41 ± 0.63 μM; not significantly different from that obtained for the parent ALL-1 cell line). However, these ALL-1R cells had a reduced rate of uptake of 6-MP (determined using the ∼K_m concentration of 100 μM) relative to the parent cell line (Fig. 6B). In terms of gene expression, the ALL-1R cells had reduced expression of *TPMT* and *SLC43A3* and an increased expression of *SLC29A4* and *ABCC5* (Fig. 6C). Immunoblotting confirmed a decrease in ENBT1 protein expression in ALL-1R cells when normalized to β-actin levels (Fig. 6D).

shRNAi Suppression of *SLC43A3*. The initial shRNAi suppression studies were done using the *SLC43A3*-HEK293 cells that we created and reported on previously (Ruel et al., 2019). *SLC43A3*-HEK293 cells were stably transduced with a lentiviral vector containing *SLC43A3*-targeting shRNAi under the control of a doxycycline-sensitive promoter (Fig. 7A). Immunoblotting and PCR indicated that *SLC43A3* transcript was suppressed upon incubation of these cells with doxycycline for 72 hours (Fig. 7, B and C), with a ∼fivefold reduction in expression observed. This is corroborated by the uptake data, which showed a fivefold reduction in the rate of 6-MP uptake upon treatment with doxycycline to induce the shRNAi expression (Rate: −Dox: 0.77 ± 0.08 second^{-1}; +Dox: 0.14 ± 0.02 second^{-1} (Fig. 7D). Furthermore, upon activation of the shRNAi expression, the *SLC43A3*-HEK293 cells were significantly less sensitive to 6-MP–induced cytotoxicity (EC_{50}: −Dox= 0.54 ±

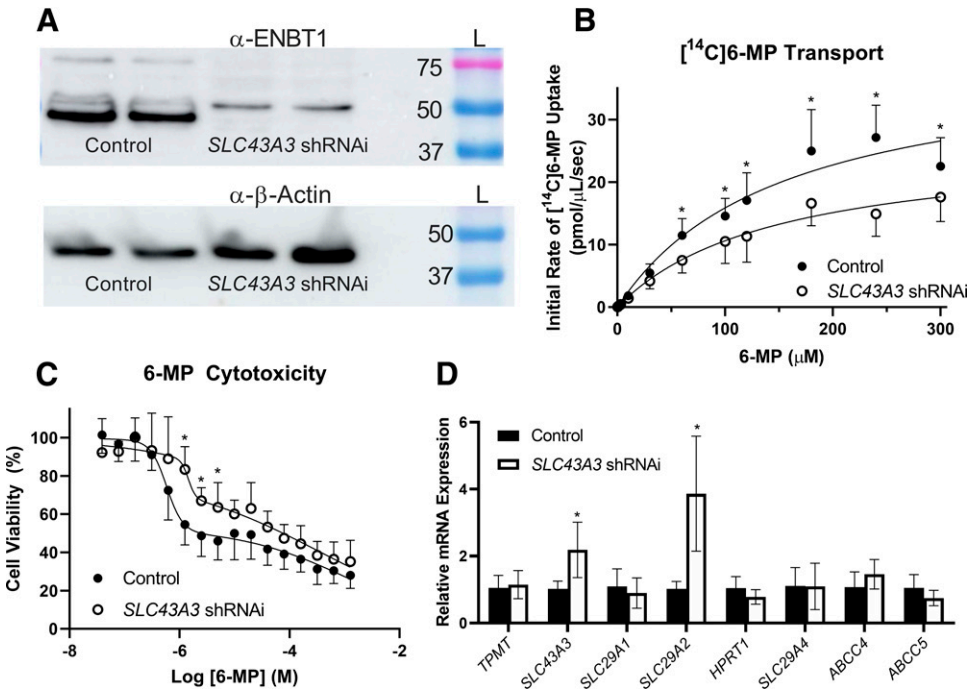

Fig. 8. shRNAi knockdown of *SLC43A3* in RS4;11 leukemia cells decreases both ENBT1-mediated uptake of 6-MP and 6-MP cytotoxicity. (A) ENBT1 protein expression in nontargeting shRNAi–transfected (Control) and *SLC43A3*-targeting shRNAi (*SLC43A3*-shRNAi)–transfected RS4;11 cells. Cell membrane samples from two independent experiments were resolved on SDS-PAGE gels, transferred to polyvinyl membranes, and probed with anti-ENBT1 (upper panel) and anti–β-actin (lower panel) antibodies. (B) Kinetic analysis of [^{14}C]6-MP uptake by RS4;11 cells transfected with nontargeting shRNAi (Control) or with *SLC43A3*-targeted shRNAi was conducted as described in Fig. 3. Points represent the mean plus or minus S.D. of 6–10 experiments done in duplicate. *Indicates a significant effect plus or minus shRNAi (two-way ANOVA with a Holm-Sidak post hoc test, P = 0.021, 0.021, 0.0003, <0.0001, <0.0001, and 0.0174 for 60, 100, 120, 180, 240, and 300 μM 6-MP, respectively). (C) Cytotoxicity of 6-MP in RS4;11 cells transfected with nontargeting shRNAi (Control) or *SLC43A3*-targeting shRNAi. Assays were conducted as described for Fig. 4. Points represent the mean plus or minus S.D. of 6–9 experiments. *Indicates a significant effect of *SLC43A3* knockdown [two-way ANOVA with a Holm-Sidak post hoc test, P = 0.039, 0.028, and <0.0001 for −5.3, −5.6, and −5.9 (log molar 6-MP), respectively]. (D) Gene expression profiles of enzymes and transporters associated with 6-MP metabolism in RS4;11 cells transfected with nontargeting shRNAi (Control) or with *SLC43A3*-targeted shRNAi. Bars represent the mean plus or minus S.D. of 5–6 independent samples done in triplicate. *Indicates a significant effect of shRNAi suppression of *SLC43A3* (unpaired t test, $P < 0.05$).

0.05 μM; +Dox = 1.58 ± 0.07 μM) (Fig. 7E). Interestingly, exposure of the untransfected base HEK293 cells to doxycycline for 72 hours actually enhanced their sensitivity to 6-MP (Fig. 7E). This finding indicates that the decrease in 6-MP sensitivity upon activation of the *SLC43A3*-shRNAi expression was due to the knockdown of *SLC43A3* and not a nonspecific effect of the doxycycline exposure.

For reasons that remain obscure, this same inducible knockdown approach was not successful in the leukemia cell lines. Therefore, we transiently transfected the RS4;11 cell line with the shRNAi–containing vectors(*SLC43A3* targeting and control) as described in *Methods*. This resulted in robust expression of the shRNAi, based on GFP reporter levels, as well as a significant reduction in ENBT1 immunoreactivity, even in the absence of doxycycline (Fig. 8A). Cells transfected with the *SLC43A3* shRNAi–containing vector displayed a 35% reduction in ENBT1-mediated 6-MP uptake (V_{max}: Control − 40 ± 4 pmol/μl per second; shRNAi − 26 ± 3 pmol/μl per second) (Fig. 8B). The *SLC43A3* shRNAi–expressing cells were also significantly less sensitive to the cytotoxic effects of 6-MP (EC_{50}: Control: 0.58 ± 0.05 μM; shRNAi: 1.44 ± 0.59 μM) (Fig. 8C). Gene expression analysis showed a significant paradoxical (in spite of the decrease in ENBT1 protein and function) twofold increase in *SLC43A3* transcript and also a fourfold increase in *SLC29A2* (equilibrative nucleoside transporter 2) transcript (Fig. 8D).

Discussion

This study clearly shows that *SLC43A3* is highly expressed in acute lymphoblastic leukemia cell lines and that it mediates the uptake of 6-MP by these cells. This is consistent with the relatively high expression of *SLC43A3* in bone marrow and lymphoid tissues as reported by the Human Protein Atlas (proteinatlas.org) (Uhlen et al., 2015). There is a strong correlation between the expression of *SLC43A3* and the rate of 6-MP uptake among these cell lines (Fig. 5A). This suggests that the protein encoded by *SLC43A3* is a major player in the cellular accumulation of 6-MP by leukemic lymphoblasts. Although 6-MP uptake correlated with the expression of *SLC43A3*, this did not translate to a correlation with 6-MP–mediated cytotoxicity in the cell panel studied. This suggests that relatively small changes (∼twofold in this case) in *SLC43A3*/ENBT1 activity may not be sufficient to be a factor in 6-MP cytotoxicity. The difference in 6-MP cytotoxicity among cell lines was likely more dependent on differences in the activity of intracellular metabolic enzymes such as *TPMT*. Nevertheless, larger changes in *SLC43A3* expression may be clinically relevant. Over 1600 coding sequence variants (nonsynonymous single nucleotide polymorphisms) and 26 splice variants have been identified to date for *SLC43A3* (Cunningham et al., 2022). Many of these are predicted to affect protein function (Poly-Phen score > 0.9)

(Adzhubei et al., 2010). However, none have been characterized, and there is limited information on allele frequency in human populations.

We showed in a previous study that there was a significant enhancement of 6-MP cytotoxicity when *SLC43A3* was recombinantly expressed in an HEK293 cell model, which normally has low endogenous levels of *SLC43A3* (Ruel et al., 2019). We also showed, in the present study, that knockdown of *SLC43A3* via transfection of these *SLC43A3*-HEK293 cells with shRNAi targeting *SLC43A3* led to a dramatic reduction in 6-MP uptake and cytotoxicity (Fig. 7). A similar result was obtained when RS4;11 leukemia cells were transfected with this same *SLC43A3* shRNAi expression vector. The *SLC43A3* shRNAi transfected RS4;11 cells showed a dramatic decrease in the rate of ENBT1-mediated 6-MP uptake and the amount of ENBT1 protein, as well as a significant decrease in their sensitivity to the cytotoxic activity of 6-MP, relative to cells transfected with the nontargeting control shRNAi (Fig. 8). However, in the RS4;11 cells, the shRNAi was expressed, based on the GFP reporter, even in the absence of doxycycline. This may reflect the higher level of the construct internalized in the RS4;11 cells (likely due to the inclusion of Leu-Fect-A in the transfection media). Therefore, *SLC43A3*-encoded ENBT1 is clearly a major contributor to the uptake of 6-MP and its cytotoxicity in the RS4;11 cell line. The paradoxical increase in *SLC43A3* expression levels observed in the *SLC43A3* shRNA–transfected RS4;11 cells may be due to an effect of the high level of expression of the shRNAi in this model, which can result in a compensatory increase in the target mRNA, with the shRNAi suppressing mRNA translation (Neumeier and Meister, 2021). There was also a significant increase in the expression of *SLC29A2*. This gene encodes for equilibrative nucleoside transporter 2, which can transport nucleobases with low affinity. So, this may be a compensatory response in these cells to the loss of the primary purine nucleobase transporter ENBT1.

To examine the potential contribution of *SLC43A3* downregulation to the development of cellular resistance to 6-MP, we selected a 6-MP–resistant subclone (ALL-1R) from the ALL-1 cells by incubation with 640 μM 6-MP for 48 hours. The resistant cell line showed a modest reduction in the expression of *SLC43A3* as well as a reduced rate of uptake of 6-MP (Fig. 6). However, the ALL-1R cells also had a reduced expression of *TPMT*, which would contribute to a reduced cellular accumulation of 6-MP metabolites, and an upregulation of the efflux pump *ABCC5* (MRP5). These combined changes, and further metabolic alterations not assessed in this study, likely led to the observed profound level of resistance of the ALL-1R cells to 6-MP. The 6-MP–resistant cells also had a dramatic upregulation of *SLC29A4* (Fig. 6C). This finding is congruent with our correlation data that shows a strong negative association between *SLC29A4* expression and the cytotoxicity of 6-MP in the cell lines examined (Fig. 5B). *SLC29A4* encodes for ENT4, an acidic pH-activated adenosine and monoamine transporter (Barnes et al., 2006; Tandio et al., 2019). ENT4 does not transport nucleobases, and the role of ENT4 in lymphoblasts has not been investigated. Although there is no known direct link between 6-MP and ENT4, it has been noted in the literature that 5-HT_{2A} receptor stimulation and/or serotonin uptake have been attributed to the activation of Rac1 in certain conditions (Dai et al., 2008; Saponara et al., 2018). Rac1 is known to be inhibited by 6-MP. Therefore, one may speculate that *SLC29A4* upregulation could be a novel resistance mechanism, and further investigation is clearly warranted.

In summary, our study shows that *SLC43A3*-encoded ENBT1 is highly expressed in acute lymphoblastic leukemia cells and is the predominant mechanism for 6-MP accumulation by these cells. Although minor differences in *SLC43A3*/ENBT1 expression do not appear to affect 6-MP cytotoxicity, major changes in the expression of *SLC43A3* due to, for example, gain or loss of function polymorphisms may have a significant impact on 6-MP therapeutic efficacy in ALL and other clinical indications.

Authorship Contributions

Participated in research design: Ruel, Nguyen, Hammond.
Conducted experiments: Ruel, Nguyen, Kim, Andrade.
Performed data analysis: Ruel, Nguyen, Hammond.
Wrote or contributed to the writing of the manuscript: Ruel, Hammond.

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Impact of *SLC43A3*/ENBT1 expression and function on 6-mercaptopurine transport and cytotoxicity in human acute lymphoblastic leukemia cells

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Supplemental Table 1: Leukemia cell line information

Cell Line	Leukemia Type	Donor[a]	
		Sex (M/F)	Age (Years)
MOLT-4	T-ALL	M	19
ALL-1	B-ALL	F	7
RS4;11	B-ALL	F	32
REH	B-ALL	F	15
NALM-6	B-ALL	M	19
SUP-B15	B-ALL	M	9

[a] cell origin data from Cellosaurus – ExPASy (https://web.expasy.org/cellosaurus/)

Impact of *SLC43A3*/ENBT1 expression and function on 6-mercaptopurine transport and cytotoxicity in human acute lymphoblastic leukemia cells

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Supplemental Table 2: PCR Primer Sequences

Gene Name	Primer Sequence (5' to 3')	Expected Product (bp)
SLC43A3 *RT-PCR* [a,c]	Fwd – CTGTGTGGACCAGATGC Rev – TGCAGAGGTGAAGGCTA	225 – SLC43A3_1 264 – SLC43A3_2
SLC43A3_2 *RT-PCR* [a,d]	Fwd – GAGACCTTTTTTCTACTCC Rev – TGCAGAGGTGAAGGCTA	197
SLC43A3 *qPCR* [b,c]	Fwd – CTTCTGGAGCTACGCTTTCT Rev – GGAGTTGAGAGTGCCAATGA	100
GAPDH *qPCR*	Fwd – GATTCCACCCATGGCAAATTC Rev – CTGGAAGATGGTGATGGGATT	87
TPMT	Fwd – GGAACAAGGACATCAGCTATTA Rev – CTGATTTCCACACCAACTACA	150

HPRT	Fwd – GTCTTGCTCGAGATGTGATG Rev – TCTACAGTCATAGGAATGGATCT	151
SLC29A1	Fwd – GAGCAGGCAAAGAGGAATCT Rev – GAGAAAGCCAGGACTGAGATATT	106
SLC29A2	Fwd – AAGTAGCTCTGACCCTGGAT Rev – GGAAGACAGTGAAGACTGAAGG	99
SLC29A4	Fwd – CAGACTTCGTGGGCAAGAT Rev – CAGGATGAAGAGGGTGATGAAG	107
ABCC4	Fwd – GGAGAGCCAAGATACAGAGAATG Rev – GAGCACCAGCTCTGAAGTAAT	107
ABCC5	Fwd – ACCATCCACGCCTACAATAAA Rev – GCATCGCACACGTAAACAAA	100

[a] Qualitative reverse transcriptase PCR.

[b] Real-time semi-quantitative PCR

[c] These primer pairs amplify the same sequence for both SLC43A3_1 and SLC43A3_2.

[d] This primer pair amplifies the sequence for SLC43A3_2.