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Research Paper

Constitutive Overexpression of P-glycoprotein, Rather than Breast Cancer Resistance Protein or Organic Cation Transporter 1, Contributes to Acquisition of Imatinib-Resistance in K562 Cells

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Purpose. The purpose of this study was to investigate the contribution of drug transporters in acquired imatinib-resistance. Specifically, we focused on the efflux transporters, P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), and an influx transporter, organic cation transporter 1 (OCT1).
Materials and methods. We established imatinib-resistant K562 cells (K562/IM). Real-time PCR or Western blot analyses were performed to examine the mRNA or protein levels. Alamar blue method was used in the cytotoxicity assay. The transport activities and intracellular imatinib levels were measured by flow cytometry and HPLC, respectively.
Results. K562/IM displayed a 47-fold increase in resistance to imatinib over the parent K562 cells. Both P-gp and BCRP were overexpressed in K562/IM relative to K562. Furthermore, the intracellular imatinib level was markedly reduced in K562/IM. Interestingly, cyclosporin A, a P-gp inhibitor, but not fumitremorgin C, a BCRP inhibitor, restored both imatinib-sensitivity and the intracellular imatinib level. By contrast, no significant difference in the expression and function of OCT1 was observed between K562/IM and K562.
Conclusions. P-gp, rather than BCRP or OCT1, is partially responsible for the development of imatinib-resistance due to constitutive and functional overexpression, leading to reduced intracellular accumulation of imatinib in K562/IM.

KEY WORDS: breast cancer resistance protein; drug resistance; imatinib; organic cation transporter 1; P-glycoprotein.

INTRODUCTION

Approximately 95% of chronic myelogenous leukemia (CML) and about 25% of acute lymphoblastic leukemia (ALL) patients are positive for the Philadelphia chromosome (Ph), which results from a reciprocal translocation between chromosomes 9 and 22 (1). This translocation results in the formation of a fusion transcript (BCR-ABL protein) possessing a constitutively activated tyrosine kinase domain, the activity of which is crucial to the pathogenesis of Ph^+ CML and ALL (1). Imatinib selectively inhibits the tyrosine kinase activity, and is successfully used for the treatment of BCR-ABL-dependent CML and ALL as well as c-KIT-dependent gastrointestinal stromal tumors (2). However, in advanced-phase CML and Ph^+ ALL, the response to imatinib may be of a short duration. Therefore, the efficacy of the drug declines with use in more advanced stages of the disease and most patients treated with imatinib in accelerated or blast phase have been reported to develop resistance (3–5).

Several different mechanisms for imatinib-resistance have been proposed including amplification of the *BCR-ABL* gene (6) and clonal evolution (7,8). However, the most significant mechanism of imatinib-resistance involves *BCR-ABL* gene mutations, which interfere with binding of imatinib to the drug target (9,10). Consequently, alternative BCR-ABL kinase inhibitors, such as PD166326 (11), nilotinib (AMN107; 12) or dasatinib (BMS-354825; 13), have been developed. However, not all leukemic samples from imatinib-resistant patients displayed amplification and/or mutation of *BCR/ABL* (14–16). Other mechanisms of resistance to imatinib are thought to be related to pharmacokinetic factors of drug disposition.

Recent papers have shown that imatinib is a substrate of P-glycoprotein (P-gp; MDR1, ABCB1), a drug efflux transporter commonly involved in multidrug resistance (14,17–22). Thus, P-gp-mediated active transport of imatinib out of the

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ABBREVIATIONS: BCRP, breast cancer resistance protein; IM, imatinib; OCT1, organic cation transporter 1; P-gp, P-glycoprotein.

target cells has been proposed as a process that prevents imatinib from reaching its intracellular target, BCR-ABL. Giles *et al.* (23) showed that P-gp expression in CML cells was observed more frequently in patients with advanced stage CML. Therefore these data suggest that P-gp could be a potential candidate contributing to imatinib-resistance. Additionally, recent studies indicate an interaction of imatinib with breast cancer resistance protein (BCRP; ABCG2). Imatinib acts as both substrate and inhibitor of BCRP (24). Furthermore, BCRP appears to be involved in the bioavailability, pharmacokinetics and disposition of imatinib, suggesting that BCRP functions as an efflux transporter of imatinib *in vivo* (25). However, it was recently shown that imatinib directly inhibits BCRP-mediated transport without being a competitive substrate (26,27). Thus, it is unclear whether BCRP is responsible for imatinib efflux, and thereby contributes to imatinib-resistance.

Cellular uptake of imatinib appears to be mediated by human organic cation transporter 1 (OCT1; 28–30). A recent clinical study conducted by Crossman *et al.* (29) reported that the expression level of *OCT1* mRNA prior to treatment with imatinib in non-responders was only one eighth that seen in responders. Elevated intracellular levels of imatinib should therefore result in a better elimination of BCR-ABL positive cells. This finding is supported by the clinical results of a recent trial involving resistant CML patients. Kantarjian *et al.* (31) reported that dose escalation of imatinib could overcome resistance to standard-dose therapy in patients with CML. Thus, intracellular levels of imatinib may be crucial even for the treatment of resistant CML patients.

Thus, several independent lines of evidence suggest the involvement of P-gp, BCRP and OCT1 in imatinib-resistance. However, it is not known which of these transporters is the most crucial for acquired resistance of CML cells to imatinib. This question has been difficult to resolve, in part, because the majority of previous studies used cell lines engineered to overexpress a single transporter. To specifically address this issue, we have established imatinib-resistant K562 (K562/IM) cells, derived from human CML, by culturing parent K562 cells with gradually increasing concentrations of imatinib. We then analyzed whether imatinib-resistance in K562/IM cells can be attributed to the functional variation between P-gp, BCRP and OCT1.

MATERIALS AND METHODS

Cells, Media and Reagents

BCRP cDNA-transfected K562 cells (K562/BCRP) and vincristine-resistant K562 cells (K562/VCR) were kindly provided by Dr. Y. Sugimoto (Japan Foundation for Cancer Research, Tokyo, Japan) and Dr. T. Tsuruo (University of Tokyo, Tokyo, Japan), respectively. Imatinib-resistant K562 cells (K562/IM) were generated by culturing K562 cells in media with gradually increasing concentrations of imatinib. The start concentration of imatinib was 0.3 μM, which caused almost 50% inhibition of cell growth. The concentration of imatinib was then increased in increments of 0.3 μM up to a maximum of 10 μM. K562, K562/VCR and K562/BCRP cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum under an atmosphere of 5% CO_2–95% air at 37°C. K562/IM cells were cultured under the same conditions in the presence of 5 μM imatinib. The drug was depleted from the medium 3 days prior to all experimental procedures to allow complete efflux of intracellular imatinib in K562/IM cells. Imatinib and cyclosporin A (CsA) were a kind gift from Novartis Pharma AG (Basel, Switzerland). Fumitremorgin C (FTC) and pheophorbide A (PhA) were provided from Dr. Robert W. Robey and Dr. Susan E. Bate (National Cancer Institute, Betheda, MD). Amantadine and alamar blue were purchased from Sigma-Aldrich (St. Louis, MO) and Alamar Bioscience, Inc. (Sacramento, CA), respectively. All other chemicals were of the highest purity available.

Cytotoxicity Assay

To quantify the cytotoxic effect of imatinib on cell growth, alamar blue was used in a semiautomatic fluorescence method (32). Alamar blue is nonfluorescent and is cleaved to a fluorescent product by living cells. Because this activity is dependent on cell viability, the amount of fluorescence is directly correlated to the number of living cells. Cells were harvested and plated at 5.0×10^4 cells/well in a volume of 50 μl in 96-well plates. Fifty microliters of culture medium containing the drug at the desired concentration was added, and the cells were then cultured for 48 h under the conditions described above. Ten microliters of alamar blue was added to each well, which were then incubated under the same culture conditions for an additional 4 h. Fluorescence was measured using a fluorescence plate reader (Fluoroscan Ascent; Labsystem, Helsinki, Finland) with excitation at 485 nm and emission at 528 nm. Relative cell viability (%) was expressed as a percentage of the fluorescence observed using cells untreated with imatinib. IC_{50} (inhibitory concentration at 50%), derived from the dose response curve, was plotted against the corresponding drug concentration and calculated from the four-parameter logistic model using the following equation:

$$IC_{50} = 10^{f(\chi)}$$
$$f(\chi) = \log(A/B) \times (50 - C)/(D - C) + \log(B)$$

Two plot points which contain an inhibitory concentration at 50% were used. A is the concentration of drug that exceeded the amount required to give 50% cell viability. B is the concentration of drug lower than the amount required to give 50% cell viability. C is cell viability (%) at B. D is cell viability (%) at A.

Fluorescence *in Situ* Hybridization (FISH)

FISH analysis of fusion genes in K562 and K562/IM cells was commissioned by SRL, Inc. (Tokyo, Japan). Briefly, FISH analysis for rearranged *BCR/ABL* fusion gene was performed using LSI *BCR/ABL* dual color DNA probe (Vysis, Downers Grove, IL) to detect t(9;22)(q34;q11.2) in interphase cells according to the manufacturer's instructions. The *ABL* probe consisted of two overlapping biotin labeled cosmids that hybridize to 9q34. The MBCR probe consisted of three overlapping digoxigenin-labeled cosmids that hybridize to the MBCR at 22q11.2. The hybridization, washing and analysis procedures have been described in a previous report (33).

Sequence of ABL Kinase Domain

The kinase domain of *ABL* was amplified by PCR. The nested version was chosen using the forward primer, 5′-GGGAGGGTGACCATTACAGG-3′, and the reverse primer, 5′ -TACTAGAGAAGGACTACCGCA-3′, for the first reaction, as well as the forward primer, 5′ -GGGAGGGTG TACCATTACAGG-3′, and the reverse primer, 5′ -ACAGG GGACACCTACACAGC-3′, for the second one. The mutation was confirmed by DNA sequencing of the entire kinase domain of the *ABL* coding region using an Applied Biosystems 3130 Genetic Analyzer (Foster City, CA).

mRNA Isolation and cDNA Synthesis

For mRNA extraction, MagNA Pure LC mRNA Isolation Kit II (Roche Diagnostics, Tokyo, Japan) was used according to the instruction manual. In this automated process, the cell samples were dissolved in a buffer containing a chaotropic salt and an RNase inactivator. The samples were homogenized with Lysing Matrix D (Qbiogene Inc., Carlsbad, CA) and FastPrep (Qbiogene Inc.) at an oscillation speed 4.5 for 30 s. The 3′-poly (A^+) from the released mRNA hybridizes to the added biotin-labeled oligo (dT). This complex is immobilized onto the surface of streptavidin-coated magnetic beads. After a DNase digestion step, unbound substances were removed by several washing steps, and purified mRNA was eluted (elution volume, 50 μl) with a low-salt buffer. cDNA was synthesized by using High-Capacity cDNA Archive Kit (Applied Biosystems Inc.). Briefly, 50 μl of mRNA substrate, 10 μl of RT buffer, 4 μl of dNTP mixture, 10 μl of RT random primers, 5 μl of MultiScribe reverse transcriptase and 21 μl of nuclease-free water were used for cDNA synthesis. After each reverse transcription, cDNA was stored at −30°C.

Real-Time PCR

We performed a TaqMan quantitative real-time RT-PCR using ABI PRISM 7900 sequence detection system (Applied Biosystems Inc.) to determine the expression level of *hMdr1a*, *hABCG2*, *hOCT1* and *hβ-actin*, by means of the standard protocol described by the manufacturer. TaqMan *hβ-actin* control reagents, the primer sets and products of TaqMan Gene Expression Assays were purchased from Applied Biosystems Inc. as follows: *hMdr1a*, Hs00184491_m1; *hABCG2*, Hs00184979_m1; *hOCT1*, Hs00231250_m1; and *hβ-actin*, 4310881E.

Western Blots

K562, K562/VCR, K562/BCRP and K562/IM cells were sonicated in a buffer comprising of 230 mM sucrose, 5 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, 1 mg/ml leupeptin, and 1 mg/ml pepstatin A. The homogenate is referred to as the whole fraction. To separate the crude membrane fraction, the homogenate was centrifuged twice at 3,000×*g*×15 min and the supernatant was further centrifuged at 100,000×*g*×30 min. The resultant pellet is referred to as the crude membrane fraction. After measurement of the protein content using a BCA protein assay reagent (Pierce, Rockford, IL), each sample was mixed in a loading buffer (2% SDS, 125 mM Tris-HCl, 20% glycerol, 5% 2-mercaptoethanol) and heated at 100°C for 2 min. The samples were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA) by semi-dry electroblotting. The blots were blocked overnight at 4°C with 2% ECL advance Blocking agents (Amersham Biosciences Corp., Piscataway, NJ) in Tris-buffered saline (TBS) containing 0.3% Tween 20 (TBS-T), and incubated for 1 h at room temperature with primary antibody specific for P-gp (C219 monoclonal antibody, Signet Laboratories, Dedham, MA), BCRP (BXP-21 monoclonal antibody, Alexis biochemicals, San Diego, CA), OCT1 (a gift for Prof. K. Inui, Kyoto University, Kyoto, Japan), Na^+/K^+-ATPase α-1 subunit (Upstate Biotechnology Inc., Lake Placid, NY), c-Abl and BCR-Abl (Santa Cruz Biotechnology, Inc., CA), and β-actin (Sigma Aldrich, Japan). The blots were washed with TBS-T and incubated with the secondary antibody (horseradish peroxidase linked antirabbit immunoglobulin F $(ab)_2$ or horseradish peroxidase linked antimouse immunoglobulin $F(ab)_2$, Amersham Biosciences Corp.) for 1 h at room temperature. Immunoblots were visualized with an ECL system (ECL Advance Western Blotting Detection Kit, Amersham Biosciences Corp.). Na^+/K^+-ATPase α-1 subunit was used to confirm equal loading of crude membrane preparation.

Flow Cytometry

The flow cytometry studies presented here are based on those described previously (34). Briefly, suspended cells were incubated in complete medium (RPMI with 10% FCS) with 1 μM rhodamine 123 (R123) with or without 10 μM CsA, a P-gp inhibitor, for 30 min at 37°C in 5% CO_2. Cells were then washed with cold complete medium and incubated for 1 h at 37°C in R123-free complete medium containing 10 μM CsA to generate the CsA /Efflux histogram, or without CsA to generate the efflux histogram. The cells were washed twice with cold PBS and analyzed by flow cytometry. P-gp mediated transport was assessed using 1 μM R123 with imatinib (5, 10 and 50 μM). When assessing BCRP transport, 10 μM PhA was used as a substrate, and FTC (10 μM) was used as the inhibitor. BCRP mediated transport was assessed using PhA (10 μM) with imatinib (1, 5 and 10 μM). Results are representative of at least three separate experiments. Samples were analyzed on a FACS Calibur (Becton Dickinson, San Jose, CA). R123 was detected with a 488 nm argon laser, whereas PhA was detected using a 635 nm red diode laser. At least 10,000 events were collected for all of flow cytometry studies. Mean intensities of fluorescence (MIF) were obtained from control experiments using R123 or PhA alone incubated in each cell and normalized to 100%.

Intracellular Imatinib Accumulation

For single measurements, 1×10^7 cells were incubated in 5 ml RPMI containing 5 μM of imatinib with or without 5 μM of CsA or 1 mM of amantadine. After an incubation time of 5 h at 37°C and 5% CO_2, cells were centrifuged at 700×*g*, 4°C, for 5 min. The resultant cell pellet was washed once in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), centrifuged and the pellet washed twice with

BSA-free ice-cold PBS. The cells were then lysed by incubating in 300 μl of 50% methanol (HPLC mobile phase/methanol=1/1) for 30 min. The resultant solution was centrifuged at 10,000×*g*, 4°C for 10 min. The supernatant was used for quantification of imatinib using the high performance liquid chromatography (HPLC) method described below (18). The pellet was added with 1N NaOH to solubilize the cells. The protein content of the solubilized cells was determined by the method of Bradford, using a Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA) with bovine γ-globulin as a standard.

HPLC Analysis

The concentration of imatinib was determined using HPLC (model LC-6A; Shimadzu, Kyoto, Japan). The HPLC analysis was performed as described previously (18). The separation was performed using a reversed-phase column (Lichrospher 100 RP-18, 5-μm particle size, 250×4 mm i.d.) at 40°C. The mobile phase was a 4:6:0.1 mixture of acetonitrile, water and 0.145 mM phosphoric acid, which contained 2.3 g/l of 1-octanesulfonic acid sodium salt. The flow rate was 1.0 ml/min. Imatinib was detected by UV absorption at 267 nm.

Statistical Analysis

Data were analyzed statistically by analysis of two-way factorial ANOVA with post hoc comparison by Fisher's PLSD and Student's *t* test to evaluate the significance of differences between groups. A *p* value of 0.05 or less was considered significant.

RESULTS

Establishment of Imatinib-Resistant K562 Cells

Imatinib-resistant K562 (K562/IM) cells were established by exposure of K562 cells to gradually increasing concentrations of imatinib (up to 10 μM). In addition, we used P-gp-overexpressed vincristin-resistant (K562/VCR) cells and BCRP-overexpressed BCRP cDNA-transfected K562 (K562/BCRP) cells. To compare the sensitivity of K562, K562/VCR, K562/BCRP and K562/IM cells to imatinib, cell viability was assessed by the alamar blue method. K562/IM cells were 46.7-fold as more resistant to imatinib as the parent K562 cells (Table I). K562/VCR cells showed a 4.4-fold increase in resistance to imatinib over K562 cells, whereas K562/BCRP cells showed comparable sensitivity to K562 cells.

To exclude potential interference from *BCR-ABL* gene-related abnormalities that cause imatinib-resistance in K562/IM cells, we examined the *BCR-ABL* gene amplification by FISH, BCR-ABL protein expression by Western blotting and *ABL* kinase domain mutations of the *BCR-ABL* gene by sequencing. FISH analysis from both K562 and K562/IM cells showed Ph^+ with a similar amplified *BCR-ABL* rearrangement segment (data not shown). Western blot analysis showed that both cell lines expressed comparable amounts of the 210 kDa band corresponding to BCR-ABL (Fig. 1a). The 120 kDa band corresponding to c-ABL protein also showed no difference in the cells (Fig. 1a). Furthermore, no mutations were found in a 669 bp fragment, containing the p-loop and *ABL* kinase domain, derived from either the K562 and K562/IM cells (data not shown).

Up-Regulation of Drug Efflux Transporters in K562/IM Cells

mRNA and protein expression levels of P-gp (MDR1, ABCB1), BCRP (ABCG2) and OCT1 in K562, K562/VCR, K562/BCRP and K562/IM cells were examined by real-time PCR and Western blot analysis. *MDR1* and *ABCG2* mRNA levels in K562/IM cells were 335- and eightfold higher than in parent K562 cells, respectively (Fig. 1b–c). Furthermore, the protein levels of both P-gp and BCRP were increased in K562/IM cells (Fig. 1e–f). The mRNA and protein levels of *OCT1*/OCT1 in K562/IM cells were similar to those in K562 cells (Fig. 1d and g). These findings indicated that both P-gp and BCRP, but not OCT1, are up-regulated during the development of imatinib-resistance.

Increased mRNA and protein expression of *MDR1*/P-gp or *ABCG2*/BCRP were detected in K562/VCR or K562/BCRP cells compared to those in the K562 cells, respectively (Fig. 1b–c and e–f). The level of *MDR1* mRNA in K562/BCRP cells was eightfold higher than in K562 cells, whereas the protein level of P-gp was unchanged (Fig. 1b and e). In addition, *OCT1* mRNA and OCT1 protein expression levels in K562/VCR or K562/BCRP cells were similar to those in K562 cells (Fig. 1d and g).

Higher Activity of P-gp and Negligible Activity of BCRP in K562/IM Cells

To determine the efflux transport activity of P-gp and BCRP in K562/IM cells, cellular accumulation of R123 (a substrate of P-gp) and PhA (a substrate of BCRP) were measured in the presence or absence of P-gp inhibitor (10 μM

Table I. IC50 Values (μM) of Imatinib Determined by Alamar Blue Assay in K562, K562/VCR, K562/BCRP and K562/IM Cells

	K562	K562/VCR	K562/BCRP	K562/IM
Imatinib	0.18±0.04	0.79±0.12[a]	0.22±0.02	8.41±0.76[b]
+Cyclosporin A	0.06±0.01	0.12±0.01[c]	0.13±0.03	4.82±0.59[b, d]
+Fumitremorgin C	0.19±0.05	0.66±0.09	0.23±0.07	8.81±0.95[b]
+Amantadine	0.42±0.03	1.18±0.10[a]	0.47±0.02	18.42±0.02[b, d]

Each value represents the mean value±SD (*n*=3). Data were analyzed statistically by analysis of two-way factorial ANOVA with post hoc comparison by Fisher's PLSD.
[a] Significant difference at 0.05 level in comparison with K562 wild type
[b] Significant difference at 0.01 level in comparison with K562 wild type
[c] Significant difference at 0.05 level in comparison with no inhibitor
[d] Significant difference at 0.01 level in comparison with no inhibitor

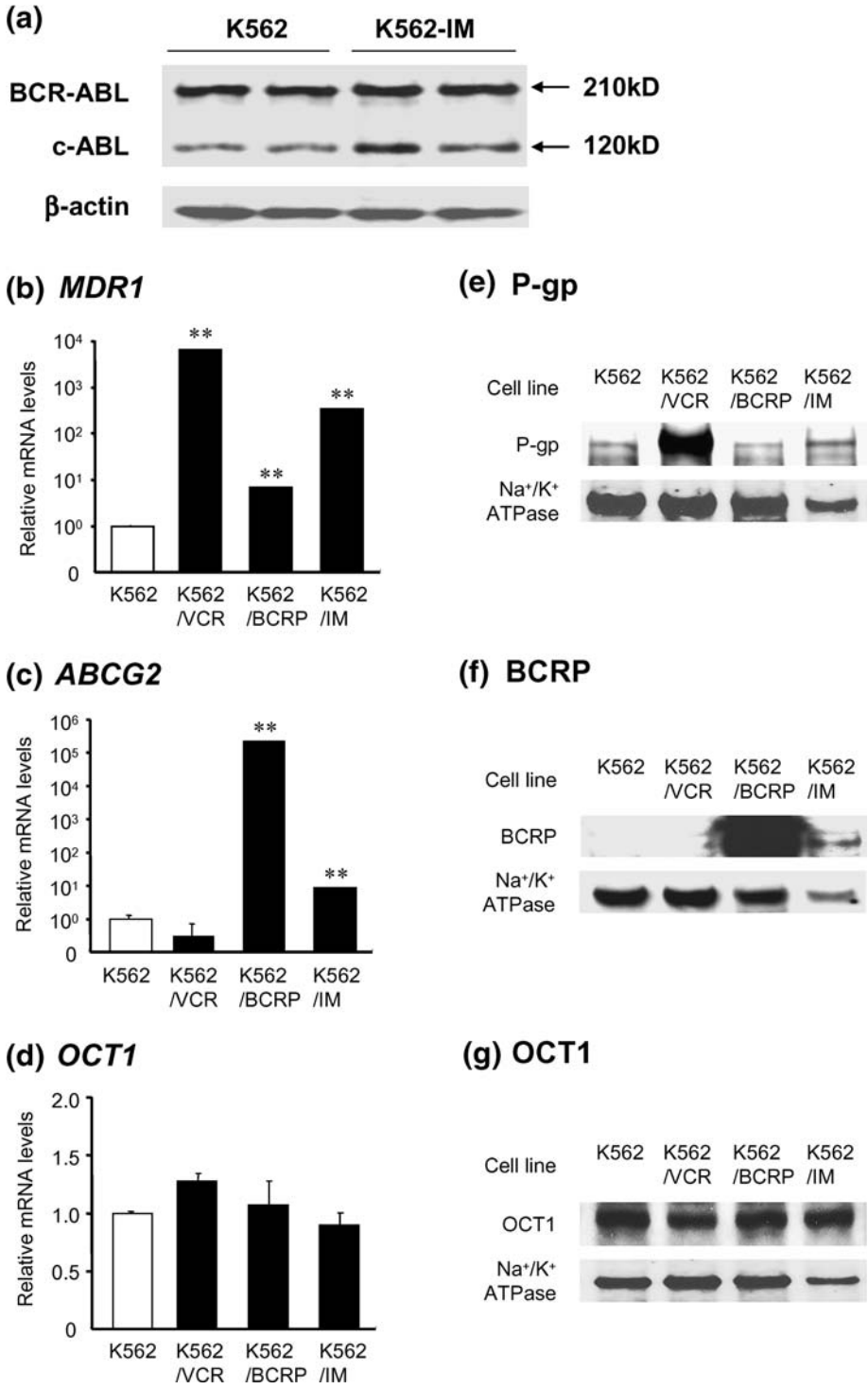

Fig. 1. Effect of imatinib exposure on P-gp, BCRP and OCT1 expression. The effect of imatinib selection on protein expression levels of **a** BCR-ABL and c-ABL, **b** *MDR1*, **c** *ABCG2*, **d** *OCT1* mRNA levels, or on **e** P-gp, **f** BCRP, **g** OCT1 protein levels were examined by real-time PCR or Western blots analysis, respectively. *Double asterisks* indicate $P<0.01$, compared to K562 (Student's *t* test)

CsA) or BCRP inhibitor (10 μM FTC) by flow cytometry. CsA had no effect on the accumulation of R123 in parent K562 cells, indicating negligible P-gp activity in K562 cells (Fig. 2a). However, CsA increased the accumulation of R123 by about 200-fold in P-gp-overexpressed K562/VCR cells (Fig. 2b), suggesting that these cells possess considerably higher P-gp activity. In K562/IM cells, CsA increased the accumulation of R123 by about threefold (Fig. 2c), suggesting that K562/IM cells display higher P-gp activity compared to the parent K562 cells. Furthermore, in K562 and K562/IM cells,

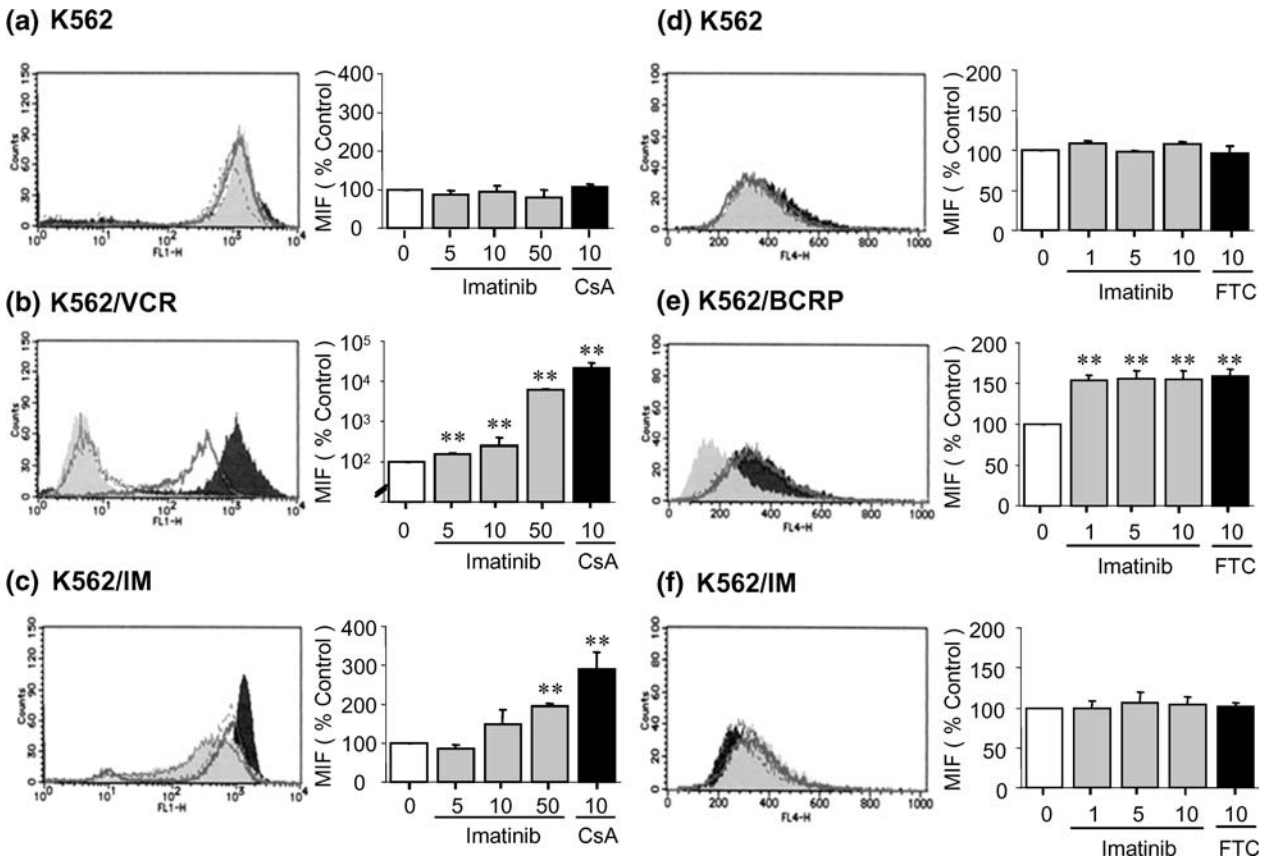

Fig. 2. Transport activity of P-gp and BCRP by flow cytometry. Cell lines were tested for their capacity to efflux R123 and PhA. **a–c** Cells were incubated with R123 (*light gray fill*), R123+10 μM CsA (*black fill*), R123+5 μM imatinib (*dashed lines*), +10 μM imatinib (*dotted lines*) and +50 μM imatinib (*solid lines*). **d–f** Cells were incubated with PhA (*light gray fill*), PhA+10 μM FTC (*black fill*), PhA+1 μM imatinib (*dashed lines*), +5 μM imatinib (*dotted lines*) and +10 μM imatinib (*solid lines*). Mean intensities of fluorescence (*MIF*) were obtained from control experiments in which R123 or PhA alone was incubated with each cell type. Values were normalized to 100%. *Double asterisks* indicate $P<0.01$, difference from no inhibitor (Student's *t* test)

treatment with FTC had no effect on PhA accumulation, indicating that these cells have negligible BCRP activity (Fig. 2d and f). In K562/BCRP cells, FTC increased PhA accumulation by about 1.5-fold (Fig. 2e), suggesting that K562/BCRP cells have higher BCRP activity compared to K562 and K562/IM cells.

Next, we examined the effect of imatinib on the intracellular accumulation of R123 or PhA. In K562/VCR and K562/IM cells, imatinib increased the accumulation of R123 in a dose-dependent manner, but treatment with imatinib showed no effect on R123 accumulation in parent K562 cells (Fig. 2a–c). By contrast, imatinib had no effect on PhA accumulation in K562 and K562/IM cells, but did increase PhA accumulation with an inhibition equivalent to 10 μM FTC seen at 1 μM imatinib in K562/BCRP cells (Fig. 2d–f). These findings suggest that imatinib inhibits both R123 and PhA transport via P-gp and BCRP.

Impaired Intracellular Imatinib Accumulation in K562/IM Cells

Intracellular imatinib levels were measured by HPLC analysis. The imatinib levels were reduced to 72 and 42% in K562/VCR and K562/IM cells compared to that in K562

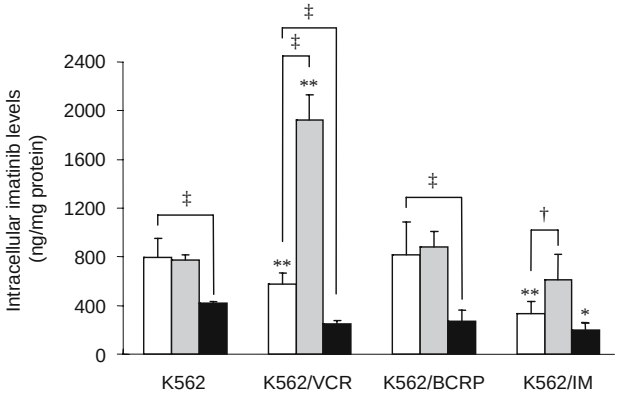

Fig. 3. Intracellular imatinib accumulation measured by HPLC analysis. The intracellular imatinib levels without (*white column*) or with 5 μM CsA (*light gray column*) or 1 mM amantadine (*black column*) were examined. Cells were exposed to imatinib with or without inhibitors at 37°C for 5 h, and the intracellular imatinib levels were measured by HPLC. *Asterisk* indicates $P<0.05$; *double asterisks* indicate $P<0.01$, differences from K562 wild type. *Dagger* indicates $P<0.05$; *double dagger* indicates $P<0.01$, differences from no inhibitor (Fisher's PLSD)

cells, respectively (Fig. 3). By contrast, K562/BCRP cells had a similar intracellular imatinib level to K562 cells.

P-gp Inhibitor Restored Imatinib Sensitivity and Intracellular Accumulation in K562/IM Cells

To determine the contribution of P-gp, BCRP and OCT1 in the acquired resistance to imatinib, the effect of transporter-specific inhibitors on imatinib sensitivity was studied in the four cell lines (Table I). CsA (5 μM) significantly sensitized K562/VCR and K562/IM cells to imatinib (i.e. by 6.6- and 1.7-fold, respectively). As a result, K562/VCR cells showed a similar sensitivity towards imatinib as K562 cells. However, K562/IM cells were still 26.8-fold as insensitive as K562 cells prior to CsA treatment. FTC (5 μM) had no effect on restoring sensitivity to imatinib in the four cell lines studied. Amantadine (100 μM), an OCT1 inhibitor, decreased the sensitivity to imatinib by twofold in all four cell lines.

To determine whether the intracellular imatinib levels were affected by P-gp, BCRP and OCT1, the four cell lines were treated with imatinib combined with CsA, FTC or amantadine (Fig. 3). CsA (5 μM) restored the imatinib levels in K562/VCR and K562/IM. In K562/VCR cells, the intracellular imatinib level with CsA was 3.4-times as high as that without CsA. In K562/IM cells, imatinib levels with CsA were twofold greater than that without CsA (i.e. similar to K562 cells). Amantadine (1 mM) decreased imatinib uptake in all four cell lines. The effect of FTC could not be evaluated for the HPLC analysis, because the UV absorption of FTC overlapped with that of imatinib.

DISCUSSION

In the present study, we explored the contribution of P-gp, BCRP and/or OCT1 in the acquired resistance to imatinib using K562/IM cells. Our findings indicate that one of these transporters is critically important in terms of sensitivity towards imatinib.

Our previous study demonstrated that imatinib is a substrate for P-gp (18), which was confirmed in the present investigation. Furthermore, P-gp was induced by imatinib exposure in K562 cells, suggesting that P-gp contributes to imatinib-resistance. In a previous report, Illmer *et al.* (20) demonstrated that modulation of P-gp by CsA prevented the P-gp-dependent decline of intracellular imatinib levels and restored imatinib sensitivity in P-gp-expressed K562/VCR cells. In addition, they demonstrated the biological effect of P-gp modulation during imatinib treatment of a patient with BCR-ABL-positive ALL. Radujkovic *et al.* (21) showed that imatinib monotherapy with 17-allylamino-17-demmethoxygeldanamycin lowered P-gp activity, which may increase intracellular imatinib levels and contribute to the sensitization of imatinib-resistant K562 and LAMA84 cells, from human CML. In our cytotoxicity assay, sensitivity to imatinib was almost restored with CsA in K562/VCR cells, and was partially restored with CsA in K562/IM cells compared to that without CsA (Table I). In addition, intracellular imatinib levels in K562/VCR and K562/IM cells decreased to about 70 and 40% of that in parent K562 cells, respectively, which were almost restored by treatment with CsA (Fig. 3). These data suggest that P-gp contributes to imatinib-resistance in K562/IM cells.

The interaction of imatinib with BCRP was demonstrated by several groups (24–27,35–38). Recently, imatinib was reported to be a substrate for BCRP *in vitro* and *in vivo*. Additionally, it was shown that chronic imatinib exposure *in vitro* causes reduced intracellular imatinib accumulation by induction of BCRP (36). Nakanishi *et al.* (37) showed that BCR-ABL regulates BCRP protein expression via the PI3K/Akt pathway, and suggested that BCRP is a mediator of imatinib resistance. However, it was recently shown that imatinib directly inhibits BCRP-mediated transport without being a competitive substrate in Saos2 cells (26). Jordanides *et al.* (27) demonstrated that imatinib inhibits BCRP, but does not act as a substrate for BCRP in primary $CD34^+$ CML cells. Furthermore, Gardner *et al.* (38) suggested that the pharmacokinetic parameters of imatinib *in vivo* were not statistically different in 16 patients who were heterozygous for *ABCG2* 421C>A compared with 66 patients carrying the wild-type sequence. Therefore, it is still unclear whether BCRP is a mediator of imatinib-resistance, or indeed whether BCRP recognizes imatinib as a substrate and/or inhibitor. In this study, we found that imatinib exposure induced *ABCG2* mRNA in K562/IM cells by eightfold compared to K562 cells, and that the protein level of BCRP was slightly increased in K562/IM cells (Fig. 1). However, K562/BCRP cells showed similar sensitivity to imatinib compared to K562 cells, and FTC did not affect the cell viability in four cell lines compared to without FTC (Table I). In addition, the intracellular imatinib level in K562/BCRP cells was similar to that in K562 cells (Fig. 3). In flow cytometry, imatinib increased PhA accumulation in K562/BCRP cells, but not in K562 and K562/IM cells. Thus, a slightly increased level of BCRP protein was not found to be functionally significant in K562/IM cells. Furthermore, these findings demonstrate that imatinib is an inhibitor, rather than a substrate, for BCRP. Therefore, we propose that BCRP does not contribute to imatinib-resistance in K562/IM cells.

Thomas *et al.* (28) reported that the influx of imatinib into the cell is an active process initially mediated by OCT1. Our results also indicate that imatinib is a substrate for OCT1. Amantadine, an OCT1 inhibitor, increased the IC_{50} values of imatinib (Table I), and decreased intracellular imatinib levels in all four cell lines (Fig. 3). Furthermore, the all four cell lines had similar expression levels of OCT1 mRNA and protein (Fig. 1c and f). Additionally, it was observed that mRNA and protein expression of OCT1 was not affected by imatinib exposure in K562 cells. In previous reports, it was shown that some inter-individual variation in the expression of OCT1 in CML cells can result in varying clinical outcomes (29,30). Our present data, however, suggests that imatinib treatment would not affect the expression of OCT1 in CML cells of patients.

We showed that P-gp, but not BCRP and OCT1, contributes to the acquired resistance to imatinib in K562/IM cells. However, 5 μM CsA, a concentration to fully inhibit P-gp activity, could not sensitize K562/IM cells to imatinib compared with K562 cells (Table I). It is conceivable that other factors are involved in the development of imatinib-resistance. To our knowledge, there is little information or reports indicating that MRP (multidrug resistance-related proteins) family, OCT2 and/or OCT3, other organic cation transporters, contribute to imatinib-resistance. Our preliminary data suggest that the expression of OCT2 or OCT3 is not detected in K562 cells. However, it might be possible that member(s) of MRP family could be involved in the

resistance. In addition, up-regulation of other transporter(s) might be involved in the acquisition of resistance. To determine the relative contribution of these efflux transporters to the acquisition of imatinib resistance, we further need to use a more specific inhibitor against P-glycoprotein rather than cyclosporine A. Alternatively, we examined the expression levels of the BCR-ABL gene and protein, in addition to mutations in the *ABL* kinase domain of K562/IM cells. Using FISH analysis, we found that the level of *BCR-ABL* gene expression in K562/IM cells was almost identical to that in K562 cells. In addition, BCR-ABL protein levels were unchanged between K562 and K562/IM cells (Fig. 1a). No *ABL* kinase domain mutations were found in the K562 and K562/IM cell lines. Dai *et al.* (16) demonstrated that BCR-ABL-independent, LYN-dependent forms of imatinib-resistance are associated with up-regulation of BCL-2 in imatinib-resistant K562 cells. Dasatinib, a dual SRC/ABL kinase inhibitor, has been used in clinical trials (13) and another SRC kinase inhibitor, CGP76030, has recently been developed (39). In this study, we did not examine whether SRC kinase, LYN and associated downstream factors are related to imatinib-resistance in our established K562/IM cells. Although the level of BCR-ABL protein in K562/IM cells was similar to that in K562 cells, it is possible that Lyn kinase and downstream factors might contribute to the observed increased resistance to imatinib. In the future study, we will explore how the K562/IM cells and K562 cells differ globally by a proteomics approach.

CONCLUSION

This study suggests that P-gp, but not BCRP and OCT1, is involved in imatinib-resistance in K562/IM cells. Therefore, P-gp could be a potential target molecule to overcome the development of imatinib-resistance in advanced CML and Ph-positive ALL. A preliminary clinical study in our hospital has shown that *MDR1* mRNA from bone marrow mononuclear cells of two CML patients with acquired resistance to imatinib, who did not display amplification and mutation of *BCR/ABL*, was significantly increased compared to that before imatinib treatment (data not shown). Further clinical investigations are needed to clarify the involvement of P-gp or other transporters in imatinib-resistance.

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REFERENCES

1. M. Kalidas, H. Kantarjian, and M. Talpaz. Chronic myelogenous leukemia. *JAMA* **286**:895–898 (2001).
2. R. Capdeville, E. Buchdunger, J. Zimmermann, and A. Matter. Glivec (STI571, imatinib), a rationally developed, targeted anticancer drug. *Nat. Rev. Drug Discov.* **1**:493–502 (2002).
3. B. J. Druker, C. L. Sawyers, H. Kantarjian, D. J. Resta, S. F. Reese, J. M. Ford, R. Capdeville, and M. Talpaz. Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. *N. Engl. J. Med.* **344**:1038–1042 (2001).
4. C. L. Sawyers, A. Hochhaus, E. Feldman, J. M. Goldman, C. B. Miller, O. G. Ottmann, C. A. Schiffer, M. Talpaz, F. Guilhot, M. W. N. Deininger, T. Fischer, S. G. O'Brien, R. M. Stone, C. B. Gambacorti-Passerini, N. H. Russell, J. J. Reiffers, T. C. Shea, B. Chapuis, S. Coutre, S. Tura, E. Morra, R. A. Larson, A. Saven, C. Peschel, A. Gratwohl, F. Mandelli, M. Ben-Am, I. Gathmann, R. Capdeville, R. L. Paquette, and B. J. Druker. Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. *Blood* **99**:3530–3539 (2002).
5. O. G. Ottmann, B. J. Druker, C. L. Sawyers, J. M. Goldman, J. Reiffers, R. T. Silver, S. Tura, T. Fischer, M. W. Deininger, C. A. Schiffer, M. Baccarani, A. Gratwohl, A. Hochhaus, D. Hoelzer, S. Fernandes-Reese, I. Gathmann, R. Capdeville, and S. G. O'Brien. A phase 2 study of imatinib in patients with relapsed or refractory Philadelphia chromosome-positive acute lymphoid leukemias. *Blood* **100**:1965–1971 (2002).
6. M. E. Gorre, M. Mohammed, K. Ellwood, N. Hsu, R. Paquette, P. N. Rao, and C. L. Sawyers. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science* **293**:876–880 (2001).
7. M. E. O'Dwyer, M. J. Mauro, G. Kurilik, M. Mori, S. Balleisen, S. Olson, E. Magenis, R. Capdeville, and B. J. Druker. The impact of clonal evolution on response to imatinib mesylate (STI571) in accelerated phase CML. *Blood* **100**:1628–1633 (2002).
8. J. E. Cortes, M. Talpaz, F. Giles, S. O'Brien, M. B. Rios, J. Shan, G. Garcia-Manero, S. Faderl, D. A. Thomas, W. Wierda, A. Ferrajoli, S. Jeha, and H. M. Kantarjian. Prognostic significance of cytogenetic clonal evolution in patients with chronic myelogenous leukemia on imatinib mesylate therapy. *Blood* **101**:3794–3800 (2003).
9. S. Branford, Z. Rudzki, S. Walsh, A. Grigg, C. Arthur, K. Taylor, R. Herrmann, K. P. Lynch, and T. P. Hughes. High frequency of point mutations clustered within the adenosine triphosphate-binding region of BCR/ABL in patients with chronic myeloid leukemia or Ph-positive acute lymphoblastic leukemia who develop imatinib (STI571) resistance. *Blood* **99**:3472–3475 (2002).
10. C. Roche-Lestienne, V. Soenen-Cornu, N. Grardel-Duflos, J.-L. Lai, N. Philippe, T. Facon, P. Fenaux, and C. Preudhomme. Several types of mutations of the Abl gene can be found in chronic myeloid leukemia patients resistant to STI571, and they can pre-exist to the onset of treatment. *Blood* **100**:1014–1018 (2002).
11. N. C. Wolff, D. R. Veach, W. P. Tong, W. G. Bornmann, B. Clarkson, and R. L. Ilaria Jr. PD166326, a novel tyrosine kinase inhibitor, has greater antileukemic activity than imatinib mesylate in a murine model of chronic myeloid leukemia. *Blood* **105**:3995–4003 (2005).
12. H. Kantarjian, F. Giles, L. Wunderle, K. Bhalla, S. O'Brien, B. Wassmann, C. Tanaka, P. Manley, P. Rae, W. Mietlowski, K. Bochinski, A. Hochhaus, J. D. Griffin, D. Hoelzer, M. Albitar, M. Dugan, J. Cortes, L. Alland, and O. G. Ottmann. Nilotinib in imatinib-resistant CML and Philadelphia chromosome-positive ALL. *N. Engl. J. Med.* **354**:2542–2551 (2006).
13. M. Talpaz, N. P. Shah, H. Kantarjian, N. Donato, J. Nicoll, R. Paquette, J. Cortes, S. O'Brien, C. Nicaise, E. Bleickardt, M. A. Blackwood-Chirchir, V. Iyer, T.-T. Chen, F. Huang, A. P. Decillis, and C. L. Sawyers. Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias. *N. Engl. J. Med.* **354**:2531–2541 (2006).
14. F. X. Mahon, M. W. Deininger, B. Schultheis, J. Chabrol, J. Reiffers, J. M. Goldman, and J. V. Melo. Selection and characterization of BCR-ABL positive cell lines with differential sensitivity to the tyrosine kinase inhibitor STI571: diverse mechanisms of resistance. *Blood* **96**:1070–1079 (2000).
15. N. J. Donato, J. Y. Wu, J. Stapley, G. Gallick, H. Lin, R. Arlinghaus, and M. Talpaz. BCR-ABL independence and LYN kinase overexpression in chronic myelogenous leukemia cells selected for resistance to STI571. *Blood* **101**:690–698 (2003).
16. Y. Dai, M. Rahmani, S. J. Corey, P. Dent, and S. Grant. A Bcr/Abl-independent, Lyn-dependent form of imatinib mesylate

(STI-571) resistance is associated with altered expression of Bcl-2. *J. Biol. Chem.* **279**:34227–34239 (2004).
17. F.-X. Mahon, F. Belloc, V. Lagarde, C. Chollet, F. Moreau-Gaudry, J. Reiffers, J. M. Goldman, and J. V. Melo. MDR1 gene overexpression confers resistance to imatinib mesylate in leukemia cell line models. *Blood* **101**:2368–2373 (2003).
18. A. Hamada, H. Miyano, H. Watanabe, and H. Saito. Interaction of imatinib mesilate with human P-glycoprotein. *J. Pharmacol. Exp. Ther.* **307**:824–828 (2003).
19. N. Widmer, S. Colombo, T. Buclin, and L. A. Decosterd . Functional consequence of MDR1 expression on imatinib intracellular concentrations. *Blood* **102**:1142, (2003).
20. T. Illmer, M. Schaich, U. Platzbecker, J. Freiberg-Richter, U. Oelschlagel, M. von Bonin, S. Pursche, T. Bergemann, G. Ehninger, and E. Schleyer. P-glycoprotein-mediated drug efflux is a resistance mechanism of chronic myelogenous leukemia cells to treatment with imatinib mesylate. *Leukemia* **18**:401–408 (2004).
21. A. Radujkovic, M. Schad, J. Topaly, M. R. Veldwijk, S. Laufs, B. S. Schultheis, A. Jauch, J. V. Melo, S. Fruehauf, and W. J. Zeller. Synergistic activity of imatinib and 17-AAG in imatinib-resistant CML cells overexpressing BCR-ABL—inhibition of P-glycoprotein function by 17-AAG. *Leukemia* **19**:1198–1206 (2005).
22. M. Mukai, X.-F. Che, T. Furukawa, T. Sumizawa, S. Aoki, X.-Q. Ren, M. Haraguchi, Y. Sugimoto, M. Kobayashi, H. Takamatsu, and S.-i. Akiyama. Reversal of the resistance to STI571 in human chronic myelogenous leukemia K562 cells. *Cancer Sci.* **94**:557–563 (2003).
23. F. J. Giles, H. Kantarjian, J. Cortes, D. Thomas, M. Talpaz, T. Manshouri, and M. Albitar. Multidrug resistance protein expression in chronic myeloid leukemia. *Cancer* **86**:805–813 (1999).
24. H. Burger, H. Tolvan, A. W. Boersma, M. Brok, E. A. Wiemer, G. Stoter, and K. Nooter. Imatinib mesylate (STI571) is a substrate for the breast cancer resistance protein (BCRP)/ABCG2 drug pump. *Blood* **104**:2940–2942 (2004).
25. P. Breedveld, D. Pluim, G. Cipriani, P. Wielinga, O. vanTellingen, A. H. Schinkel, and J. H. M. Schellens. The Effect of Bcrp1 (Abcg2) on the *In vivo* pharmacokinetics and brain penetration of imatinib mesylate (Gleevec): implications for the use of breast cancer resistance protein and P-glycoprotein inhibitors to enable the brain penetration of imatinib in patients. *Cancer Res.* **65**:2577–2582 (2005).
26. P. J. Houghton, G. S. Germain, F. C. Harwood, J. D. Schuetz, C. F. Stewart, E. Buchdunger, and P. Traxler. Imatinib mesylate is a potent inhibitor of the ABCG2 (BCRP) transporter and reverses resistance to topotecan and SN-38 *in vitro*. *Cancer Res.* **64**:2333–2337 (2004).
27. N. E. Jordanides, H. G. Jorgensen, T. L. Holyoake, and J. C. Mountford. Functional ABCG2 is overexpressed on primary CML CD34+ cells and is inhibited by imatinib mesylate. *Blood* **108**:1370–1373 (2006).
28. J. Thomas, L. Wang, R. E. Clark, and M. Pirmohamed. Active transport of imatinib into and out of cells: implications for drug resistance. *Blood* **104**:3739–3745 (2004).
29. L. C. Crossman, B. J. Druker, M. W. N. Deininger, M. Pirmohamed, L. Wang, and R. E. Clark. hOCT 1 and resistance to imatinib. *Blood* **106**:1133–1134 (2005).
30. D. L. White, V. A. Saunders, P. Dang, J. Engler, A. C. Zannettino, A. C. Cambareri, S. R. Quinn, P. W. Manley, and T. P. Hughes. OCT-1-mediated influx is a key determinant of the intracellular uptake of imatinib but not nilotinib (AMN107): reduced OCT-1 activity is the cause of low *in vitro* sensitivity to imatinib. *Blood* **108**:697–704 (2006).
31. H. M. Kantarjian, M. Talpaz, S. O'Brien, F. Giles, G. Garcia-Manero, S. Faderl, D. Thomas, J. Shan, M. B. Rios, and J. Cortes. Dose escalation of imatinib mesylate can overcome resistance to standard-dose therapy in patients with chronic myelogenous leukemia. *Blood* **101**:473–475 (2003).
32. J. O'Brien, I. Wilson, T. Orton, and F. Pognan. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur. J. Biochem.* **267**:5421–5426 (2000).
33. G. W. Dewald, W. A. Wyatt, A. L. Juneau, R. O. Carlson, A. R. Zinsmeister, S. M. Jalal, J. L. Spurbeck, and R. T. Silver. Highly sensitive fluorescence *in situ* hybridization method to detect double BCR/ABL fusion and monitor response to therapy in chronic myeloid leukemia. *Blood* **91**:3357–3365 (1998).
34. R. W. Robey, K. Steadman, O. Polgar, K. Morisaki, M. Blayney, P. Mistry, and S. E. Bates. Pheophorbide a is a specific probe for ABCG2 function and inhibition. *Cancer Res.* **64**:1242–1246 (2004).
35. C. Özvegy-Laczka, T. Hegedüs, G. Varady, O. Ujhelly, J. D. Schuetz, A. Varadi, G. Keri, L. Örfi, K. Nemet, and B. Sarkadi. High-affinity interaction of tyrosine kinase inhibitors with the abcg2 multidrug transporter. *Mol. Pharmacol.* **65**:1485–1495 (2004).
36. H. Burger, H. vanTol, M. Brok, E. A. Wiemer, E. A. deBruijn, G. Guetens, G. deBoeck, A. Sparreboom, J. Verweij, and K. Nooter. Chronic imatinib mesylate exposure leads to reduced intracellular drug accumulation by induction of the ABCG2 (BCRP) and ABCB1 (MDR1) drug transport pumps. *Cancer Biol. Ther.* **4**:747–752 (2005).
37. T. Nakanishi, K. Shiozawa, B. A. Hassel, and D. D. Ross. Complex interaction of BCRP/ABCG2 and imatinib in BCR-ABL-expressing cells: BCRP-mediated resistance to imatinib is attenuated by imatinib-induced reduction of BCRP expression. *Blood* **108**:678–684 (2006).
38. E. R. Gardner, H. Burger, R. H. vanSchaik, A. T. vanOosterom, E. A. deBruijn, G. Guetens, H. Prenen, F. A. deJong, S. D. Baker, S. E. Bates, W. D. Figg, J. Verweij, A. Sparreboom, and K. Nooter. Association of enzyme and transporter genotypes with the pharmacokinetics of imatinib. *Clin. Pharmacol. Ther.* **80**:192–201 (2006).
39. M. Warmuth, N. Simon, O. Mitina, R. Mathes, D. Fabbro, P. W. Manley, E. Buchdunger, K. Forster, I. Moarefi, and M. Hallek. Dual-specific Src and Abl kinase inhibitors, PP1 and CGP76030, inhibit growth and survival of cells expressing imatinib mesylate-resistant Bcr-Abl kinases. *Blood* **101**:664–672 (2003).