

# Up-regulation of P-glycoprotein confers acquired resistance to 6-mercaptopurine in human chronic myeloid leukemia cells

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**Abstract.** To investigate the mechanisms of cellular resistance to 6-mercaptopurine (6-MP) in chronic myeloid leukemia (CML), a 6-MP resistant cell line (K562-MP5) was established by stepwise selection of the CML cell line (K562). The results of the drug sensitivity analysis of the K562-MP5 cell line revealed the cells to be 339-fold more resistant to 6-MP compared with the parental K562 cells. K562-MP5 cells exhibited decreased accumulation and increased efflux of [<sup>14</sup>C]6-MP and its metabolites. In addition, K562-MP5 cells showed increased [<sup>3</sup>H]MTX transport. K562-MP5 cells over-expressed P-glycoprotein (P-gp) and up-regulated MDR1 mRNA levels. Taken together, these results suggest that the up-regulation

of P-gp, which contributes to the decreased accumulation by increasing the efflux of 6-MP and its metabolites, underlies the mechanism of 6-MP resistance in K562 cells.

## Introduction

The clinical development of resistance to chemotherapeutic drugs is one of the major factors responsible for the failure of cancer chemotherapy. Cancer cells may become resistant to a variety of drugs with different structures or cellular targets, a phenomenon called multidrug resistance (MDR) (1-4). As a nucleobase anti-cancer drug, 6-mercaptopurine (6-MP) is widely used in maintenance therapy for childhood acute lymphoblastic leukemia (ALL) (1,5-7). Recently, we explored the possible mechanisms underlying clinical resistance to 6-MP in ALL, including the up-regulation of ATP-binding cassette (ABC), down-regulation of plasma membrane nucleoside transporters (NTs) and alterations in activities of metabolic enzymes.

P-glycoprotein (P-gp/MDR1) is a member of the ABC superfamily of transmembrane transporters, and it functions as a direct active transporter of a variety of drugs (8). The overexpression of P-gp and MRP1 was shown to correlate with short survival in patients with adult T-cell leukemia (9,10). However, over the last two decades, it has become evident that P-gp is not the only human ABC transporter that, at least *in vitro*, is able to confer resistance to clinically significant chemotherapeutic agents leading to MDR (1,3, 11-16). In humans, the ABC transporter superfamily comprises 49 genes that belong to a 'family tree' with 7 designated branches (A to G) (17,18). The human multidrug resistance protein (MRP) family consists of 10 members and MRPs 1-8 have been isolated and proven to be involved in drug resistance (19-21). Extensive studies showed that over-expression of MRP4, MRP5, MRP8 and breast cancer resistance protein (BCRP/ABCG2) confers resistance to nucleobase and nucleoside analogs (13,14,16,22-27). In a recent study, it was observed that over-expressed MRP4 plays a significant role in conferring resistance to 6-MP in ALL by reducing accumulation of [<sup>14</sup>C]6-MP and its metabolites in cells by acting as an efflux pump (28).

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**Abbreviations:** ABC, ATP-binding cassette; ALL, acute lymphoblastic leukemia; BCRP/ABCG2, breast cancer resistance protein; CBB, Coomassie brilliant blue; CML, chronic myeloid leukemia; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hENT, human equilibrative nucleoside transporter; hCNT, human concentrative nucleoside transporter; HGPRT, hypoxanthine guanine phosphoribosyltransferase; IMPDH, inosine monophosphate dehydrogenase; MDR, multidrug resistance; 6-MMPR, 6-methylmercaptopurine riboside; 2-MP, 2-mercaptopurine; 6-MP, 6-mercaptopurine; MRP, multidrug resistance protein; MTT, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan; MTX, methotrexate; MX, mitoxantrone; NT(s), nucleoside transporter(s); PBS, phosphate-buffered saline; P-gp, P-glycoprotein; PMEAs, 9-(2-phosphonylmethoxyethyl) adenine; 6-TG, 6-thioguanine; TPMT, thiopurine methyltransferase

**Key words:** chronic myeloid leukemia, multidrug resistance, 6-mercaptopurine, P-glycoprotein, ATP binding cassette transporter

Most nucleoside analogs enter cells via the plasma membrane nucleoside transporters; a decrease of nucleoside transporters leads to decreased drug uptake (29-31). Our previous study revealed that down-regulation of human equilibrative nucleoside transporter 1 (hENT1) and human concentrative nucleoside transporters 2 and 3 (hCNT2 and hCNT3), leading to decreased accumulation of 6-MP in cells with acquired resistance to 6-MP, was involved in 6-MP resistance in ALL (28).

As a pro-drug, 6-MP undergoes extensive metabolism inside cells to become active metabolites. The effects of 6-MP are mediated via its intracellular conversion to the 6-thioguanine nucleotides and 6-methyl-thioinosine 5'-monophosphate, which are active metabolites of 6-MP. Thiopurine methyltransferase (TPMT) and hypoxanthine guanine phosphoribosyl transferase (HGPRT) are the two key enzymes responsible for catalyzing these reactions. In the previous study, it was found that the activity of 6-MP metabolic enzyme TPMT was increased in the 6-MP-resistant cells in ALL (28). This study aimed to investigate whether the mechanisms involved in 6-MP-resistant cells apply to chronic myeloid leukemia (CML).

## Materials and methods

**Reagents.** [ $^{14}\text{C}$ ]6-MP (51 mCi/mmol), [ $^{14}\text{C}$ ]inosine monophosphate (50 mCi/mmol) and [ $^{14}\text{C}$ ]hypoxanthine (47 mCi/mmol) (Moravsek Biochemicals, Brea, CA, USA), S-[methyl- $^{14}\text{C}$ ]-adenosyl-L-methionine (55 mCi/mmol) (American Radio-labeled Chemicals Inc., St. Louis, MO, USA), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 9-(2-phosphorylmethoxyethyl) adenine (PMEA) (Gilead, Forest City, CA, USA), Coomassie brilliant blue (CBB) stain solution (Bio-Rad, Hercules, CA, USA), monoclonal antibodies against P-gp (Signet Laboratories Inc., Dedham, MA, USA) and against BCRP/ABCG2 (A.G. Scientific Inc., San Diego, CA, USA) were purchased. Allopurinol, 2-mercaptapurine (2-MP), 6-MP, 6-TG, cisplatin, creatine phosphokinase, Cytarabine (AraC), 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT), ethylenediaminetetraacetic acid (EDTA), etoposide, glutathione, glycine, mitoxantrone (MX), 6-methylmercaptapurine riboside (6-MMPR), MTX, phosphocreatine, 5-phosphoribosyl-1-pyrophosphate, potassium phosphate and vincristine were obtained from Sigma-Aldrich (St. Louis, MO, USA). The monoclonal antibodies against MRP1 (32), MRP4 (13,14), hENT1, hENT2, hCNT2 and hCNT3 (20,34,35) and polyclonal antibodies against MRP5 (33) and MRP8 (16) were previously described.

**Cell culture.** BCR/ABL-positive CML cell line K562 (American Type Culture Collection, Manassas, VA, USA) (termed K562 cells) is a human cell line that was originally derived from a CML patient in blast crisis. A 6-MP-resistant sub-clone (K562-MP5) was selected from K562 cells by growth in the presence of increasing concentrations of 6-MP, up to a final concentration of 5 mM which was achieved over a 3-month period. K562-MP5 cells were grown in drug-free medium for at least 2 weeks prior to being used for experiments. K562-MP5 cells exhibited a stable phenotype as shown by the MTT assay after growth in the absence of the drug for 3 months. HEK293/pcDNA and HEK293/ABCG2-R2 cell lines were

kindly provided by Drs Susan Bates and Robert Robey (NCI, NIH, Bethesda, MD, USA) and were previously described (36). The cells were subcultured twice weekly at 37°C in a 5% CO<sub>2</sub> humidified atmosphere in growth medium comprising DMEM supplemented with heat-inactivated 10% FBS.

**Analysis of drug sensitivity by MTT assay.** Cell viability was determined by a modified MTT cytotoxicity assay as previously described (37). In brief, cells were plated into 96-well tissue culture plates ( $1.2 \times 10^4$  cells/well) in 0.2 ml medium. Following cell incubation at 37°C in a 5% CO<sub>2</sub> humidified atmosphere in DMEM supplemented with heat-inactivated 10% FBS for 70 h, 20  $\mu\text{l}$  of MTT (2 mg/ml PBS) was added to each well. The plates were incubated for another 2 h. The cells were collected in microcentrifuge tubes and the media were removed by centrifugation at 1,500 x g for 2 min. Cell pellets were washed twice with ice-cold phosphate-buffered saline (PBS) and 100  $\mu\text{l}$  of dimethylsulfoxide (DMSO) was added into each tube at room temperature to solubilize the formazan crystals. The dissolved formazan was then transferred into the fresh 96-well plates and the absorbance was determined at 570 nm using an Opsys microplate reader (Dynex Technologies Inc., Chantilly, VA, USA).

**Analysis of accumulation and efflux of [ $^{14}\text{C}$ ]6-MP.** Drug accumulation and efflux experiments were performed with a slight modification of methods previously described (14). In brief, for the accumulation experiments,  $2 \times 10^6$  cells/well of K562 or K562-MP5 cells were seeded in triplicate in 24-well plates and incubated at 37°C with 10  $\mu\text{M}$  [ $^{14}\text{C}$ ]6-MP in complete medium for 60 min. Cells were collected in microcentrifuge tubes and the media were removed by centrifugation at 1,500 x g for 2 min. Cell pellets were washed 3 times with ice-cold PBS, and then radioactivity was measured by liquid scintillation counting. For the efflux experiments,  $2 \times 10^6$  cells/well of K562 or K562-MP5 cells were seeded in triplicate in 24-well plates and were incubated at 37°C in an energy depletion medium (glucose-free, pyruvate-free DMEM containing 10% dialyzed FBS, 5 mM sodium azide) containing 10  $\mu\text{M}$  [ $^{14}\text{C}$ ]6-MP for 60 min. The cells were then washed 3 times with ice-cold PBS and were incubated at 37°C for 30 and 60 min in complete medium without radiolabeled drugs. Cell-associated radioactivity was determined at the end of 60-min incubation in an energy depletion medium and at various subsequent time points.

**Preparation of membrane vesicles and Western blot analysis.** Membrane vesicles were prepared by the nitrogen cavitation method as previously described (14). Briefly, cells from culture were washed twice with ice-cold PBS and once with vesicle buffer [10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 0.2 mM CaCl<sub>2</sub>], and then equilibrated at 4°C under nitrogen pressure at 400 psi (25 kg/cm<sup>2</sup>) for 15 min. The cell homogenate was added with EDTA to a final concentration of 1 mM, diluted with dilution buffer [10 mM Tris-HCl (pH 7.4), 0.25 M sucrose] and centrifuged at 1,500 x g for 10 min to remove nuclei and unlysed cells. The supernatant was layered onto a 35% sucrose cushion [10 mM Tris-HCl (pH 7.4), 35% sucrose, 1 mM EDTA] and centrifuged at 16,000 x g for 30 min. The interface was collected and then centrifuged at 100,000 x g for

45 min. The vesicle pellet was re-suspended in dilution buffer by sequentially using a 26-gauge needle. Vesicles were stored at  $-80^{\circ}\text{C}$  until use. The protein concentrations were determined using the Bradford method (38). Proteins of membrane vesicles were resolved by 4-12% SDS-PAGE and transferred to nitrocellulose filters. P-gp, BCRP/ABCG2, and MRPs 1, 4, 5 and 8 were detected using monoclonal antibodies against P-gp, BCRP/ABCG2, MRP1 and MRP4 (at dilutions of 1:200, 1:500, 1:2,000 and 1:1,000, respectively) and polyclonal antibodies against MRP5 and MRP8 (at dilutions of 1:500) and horseradish peroxidase (HRP)-conjugated secondary antibodies (all at a dilution of 1:1,000). hENT1, hENT2, hCNT2 and hCNT3 were detected using monoclonal antibodies (all at a dilution of 1:10) and HRP-conjugated secondary antibodies (all at a dilution of 1:1,000). Enhanced chemiluminescence (Amersham Biosciences Corp., Piscataway, NJ, USA) was used for visualization. Since actin, the normally used control, was not detectable in the samples prepared from the membrane vesicles, CBB staining was used to demonstrate approximately equal loading.

**RT-PCR assay.** The procedures and protocols from RNeasy® mini handbook were followed. Total cellular RNA was isolated from K562 and K562-MP5 cells using Perfect RNA™ kit from the Eppendorf Co. (Westbury, NY, USA). The total RNA concentration and purity were determined by measuring absorbance at 260 and 280 nm with the UV T60 spectrophotometer. The integrity of total RNA was then checked by agarose gel electrophoresis and ethidium bromide staining. For RT-PCR, 1  $\mu\text{g}$  total RNA samples was used for cDNA synthesis by using cMaster RTplusPCR system and cMaster RT kit. The primer sequence of MRP4 was: sense 5'-TGATGAGCCGTATGTTTTC-3' and antisense 5'-CTT CGGAACGGACTTGACAT-3'. The primer sequence of the internal control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was: sense 5'-GCCAAAAGGGTCATCATCTC-3' and antisense 5'-GTAGAGGCAGGGATGATGTTC-3'. The primer sequence of MDR1 was: sense 5'-ATATCAGCAG CCCACATCAT-3' and antisense 5'-GAAGCACTGGGATG TCCG GT-3'. One-step RT-PCR was carried out for 35 cycles as follows: reverse transcription at  $50^{\circ}\text{C}$  for 30 min, initial denaturation at  $94^{\circ}\text{C}$  for 2 min, template denaturation at  $94^{\circ}\text{C}$  for 15 sec, primer annealing at  $52^{\circ}\text{C}$  for 20 sec and primer extension/elongation at  $68^{\circ}\text{C}$  for 30 sec. The PCR products were separated by denaturing agarose gel electrophoresis. The gel was stained with 1  $\mu\text{g}/\text{ml}$  ethidium bromide and the bands were visualized using the ECL chemiluminescence system.

**Vesicular transport experiments.** The vesicular transport experiments of [ $^3\text{H}$ ]MTX were performed using the rapid filtration method as previously described (16,36). Membrane vesicles prepared from HEK293/pcDNA and HEK293/ABCG2-R2 cells were used as negative and positive controls, respectively. Transport experiments were carried out in medium containing membrane vesicles (10  $\mu\text{g}$ ), 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 10 mM  $\text{MgCl}_2$ , 4 mM ATP or 4 mM AMP, 10 mM phosphocreatine, 100  $\mu\text{g}/\text{ml}$  creatine phosphokinase, and 0.5  $\mu\text{M}$  radiolabeled MTX, in a total volume of 50  $\mu\text{l}$ . Reactions were carried out at  $37^{\circ}\text{C}$  for 10 min and were stopped by the addition of 3 ml of ice-cold stop solution [0.25 M sucrose, 100 mM NaCl, and 10 mM Tris-HCl (pH 7.4)]. For the rapid

filtration step, samples were passed through 0.22  $\mu\text{m}$  GVWP filters (Millipore Corp., Billerica, MA, USA) pre-soaked in the stop solution under vacuum. The filters were washed 3 times with 3 ml of ice-cold stop solution and dried at room temperature for 30 min. Radioactivity was measured using a liquid scintillation counter. Rates of net ATP-dependent transport were determined by subtracting the values obtained in the presence of 4 mM AMP from those obtained in the presence of 4 mM ATP.

**HGPRT activity.** HGPRT activity in cell lysates was estimated by the formation of [ $^{14}\text{C}$ ]inosine monophosphate from [ $^{14}\text{C}$ ]hypoxanthine by a modification of a previously described method (28,39) according to the following protocol: 20  $\mu\text{l}$  cell lysates, 10  $\mu\text{l}$  water, and 70  $\mu\text{l}$  cocktail (20  $\mu\text{l}$  of 0.5 M glycine buffer, 10  $\mu\text{l}$  of 50 mM  $\text{MgCl}_2$ , 10  $\mu\text{l}$  of 10 mM 5-phosphoribosyl-1-pyrophosphate, 10  $\mu\text{l}$  of 1.5 mM [ $^{14}\text{C}$ ]hypoxanthine, and 20  $\mu\text{l}$  water) were mixed and incubated for 15 min at room temperature. The reaction was stopped by placing mixtures on ice and adding 5  $\mu\text{l}$  of 0.25 M EDTA. Sample mixture or standard (20  $\mu\text{l}$ ) (containing the known amount of [ $^{14}\text{C}$ ] inosine monophosphate) were detected on polyethyleneimine cellulose paper, dried, and washed 3 times with 3 ml of 1 mM  $\text{NH}_4\text{HCO}_3$ . The bound radioactivity was counted in 5 ml of liquid scintillation solution.

**TPMT activity.** TPMT activity was analyzed by a modification of a previously described radiochemical assay (28,40). Cell lysate (100  $\mu\text{l}$ ) was incubated with 7.5 mM 6-MP, 15 mM glutathione, 50  $\mu\text{M}$  allopurinol, 1 M potassium phosphate (pH 7.5), and 23  $\mu\text{M}$  (55  $\mu\text{Ci}/\mu\text{mol}$ ) S-[methyl- $^{14}\text{C}$ ]adenosyl-L-methionine (in a total volume of 160  $\mu\text{l}$ ) for 60 min. The radiolabeled 6-MP that was produced was extracted with 20% isoamyl alcohol in toluene, and counted in 5 ml of liquid scintillation solution. Results were normalized to 1 mg protein, based on the amount of protein used for the 100  $\mu\text{l}$  of lysate. One unit of enzyme activity represents the formation of 1 nmol of 6-MP per hour of incubation.

**Statistical analysis.** The data were analyzed by the unpaired Student's t-test and  $P < 0.05$  was considered to be statistically significant.

## Results

**Drug resistance profile of 6-MP-resistant K562 cells.** To investigate the mechanisms of cellular resistance to 6-MP in CML, a 6-MP-resistant cell line was established using the CML K562 cells. K562 cells were made resistant to 6-MP by stepwise selection in 6-MP. Analysis of drug sensitivity of the resulting cell line K562-MP5 indicated that K562-MP5 cells were 339-fold more resistant to 6-MP compared with parental K562 cells (Table I, Fig. 1A). K562-MP5 cells were highly cross-resistant to other nucleobase analogs such as 6-TG and exhibited lower levels of resistance to 2-MP and PMEA (Table I, Fig. 1). K562-MP5 cells were not resistant to AraC, but were 25-fold more sensitive to 6-MMPR. In addition, K562-MP5 cells were resistant to the non-nucleobase agents MX, vincristine and cisplatin, but were more sensitive to MTX.

Table I. Drug resistance profile of K562-MP5 cells<sup>a</sup>.

Reagents	IC <sub>50</sub> (μM)		Relative resistance <sup>b</sup>
	K562	K562-MP5	
6-MP	22.6±5.2	7647±135 <sup>e</sup>	339.0
6-TG	4.3±1.3	387±123 <sup>e</sup>	91.0
2-MP	1369±158	7245±329 <sup>d</sup>	5.3
PMEA	346±111	805±127 <sup>c</sup>	2.3
AraC	6.3±2.0	7.0±2.2	1.1
6-MMPR	2.4±0.4	0.10±0.01 <sup>e</sup>	0.04
MX	0.010±0.003	0.015±0.002	1.5
Vincristine	0.13±0.04	0.41±0.11 <sup>d</sup>	3.2
Cisplatin	149±33	91±38 <sup>c</sup>	2.6
MTX	1.54±0.12	0.64±0.07 <sup>d</sup>	0.4

<sup>a</sup>Values are given as mean ± SD of at least three separate experiments. Data were analyzed by the unpaired Student's t-test. IC<sub>50</sub> of K562-MP5 cells differed significantly from K562 cells at <sup>c</sup>P<0.05, <sup>d</sup>P<0.01 and <sup>e</sup>P<0.001. <sup>b</sup>Relative resistance was obtained by dividing the IC<sub>50</sub> value K562 cells by the IC<sub>50</sub> value of K562-MP5 cells for a particular treatment.

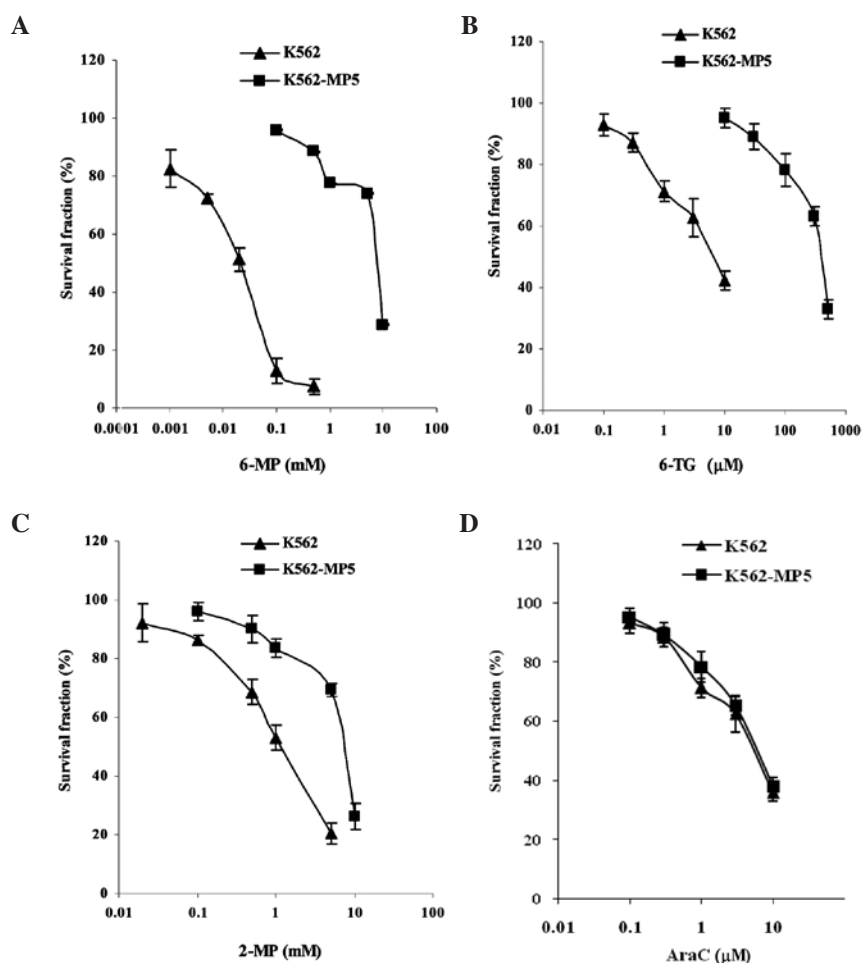


Figure 1. Sensitivity of K562 (▲) and K562-MP5 (■) cells to 6-MP, 6-TG, 2-MP or AraC was analyzed using the MTT cytotoxicity assay as described in Materials and methods. Data points are the mean ± SD of at least three separate experiments.

*Analysis of accumulation and efflux of [<sup>14</sup>C]6-MP.* To determine whether decreased accumulation of the drug was involved in the resistance of K562-MP5 cells, the accumula-

tion of radioactivity derived from [<sup>14</sup>C]6-MP was analyzed. The pilot testing had revealed that the maximum accumulation of [<sup>14</sup>C]6-MP and its metabolites was achieved at 60 min.



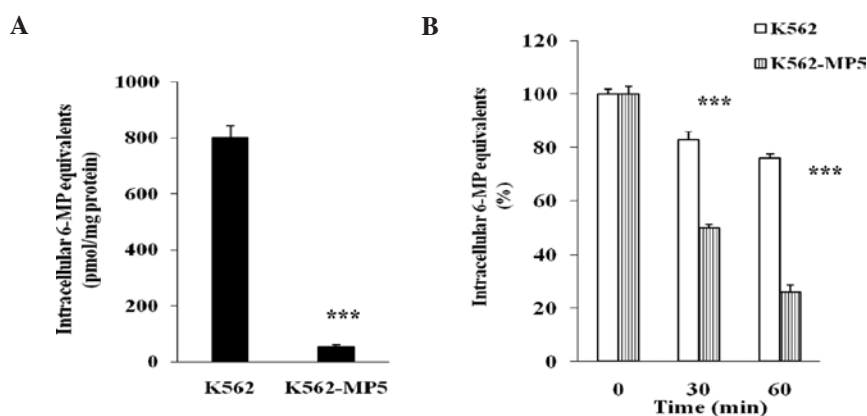


Figure 2. Time-dependent accumulation and efflux of [ $^{14}\text{C}$ ]6-MP and/or its metabolites in K562 and K562-MP5 cells. (A) Accumulation of [ $^{14}\text{C}$ ]6-MP and its metabolites at 60 min in K562 and K562-MP5 cells in complete medium. Values are given as mean  $\pm$  SD, n=3. Data were analyzed by the unpaired Student's t-test. Accumulation of [ $^{14}\text{C}$ ]6-MP and its metabolites at 60 min in K562-MP5 cells decreased significantly compared with K562 cells at \*\*\*P<0.001. (B) Time course of efflux of [ $^{14}\text{C}$ ]6-MP and its metabolites in K562 and K562-MP5 cells after various efflux times. Values are given as mean  $\pm$  SD, n=3. The percentages of retained 6-MP equivalents after 30 and 60 min efflux are indicated in K562 and K562-MP5 cells relative to that at 0 time. Data were analyzed by the unpaired Student's t-test. After 30 and 60 min efflux, the retained 6-MP equivalents in K562-MP5 cells decreased significantly compared with K562 cells at \*\*\*P<0.001.

The accumulation of [ $^{14}\text{C}$ ]6-MP equivalents was markedly reduced in K562-MP5 compared with K562 cells (Fig. 2A), with K562-MP5 cells accumulating only 6% of [ $^{14}\text{C}$ ]6-MP equivalents in comparison to K562 cells.

To further dissect the basis of the decreased accumulation of [ $^{14}\text{C}$ ]6-MP equivalents in K562-MP5 cells, separate efflux experiments were performed. K562 and K562-MP5 cells were allowed to accumulate [ $^{14}\text{C}$ ]6-MP equivalents in the energy depletion medium, which prevents the activity of ATP-dependent efflux pumps. After 60 min, the intracellular accumulation was comparable in K562 and K562-MP5 cells. Cells were then switched to complete medium to allow efflux, and intracellular radioactivity was measured after 30 and 60 min. After 30 min efflux, 50% of the accumulated 6-MP equivalents were released from K562-MP5 cells, whereas only 16% of the accumulated 6-MP equivalents were released from K562 cells. After 60 min efflux, 73% of the accumulated 6-MP equivalents were released from K562-MP5 cells, whereas only 25% of the accumulated 6-MP equivalents were released from K562 cells (Fig. 2B). These results indicate that increased efflux was involved in the reduced cellular accumulation in K562-MP5 cells.

**Expression of P-gp, MRPs, BCRP/ABCG2 and NTs.** The increased efflux exhibited by K562-MP5 cells, in combination with cross-resistance to vincristine, suggested that P-gp is involved in the resistance phenotype of the cell line. As shown in Fig. 3A, P-gp was markedly over-expressed in K562-MP5 cells. However, the expression of MRP4, a pump which was previously found to be over-expressed in ALL cells made resistant to 6-MP (26), was similar in K562 and K562-MP5 cells. Expression of MRP1, MRP5, MRP8 and BCRP/ABCG2 was undetectable (data not shown) and the levels of influx NTs (hENT1, hENT2, hCNT2 and hCNT3) were similar in the two cell lines (Fig. 3A).

**Expression of MDR1 mRNA levels in K562 and K562-MP5 cells.** The RT-PCR assay was used to ascertain whether the mRNA levels of MDR1 were up-regulated in the K562-

MP5 cells. As shown in Fig. 3B, MDR1 mRNA levels were significantly increased in K562-MP5 cells compared with K562 cells.

**Transport of [ $^3\text{H}$ ]MTX.** P-gp is an ATP-dependent membrane efflux pump, which is able to transport anti-cancer drugs such as the established P-gp substrate MTX, leading to drug resistance. P-gp-dependent transport activity was examined by analyzing the ability of the pump to transport [ $^3\text{H}$ ]MTX into inside-out membrane vesicles. The ATP-dependent MTX transport with membrane vesicles prepared from K562-MP5 cells was significantly higher than that from K562 cells with a transport rate of [ $^3\text{H}$ ]MTX 0.42 and 0.05 pmol/min/mg protein by K562-MP5 and K562 membrane vesicles, respectively (Fig. 4).

**Enzyme activities of HGPRT and TPMT in K562 and K562-MP5 cells.** Since HGPRT and TPMT are the two key enzymes associated with the metabolism of 6-MP, the activity of these enzymes was analyzed. HGPRT activity is similar in K562 and K562-MP5 cells, with activity of  $1360 \pm 200$  and  $1360 \pm 180$  pmol/min/mg protein, respectively. TPMT activity was also similar in K562 and K562-MP5 cells, with activity of  $0.489 \pm 0.063$  and  $0.556 \pm 0.110$  U/mg protein, respectively.

## Discussion

The studies of Hart *et al* and Wuchter *et al* showed that P-gp may contribute to the poor prognosis of adult T-cell leukemia and adult acute myeloid leukemia (41,42). Kuwazuru *et al* examined the P-gp expression levels in fresh leukemia cells from CML patients in blast crisis and found that 6 out of 11 patients (9 in the refractory state) were P-gp-positive. In addition, these authors showed that P-gp-expression levels correlate with the response of patients to chemotherapy (43). However, none of the aforementioned studies attempted to elucidate the mechanisms underlying the P-gp function. We recently demonstrated that the up-regulation of MRP4 and

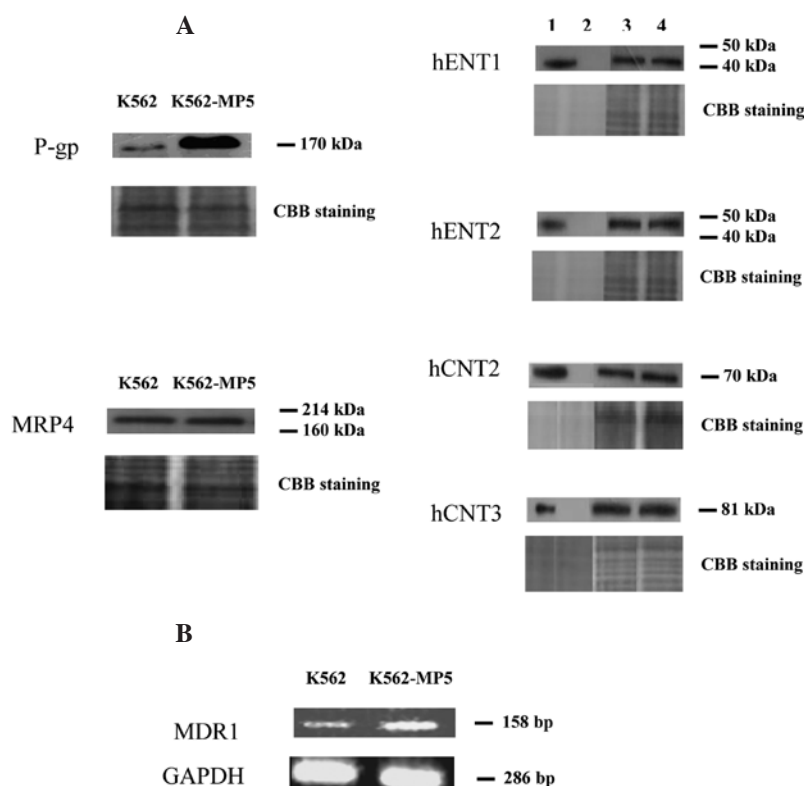


Figure 3. (A) Western blot analysis of P-gp, MRP4, hENT1/2 and hCNT2/3 in membrane vesicle preparations from K562 and K562-MP5 cells. Membrane vesicles were prepared as described in Materials and methods from sensitive and resistant cells (K562 and K562-MP5) and, as controls, from yeast cells producing recombinant hENT1/2 or hCNT2/3. Protein was resolved by SDS-PAGE on 4-12% gel and electrotransferred to nitrocellulose membranes. Mobilities of the molecular mass markers are indicated in kilodaltons. In the right panel, lanes 1, 2, 3 and 4 are yeast cells highly producing the indicated protein (positive control), yeast cells without the indicated protein (negative control), K562 and K562-MP5 cells, respectively. Proteins of 20  $\mu$ g/lane [for the positive and negative controls of hENT1/2 (0.2  $\mu$ g/lane) and hCNT2/3 (0.5  $\mu$ g/lane)] were loaded. The bottom panel is a section of an identical gel stained with Coomassie brilliant blue to demonstrate approximately equal loading. (B) RT-PCR analysis of mRNA expression levels of MDR1 in K562 and K562-MP5 cells.

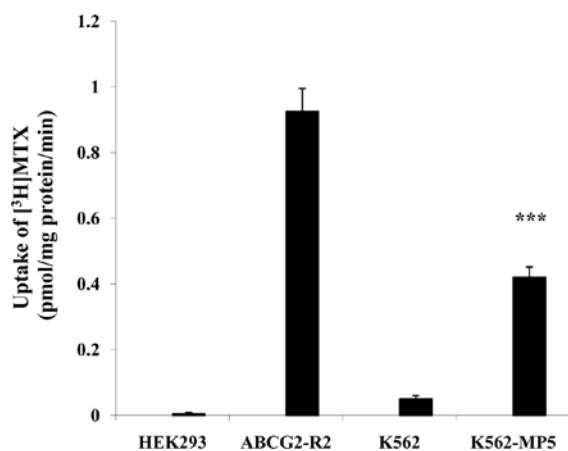


Figure 4. Uptake of [ $^3$ H]MTX into inside-out membrane vesicles derived from K562 and K562-MP5 cells. Values are given as mean  $\pm$  SD,  $n=3$ . Membrane vesicles prepared from HEK293/pcDNA and HEK293/ABCG2-R2 are used as negative and positive controls, respectively. Data were analyzed by the unpaired Student's *t*-test. The ATP-dependent uptake of [ $^3$ H]MTX in K562-MP5 cells increased significantly compared with K562 cells at \*\*\* $P<0.001$ .

down-regulation of influx transporters hENT1, hCNT2 and hCNT3, which lead to decreased accumulation of 6-MP in cells with acquired resistance to 6-MP, play a significant role in 6-MP resistance in ALL (28). To investigate whether or not these drug resistance factors also play a potential role

in CML, we established a resistant cell line (K562-MP5) by stepwise selection using a CML cell line (K562).

The present results showed that K562-MP5 cells were highly resistant to 6-MP in comparison with the parental K562 cells. In addition, K562-MP5 cells were cross-resistant to other anti-cancer drugs such as 6-TG, 2-MP, vincristine, MX, cisplatin and anti-hepatitis B agent PMEA (Table I, Fig. 1). P-gp and MDR1 mRNA levels were up-regulated in K562-MP5 cells (Fig. 3), which is consistent with the results of Zeng *et al* who showed that high expression of P-gp in the surface membranes of cells is responsible for resistance to 6-MP (44). Compared to K562 cells, K562-MP5 cells had significantly lower accumulation and higher efflux of [ $^{14}$ C]6-MP equivalents (Fig. 2). Transport of [ $^3$ H]MTX into membrane vesicles prepared from K562-MP5 cells was significantly higher than that of K562 cells (Fig. 4). Although MTX is also a substrate of MRPs 1, 2, 3, 4 and BCRP/ABCG2, only P-gp was significantly up-regulated in K562-MP5 cells (Fig. 3A). The expression levels of MRP4 were similar in K562 and K562-MP5 cells (Fig. 3A). Furthermore, MRP1 and BCRP/ABCG2 were not detected in K562 and K562-MP5 cells (data not shown). No studies are currently available showing that MRP2 and MRP3 are expressed in CML cells. The results of the present study validate the function of P-gp as an efflux transporter and suggest that over-expression of P-gp confers resistance to 6-MP in CML.

These results are in contrast to our previous results which showed that the levels of hENT1, hCNT2 and hCNT3 in 6-MP-resistant CEM-MP5 cells were decreased in comparison to levels in parental CEM cells (28), and that levels of hENT1, hENT2, hCNT2 and hCNT3 were similar in K562 and K562-MP5 cells (Fig. 2A). Therefore, influx NTs are not involved in the decreased accumulation of 6-MP and its metabolites in K562-MP5 cells. Additionally, unlike our previous study, which showed that TPMT activity was higher in 6-MP-resistant CEM-MP5 cells, neither HGPRT nor TPMT, the key enzymes involved in the metabolism of 6-MP, showed a difference in activity between K562-MP5 cells and K562 cells (data shown in Results). The results therefore suggest that plasma membrane influx NTs and the enzymes involved in 6-MP metabolism play a role in the resistance of 6-MP in ALL (28), but not in CML.

Consistent with our previous findings in CEM-MP5 and CEM cells (28), K562-MP5 cells were found to be significantly responsive to 6-MMPR and MTX compared to the wild-type K562 cells (Table I). 6-MMPR, a methylated metabolite of 6-MP, is able to bypass resistance to the parental drug. This phenomenon is explained by the recent findings that the 6-MP resistant cells have a reduced purine nucleotide synthesis and lower levels of ribonucleoside triphosphates compared with the parental cells (5,7). In contrast, the influx of 6-MMPR into these cells was not significantly altered (5,7). K562-MP5 cells, which express higher levels of P-gp, were significantly more responsive to MTX than K562 cells. Two possible explanations for this phenomenon are: i) P-gp can only efflux the MTX monoglutamate (45,46); however, after incubating the cells for 72 h, the predominance of MTX in the K562-MP5 cells had been converted to MTX polyglutamates, and ii) K562-MP5 cells produced higher levels of MTX polyglutamates than the K562 cells, and the MTX polyglutamates potentially inhibit the *de novo* biosynthesis of purines.

In conclusion, the present results indicate that the up-regulation of P-gp, which contributes to the decreased uptake and increased efflux of 6-MP and its metabolites, plays a critical role in 6-MP resistance in CML. These findings suggest that the mechanisms of 6-MP resistance in CML are different from those of ALL.

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