Reversal of Imatinib resistance in BCR-ABL-positive leukemia after inhibition of the Na⁺/H⁺ exchanger

Weina Jin, Qinghua Li, Yani Lin, Ying Lu, Huawen Li, Lihong Wang, Ronghua Hu, Li Ma, Jianxiang Wang, Tianxiang Pang

State Key Laboratory of Experimental Hematology, Institute of Hematology and Hospital of Blood Diseases, Chinese Academy of Medical Sciences and Peking Union Medical College, Nanjing Road 288, Tianjin 300020, China

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The present study was undertaken to estimate the therapeutic benefit to down-regulate the Na⁺/H⁺ exchanger 1 (NHE1) for reversing chemoresistance of BCR-ABL-positive leukemia patient cells and cell lines. As a result, after treatment with specific NHE1 inhibitor Cariporide or high K⁺ buffer to decrease intracellular pH (pHᵢ), cells from relapsed patients exhibited decreased Pgp level, enhanced Rhodamine123 and drug accumulation, decreased colony-forming ability and the modulations of mitogen-activated protein kinases (MAPKs) activities. Furthermore, we used BCR-ABL-positive cell line K562 and its resistant counterparts K562/DOX and K562/G01 cell lines for further study. Together, these findings suggest that Pgp may be associated with the reversal of drug resistance in BCR-ABL-positive leukemia patients and cell lines by the inhibition of NHE1 though MAPK pathways.

1. Introduction

The chimeric BCR-ABL gene, which is created by the formation of the Philadelphia chromosome (Ph), encodes bcr-abl fusion protein. The deregulated protein tyrosine kinase activity in bcr-abl is the cause of CML and 25% ALL [1]. Imatinib has been developed as a specific and targeted therapy for CML. It has also shown significant activity in patients with ALL who are BCR-ABL-positive. Treatment outcome with Imatinib-based regimens has improved compared with historic controls, but most patients who do not undergo allogeneic stem cell transplantation (SCT) eventually relapse [2–4].

ATP-binding cassette subfamily B member 1 (ABCB1, also known as MDR1 and P-glycoprotein) is one of the ATP-binding cassette (ABC)-superfamily multidrug efflux pumps which is known to be an efflux pump of Imatinib and responsible for chemoresistance [5]. High expression of Pgp has been observed prior to chemotherapy treatment in many different tumor types. But in hematological malignancies, such as leukemia, lymphomas and multiple myelomas, low levels of Pgp expression observed initially are markedly increased after chemotherapy treatment and relapse [5]. Furthermore, it has been reported that the intracellular levels of Imatinib decreased in Pgp-positive leukemic cells [6]. All these data indicate that Pgp overexpression is an important clinical mechanism in the diversity of resistance mechanisms in leukemia.

The NHE family is involved in numerous physiological processes in mammals, including regulation of intracellular pH, cell-volume control, cytoskeletal organization, heart disease and cancer. In our previous study, we found that Pgp-mediated multidrug resistance could be reversed by NHE1-inhibition through down-regulating the MDR1...
mRNA and protein level in a time and pH-dependent manner in BCR-ABL-positive K562/DOX cells, and the activity of Pgp was also significantly decreased [7]. We also found in our preliminary experiment that the viability of normal hematopoietic cells was not influenced by specific NHE1 inhibitor Cariporide, which led us to evaluate the effect of NHE1 on Pgp-associated leukemia patients. Furthermore, the molecular events linking the NHE1 and Pgp remain unclear. In the present study, we first compared the correlation between NHE1 activity and Pgp expression in BCR-ABL-positive and BCR-ABL-negative patients. Using specific NHE1 inhibitor Cariporide and high K+ buffer to rapidly decrease pH, we investigated the contribution of NHE1 to the Pgp-associated Imatinib resistance in BCR-ABL-positive patients, and further study the molecular pathways involved in with BCR-ABL-positive leukemic K562 as well as its resistant K562/DOX and K562/G01 cell lines.

2. Materials and methods

2.1. Materials

We obtained RPMI 1640 media from Gibco-BRL Life Technologies, Inc. (Burlington, ON, Canada); Fetal bovine serum (FBS) from HyClone (Logan, UT); Rhodamine 123 (Rh123), 2′,7′-bis (2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl (BCECF-AM), Imatinib, Doxorubicin and Cariporide from Sigma (Shanghai, China); and MAPKs inhibitors PD98059, SB203580 and SP600125 from Beyotime Technologies, Inc. (Burlington, ON, Canada); Enhanced Chemiluminescence Reagent Plus (ECL) reagents from BD Transduction Laboratories.

2.2. Patient samples

We collected born marrow samples in Hospital of Blood Diseases from October 1, 2008 to September 30, 2010. Inclusion criteria for our study were based on the European Leukemia Net (ELN) criteria. Clinical evaluation of patients was performed with physical examination and laboratory monitoring. Molecular analysis included quantitative measurement of BCR-ABL transcript level every 3–6 months. Results were expressed as a ratio of BCR-ABL to reference gene (mainly c-ABL) transcript’s copy number on the basis of an intra-laboratory standard. All the patient samples were treated in accordance with the Helsinki Declaration. Before the start of treatment, each patient gave written informed consent. The clinical characteristics of patients were shown in Table 1.

Within 24 h after sampling, mononuclear cells from bone marrow samples from patients were isolated by density gradient centrifugation using Lymphoprep, centrifuged at 480g for 15 min at room temperature. After that, initial immunophenotyping panel performed to confirm the lineage of the leukemia with monoclonal antibodies specific to lymphoid- and myeloid-associated antigens. Of the patient samples, more than 90% were of myeloid origin or lymphoid origin. Isolated mononuclear cells were washed twice and re-suspended in RPMI 1640 medium, 50 U/mL penicillin, 50 μg/mL streptomycin and 20% FBS.

2.3. Cell culture and experimental conditions

The culture of K562 and K562/DOX cell lines and experimental conditions were performed as described previously [7]. The K562/G01 cell line was provided by pharmacology laboratory at Institute of Hematology, which were cultured in RPMI1640 containing 10% fetal bovine serum (FBS), penicillin (50 U/mL), streptomycin (50 μg/mL) and L-glutamine (2 mmol/l) at 37 °C under 5% CO2. Imatinib at concentration of 1 μM was added to K562/G01 cell culture system to maintain resistance activity. Prior to use in experiments, K562/G01 cells were cultured in drug-free medium for two weeks.

2.4. Cytotoxicity assay

Cytotoxicity analysis was determined by the MTT assay. Briefly, cells were seeded into 96-well culture plates at a density of 5 × 104 cells/ml. Serial concentration of Imatinib were added with or without Cariporide in a final volume of 200 μl per well. After the drug treatment for 24 h, the medium was replaced with an equal volume of fresh medium containing 0.5 mg/mL MTT and incubated for 4 h at 37 °C.
Then, the medium was removed and 100 μl DMSO were added and incubated for 10 min at room temperature. The cytotoxic effects of drugs were determined according to the OD values using a microplate reader at absorption wavelength of 490 nm.

2.5. Measurements of intracellular concentrations of Imatinib

Isolated mononuclear cells from patients were added to 1 ml water containing 5.25 μg/ml internal standards, clozapine, and the resultant suspension was sonicated. The cellular Imatinib were then purified by solid phase extraction by Oasis HLB (Waters, Milford, MA, USA). Imatinib and clozapine were eluted with 1500 μl of methanol and evaporated to dryness under vacuum. The residue was re-suspended in 70 μl mobile phase solution of HPLC, and a volume of 50 μl was injected into the HPLC column. The flow rate was 1.0 mL/min, and the detection wavelength was 265 nm. Protein concentration was determined by the method of BCA protein assay (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard.

2.6. Colony assay

Bone marrow mononuclear cells from patients were incubated for 24 h in RPMI 1640 medium supplemented with 20% FBS and 50 ng/ml recombinant human GM-CSF (Immunex Inc, Seattle, WA). Cariporide was added at the initiation of cultures on exposure to 1 μM Imatinib at concentrations of 10 μM. After extensive washing, 1 × 10^5 cells were plated in 0.8% methylcellulose in RPMI 1640 with 20% FBS and 50 ng/ml GM-CSF. Duplicate cultures were incubated in 35-mm petri dishes for 7 days at 37 °C in a humidified atmosphere of 5% CO2 in air. Colonies were

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**Fig. 1.** The intracellular pH (pHi) in primary patient samples. (A) The box and whisker plots for normal bone marrow of primary leukemic patient samples. The whiskers above and below the box designate the 95th and 5th percentiles, respectively; the solid line within the box represents the median value; dots above or below the box indicate outliers. BCR-ABL-positive (BCR-ABL+) samples included CML (n = 19) and BCR-ABL+ ALL (n = 9); BCR-ABL-negative (BCR-ABL−) sample included AML (n = 8) and BCR-ABL+ ALL (n = 2). (B and C) correlation between pH and MDR1 expression was analyzed in BCR-ABL+ and BCR-ABL− samples. Each sample was divided into 2 aliquots, 1 for pH, and the other for real-time PCR. The graph shows the number of values for the individual samples, the linear regression and the P value. (D) The pH, in newly diagnosed and relapsed BCR-ABL+ patient samples was determined with or without 10 μM Cariporide for 3 h.
microscopically evaluated on day 7. A blast colony was defined as a cluster of 40 or more cells.

2.7. Measurement of intracellular pH and intracellular acidification treatment

Intracellular pH of cells was assessed by flow cytometry using the pH-sensitive fluorescent probe BCECF-AM as described previously. Intracellular acidification was performed as described previously [7].

2.8. Real-time RT-PCR, Western blotting, flow cytometry and confocal laser microscopy

These analyses were performed as described previously [7].

2.9. Data analysis

The correlation between pH and MDR1 level was estimated using the Pearson correlation coefficient. Other statistical analyses were made with Student's paired t-test using GraphPad Prism (San Diego, USA). Significance was assumed for P values less than 0.05.

3. Results

3.1. Effect of NHE1 on pH, and Pgp expression in primary patient samples

We first compared the pH values of patients with different leukemias. The box and whisker plots showed the higher pH values of BCR-ABL-positive (P < 0.001) and BCR-ABL-negative (P = 0.0194) patient cells than healthy donors (Fig. 1A). When pH and MDR1 mRNA expressions were further analyzed for any correlation, a positive linear regression was obtained with a correlation coefficient (r²) of 0.3439 in BCR-ABL-positive patients (Fig. 1C, P = 0.0010). According to these results, we mainly investigated the role of NHE1 activity in BCR-ABL-positive leukemia patients through comparison between newly diagnosed and relapsed patients. The pH was about 7.11 in newly diagnosed patient while 7.32 in relapsed patient cells as shown in Fig. 1D which was similar to the other report [8]. After treated with 10 μM Cariporide for 24 h, the pH decreased to 7.15 (Fig. 1D). The MDR1 mRNA and protein (Pgp) levels are higher in relapsed patients (Fig. 2A and B, P < 0.001), and the Pgp level decreased compared with untreated cells in relapse patients after Cariporide treatment. However, because of the undetectable or low Pgp levels in newly diagnosed patients, incubation with Cariporide did not significantly alter the protein levels of Pgp (P > 0.05).

3.2. NHE1-inhibition enhanced drug accumulation and sensitivity to Imatinib in relapsed patient cells

We then investigated the effects of low pH and NHE1 inhibition on drug accumulation and sensitivity to Imatinib. After incubation with 10 μM Cariporide or high K+ buffer for one hour, cells of relapsed patients (Fig. 3B) showed increased uptake of Rh123 and Doxorubicin (ADM) in pH-dependent style compared with newly diag-
Fig. 3. Representative examples for functional Pgp activity in the newly diagnosed and relapsed patients as detected by the Rhodamine 123 and doxorubicin accumulation test. The cells of patients were treated with 10 μM Cariporide, and the pH were induced to 7.2, 7.0, 6.8 with the high K+ buffer treatment for 3 h, respectively, the rhodamine123 or doxorubicin was added to the medium 1 h prior to viewing with confocal laser microscope as described under Section 2. Representative experiments are shown. Magnifications: 10 × 40.

Fig. 4. Effect of high K+ buffer on MAPK activity in cells of newly diagnosed and relapsed patients. Cells were treated with high K+ buffer for 0, 3 and 30 min. The cell lysates were prepared and analyzed for phosphorylation of p38 MAPK, ERK1/2 and JNK by Western blotting.
nosed patients (Fig. 3A). But no obvious change was found when pre-treated with Verapamil (data not shown) which implied the NHE1 might influence the Rh123 kinetics through regulating Pgp activity. Flow cytometry was also used to measure the intracellular fluorescent of Rh123, and the similar results were obtained (Supplementary Fig. S1).

Because the patients' bone marrow cells cycle very slowly in cultures, we assessed the effect of Cariporide on patient cells using colony assays. To study the inhibitory effect of NHE1 on the growth of CML cells, Cariporide was added at the initiation of cultures on exposure to 1 μM Imatinib at concentrations of 10 μM. Cells treated with Imatinib alone were used as control. Significant decreases of colony-forming units (CFUs) were observed in five of six relapsed patients (Fig. 2C, \( P = 0.003 \)). One relapsed patient showed slight reduction of CFUs (5.8%). Our studies demonstrate that inhibition of NHE1 could significantly reduce CFUs of patients' bone marrow cells dependent of patients' responses to Imatinib which may be associated with Pgp.

In order to further assess whether the effect of Cariporide are associated with specific transporter-dependent accumulation of Imatinib within the cells, HPLC assay for quantitative evaluation of intracellular concentration of Imatinib was performed. Characterization of five patient samples involved in this assay was summarized in the legend of Fig. 2D. Following 15 min pre-treatment with 10 μM Cariporide, cells were incubated with 1 μM Imatinib at 37 °C for 60 min, and prepared for HPLC detection. Although the standard error of the measurements was too large to obtain statistic significance at these very low drug concentrations (\( P = 0.1339 \)), the results were also in accordance with the colony forming assay. Intracellular concentration of Imatinib with Cariporide pre-incubation (140.2 ± 34.64 ng/mg protein) was higher than that without Cariporide pre-incubation (75.48 ± 48.49 ng/mg protein) in three of five patients.

3.3. Effect of NHE1 on the activation of MAPKs signaling pathways in patients

The phosphorylation of p38 MAPK decreased in a time-dependent manner in relapsed patients, and it was maximally inhibited at 30 min (Fig. 4A; Supplementary Fig. S2, \( P = 0.043 \)). In contrast, there was no obvious change of p38 activity in newly diagnosed patients (Fig. 4A; Supplementary Fig. S2, \( P > 0.05 \)). After stimulation with high K⁺ buffer, ERK1/2...
P = 0.022) and JNK (P = 0.041) activated in both groups (Fig. 4B and C; Supplementary Fig. S2). ERK5 was detected out in four newly diagnosed and seven relapsed patients with amount varied among distinct patient samples, suggesting that ERK5 expression may associate with a more aggressive phenotype. ERK5 phosphorylation seemed to decrease in relapsed patients after treated with high K⁺ buffer, although there was no statistical significance (data not shown, P > 0.05).

3.4. Effect of NHE1-inhibition on the sensitivity of cell lines to imatinib

We next used BCR-ABL-positive cell line K562 and its resistant counterparts K562/DOX (multidrug resistance cell line with Pgp overexpression) and K562/G01 cell line (Imatinib resistance cell line with Pgp overexpression) to reveal the role of NHE1 implicated in the reversal of Imatinib resistance [9]. We have demonstrated in our previous paper that inhibition of NHE1 or high K⁺ buffer resulted in the decreased Pgp mRNA and protein level, as well as the suppressed Pgp activity with increased Rh123 and doxorubicin accumulation in K562/DOX cell line [7]. We also found the similar results that Pgp mRNA and protein level decreased after NHE1-inhibition in Imatinib-resistant K562/G01 cells (Fig. 5A). However, the increased Rh123 and doxorubicin accumulation was not observed in K562/G01 cells. The drug efflux function of Pgp seemed to be suppressed because of the similar intracellular concentrations of Rh123 and doxorubicin between K562 and K562/G01 cells (data not shown).

The cells were incubated with 10 μM Cariporide and/or 30 μM Verapamil and then assessed for viability on exposure to Imatinib using MTT assay (Fig. 5B). The resistance of K562/DOX and K562/G01 to Imatinib was partially reversed by simultaneous incubation with 30 μM Verapamil (P = 0.01 in K562/DOX and P = 0.013 in K562/G01) or 10 μM Cariporide (P = 0.011 in K562/DOX and P = 0.014 in K562/G01). Moreover, a clear enhancement of sensitivity to Imatinib was observed when both Cariporide and Verapamil were added into the culture (P = 0.007 in K562/DOX and P = 0.001 in K562/G01). Although Verapamil could sensitize the parental K562 cells to Imatinib partially (P = 0.012), no change was detected on co-treatment with Cariporide and Verapamil compared with Verapamil alone (P > 0.05).

3.5. Effect of NHE1 on the activation of MAPKs signaling pathways in cell lines

High K⁺ buffer decreased the phosphorylation of p38 MAPK and ERK5 in resistant K562 cells in a time and pH-dependent fashion, while ERK1/2 and JNK increased in both resistant and sensitive K562 cells (Fig. 6; Supplementary Fig. S3). Then we used Cariporide, which has been proved to regulate both K562 and K562/DOX cells to pH 7.0 according to our previous results [7,10]. Phosphorylation of p38 decreased in a time-dependent manner in K562/DOX cells (P = 0.037) but did not significant changed in K562/G01 cells (P > 0.05). However, p38 MAPK activity increased in K562 cells after treatment with Cariporide persisting to 60 h (Fig. 7, Fig. 8. Inhibition of NHE1 sensitized resistant cell lines to Imatinib via p38 MAPK pathway. The MTT analysis of K562/DOX and K562/G01 cells was exposed to different concentrations of Imatinib with or without Cariporide and/or MAPKs inhibitors. Results resemble the means of three independent experiments that were determined in triplet each. OD values of untreated control cells were set 100%. All other values refer to the untreated control.
ERK1/2 activation suppress p38 activity in this process. In K562/DOX cells. Suppression of JNK and ERK1/2 activities significantly served the effect of p38 MAPK signaling in the regulation of Pgp expression. However in K562/G01 cells, because the level of Pgp after inhibition of NHE1 was too low to be detected (Fig. 5A), we could not observe the effect of p38 MAPK signaling in the regulation of Pgp expression. However in K562/DOX cells, because the level of Pgp after inhibition of NHE1 was too low to be detected (Fig. 5A), we could not observe the effect of p38 MAPK signaling in the regulation of Pgp expression. However in K562/DOX cells, because the level of Pgp after inhibition of NHE1 was too low to be detected (Fig. 5A), we could not observe the effect of p38 MAPK signaling in the regulation of Pgp expression. However in K562/DOX cells, because the level of Pgp after inhibition of NHE1 was too low to be detected (Fig. 5A), we could not observe the effect of p38 MAPK signaling in the regulation of Pgp expression.

To further elucidate the role of p38 MAPK signaling in the regulation of Pgp expression in K562/DOX cells, we analyzed Pgp levels in the presence of SB203580 (20 μM). As shown in Fig. 9A and B, Pgp expressions were reduced at both mRNA (P < 0.001) and protein (P < 0.005) level when K562/DOX cells were simultaneously incubated with SB203580 and Cariporide. This indicates that p38 MAPK possibly mediated the reversal of drug resistance induced by NHE1-inhibition through regulation of Pgp expression. However in K562/G01 cells, because the level of Pgp after inhibition of NHE1 was too low to be detected (Fig. 5A), we could not observe the effect of p38 MAPK signaling in the regulation of Pgp expression. Subsequently, we examined the cross-talk of these MAPK pathways in K562/DOX cells. Suppression of JNK and ERK1/2 activities significantly enhanced p38 phosphorylation (Fig. 10) which indicates that JNK and ERK1/2 activation suppress p38 activity in this process.

4. Discussion

Overexpression of BCR-ABL and P-glycoprotein (Pgp) are two of the known mechanisms of Imatinib resistance [6,9,11]. Compounds capable of reducing BCR-ABL protein level or inhibiting Pgp function may support the effect of Imatinib either by reduction of target molecules or increment of intracellular Imatinib concentrations [12]. Here, on the one hand, we found direct correlation between intracellular pH and Pgp gene expression in BCR-ABL-positive patient samples, indicating potential role of NHE1 in BCR-ABL-positive leukemia; On the other hand, we confirmed NHE1 as an important target protein implied in reversal of Imatinib resistance in resistant K562 cell lines and BCR-ABL-positive patient cells which is independent of Pgp protein stability (Supplementary Fig. S4). Increasing the intracellular levels of Imatinib by down-regulating Pgp may be capable of overcoming the insensitivity of the BCR-ABL kinase that is, for example, caused by mutation. According to above results, we suppose that NHE1 act in this process mainly depends on the regulation of intracellular pH. The regulation of Pgp expression and function may be related to the NHE1 effect but not the only or definite factor involved in this process, because the resistance of Imatinib can be also reversed in K562/G01 cells which express dysfunctional Pgp. Interestingly, this data is in accordance with the results we found in a group of primary patient samples (Supplementary Fig. S5). These cells without Pgp expression can also be influenced by NHE1 regulation. Above finding promotes us to speculate some other mechanisms involved in this regulation, such as drug efflux pumps. However, the related mechanisms were still obscure, and need further study. As to the different effects of Cariporide and high K⁺ buffer on MAPKs activities, we consider the rapid and transient phosphorylation of ERK1/2 and JNK as the main factor that can not be detected after sustained Cariporide treatment.

Map kinase pathways are activated by various stimuli to participate in the generation of specific biologic responses including drug resistance. Many previous studies have reported several mechanisms that reverse Pgp-mediated drug resistance via some MAPK pathways [13–16]. ERK signaling cascade has been demonstrated to be implicated in transformation by the BCR-ABL proto-oncogene [16], while ERK pharmacologic inhibitors appear to exhibit synergetic effects with Imatinib in the induction of apoptosis of BCR-ABL-expressing cells [17]. In our study, both high K⁺ buffer and Cariporide were able to activate ERK1/2 protein in a time and pH-dependent manner. But persistent activation of this kinase was not observed in either BCR-ABL-positive cell lines or primary patient cell sample which was consist with other reports [18]. The specific ERK inhibitor PD98059 had no effect on NHE1-mediated reversal of Imatinib resistance, which indicates that ERK1/2 pathway is not directly involved in this process. Biochemical pathways always operate in conjunction with each other and their interplay decides the final outcome. The existence of cross-talk among MAPK pathways themselves have been proved in several studies [19]. Inactivation of p38 MAPK, which was essential in Pgp down-
regulation and reversal of Imatinib resistance, could be induced by ERK1/2 activation. So according to above results, ERK1/2 may cross-talk with p38 MAPK pathway in this process. The possible mechanism may be that activated ERK, which in turn up-regulated MAPK phosphatase-1, thereby inactivating p38 MAPK. Depending on the stimuli and the strength and duration of JNK activation, the cellular response has diverse outcomes, which ranges from the increased survival to reversal of drug resistance and induction of apoptosis [20–22]. Cross-talk between JNK and p38 MAPK has also been widely demonstrated [23]. Increased activation of JNK on p38α inhibition has also been observed in mouse models [24]. Here, along with above reports, we also found that JNK suppressed p38 activity after incubated with Cariporide, suggesting a potential role of JNK in reversal of Imatinib resistance of BCR-ABL-positive patients and cell lines. As to whereby JNK and ERK inhibitors can not affect cells sensitivity to Imatinib therapy, we presume that other signaling pathways besides MAPKs bypass to attenuate the effect of JNK or ERK inhibitor, such as PI3-kinase/Akt and PKC pathways.

A link between abnormal levels of ERK5 expression and cancers was established by the analysis of human tumors including prostate cancer, breast cancer, multiple myeloma, and so on. We found in present studies that ERK5 expressed and activated in more relapsed patient samples than newly diagnosed patient samples which are in agreement with other reports and indicate that expression and activation of ERK5 correlates with patients’ unfavorable conditions and poor prognosis [25]. Treatment of high K+ buffer decreased ERK5 phosphorylation in K562/DOX cells without influence of K562 cells which indicates that ERK5 may play an important part in the reversal of drug resistance mediated by NHE1. One possible explanation may include the contribution of receptor tyrosine kinases of the ErbB family. Overexpression or mutations of these members in cancers have been proved to be associated with poor prognosis, shorter disease-free intervals, increased risk of metastasis, and resistance to chemotherapy. Protein microarray showed down-regulation of ErbB2 in K562/DOX cell lines after treatment with Cariporide for 24 h (paper not published), which reveals potential role of ErbB2 in this process. Based on above results, we suppose that ERK5 may participate in Pgp-associated drug resistance via ErbB-dependent mechanism [25–27].

In summary, the results presented in this study show a clear correlation between NHE1 and Imatinib resistance in BCR-ABL-positive cell lines and primary patient samples. From a clinical point of view, combination of Imatinib and Cariporide may have a favorable clinical application in BCR-ABL-positive CML and ALL patients. All these find-
ings support the hope for therapeutic improvement in CML and BCR-ABL-positive ALL using combination chemotherapy.

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**Conflict of interest**

None declare.

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**Appendix A. Supplementary material**


**References**


