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ORIGINAL ARTICLE: RESEARCH

Development of imatinib and dasatinib resistance: dynamics of expression of drug transporters *ABCB1*, *ABCC1*, *ABCG2*, *MVP*, and *SLC22A1*

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Abstract

About 20% of patients with chronic myeloid leukemia (CML) do not respond to treatment with imatinib either initially or because of acquired resistance. To study the development of CML drug resistance, an in vitro experimental system comprising cell lines with different resistance levels was established by exposing K562 cells to increasing concentrations of imatinib and dasatinib anticancer agents. The mRNA levels of *BCR– ABL1* and of genes involved in drug transport or redistribution (*ABCB1*, *ABCC1*, *ABCC3*, *ABCG2*, *MVP*, and *SLC22A1*) were measured and the ABL1 kinase domain sequenced. Results excluded *BCR– ABL1* overexpression and mutations as relevant resistance mechanisms. Most studied transporters were overexpressed in the majority of resistant cell lines. Their expression pattern was dynamic: varying with resistance level and chronic drug exposure. Studied efflux transporters may have an important role at the initial stages of resistance, but after prolonged exposure and for higher doses of drugs other mechanisms might take place.

Keywords: *Chronic myeloid leukemia, multidrug resistance, drug transporters (ABCB1, ABCC1, ABCC3, ABCG2, MVP, SLC22A1), K562 cell line*

Introduction

Chronic myeloid leukemia (CML; 15% of adult leukemia) is a clonal hematopoietic stem cell disorder, the consequence of a reciprocal translocation resulting in fusion of the *BCR* gene, on 22q11, and the *ABL1* gene, on 9q34, giving rise to the Philadelphia chromosome (Ph) and *BCR–ABL1* protein. This oncoprotein displays constitutive tyrosine kinase activity, which alters cellular homeostatic mechanisms resulting in increased proliferation, decreased apoptosis, mutation accumulation, and genomic instability [1,2].

Imatinib mesylate, like most tyrosine kinase inhibitors (TKIs) that target oncogenic kinases on which cancer cells become dependent for survival, has great efficacy and safety as first-line therapy for patients with CML due to its inhibition of BCR–ABL1 and induction of apoptosis [3]. More than 80% of newly diagnosed patients attain complete cytogenetic remission, and after 6 years of treatment the majority of patients will continue to respond well [4]. However, imatinib resistance is now a well-recognized problem, particularly in the advanced phase of the disease. One of the main mechanisms of resistance in patients with CML is the presence of

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mutations in the *BCR–ABL1* tyrosine kinase domain [2]. However, some patients may develop resistance without kinase domain mutations, whereas others develop kinase domain mutations without developing imatinib resistance, suggesting that additional factors are required to produce a fully drug-resistant phenotype. Other described resistance mechanisms include *BCR–ABL1* amplification, overexpression of the SRC family of kinases, reduced drug uptake, enhanced drug efflux, activation of DNA repair, and defective apoptotic pathways [2]. Nilotinib and dasatinib, the second-generation tyrosine kinase inhibitors, have been developed to override the phenomenon, but they do not overcome all the causes of resistance. Published data suggest that *BCR–ABL1* positive cells can evade the inhibitory effect of nilotinib and dasatinib by several mechanisms, still poorly understood, but similar to those observed with imatinib [5,6].

Cellular drug resistance is a major obstacle not only in CML but in cancer therapy in general. Cancer cells can acquire resistance to a single drug, to a class of cytotoxic or novel targeted drugs, or to a broad spectrum of unrelated drugs, a phenomenon known as multidrug resistance (MDR). Several mechanisms including those described above for imatinib resistance can contribute to the MDR phenomenon. Most commonly, however, MDR results from the active adenosine triphosphate (ATP)-dependent transport of drugs out of the cell by efflux pumps belonging to the ATP-binding cassette (ABC) family of transporters [7]. At least 18 human ABC transporters have been described as being associated with drug transport *in vitro* [8]. In addition to these, other proteins associated with redistribution of the drug from the nucleus to the cytoplasm have also been associated with the MDR phenotype, such as the major vault protein (MVP) [9]. Hematopoietic stem cells are known to express a great number of membrane efflux transporter proteins, some of them being overexpressed for protection against genetic damage caused by xenobiotics, maintenance of quiescence, and cell fate decisions [10]. Therefore, the CML initiating cell may be innately resistant to therapy due to the expression profile of some efflux proteins. It is now well known that different ABC efflux transporters such as ABCB1 (P-glycoprotein, MDR1), multidrug resistance-associated proteins (MRPs, e.g. ABCC1, ABCC3), or the breast cancer resistance protein (BCRP or ABCG2) actively regulate the traffic of small molecules across the cell membrane, being therefore key determinants of intracellular drug concentrations, including imatinib and dasatinib [11,12]. Both dasatinib and imatinib are ligands of the ABCB1 and ABCG2 efflux transporters in leukemic cells. However, imatinib has been shown to be also an inhibitor of ABCG2, and therefore some controversy exists over whether or not it can confer resistance [13–15]. Whereas the cellular uptake of imatinib has been shown to be mediated by human organic cation transporter 1 (hOCT-1 or SLC22A1) [16], dasatinib cellular uptake is predominantly passive and not SLC22A1 dependent [12].

Despite the large number of articles published thus far, it is not known which of these transporters is the most crucial for the acquired resistance of CML cells to imatinib. This difficulty is due, in part, to the fact that the majority of previous studies were performed on cell lines engineered to overexpress a single transporter [13,17–19].

There is a lack of more detailed characterization of the role of transporters; specifically, the chronic administration of TKIs strongly suggests that any possible interactions with MDR transporters should be studied, analyzing periods of prolonged drug exposure.

With the purpose of elucidating the role of drug transporters in acquired resistance, we established imatinib- and dasatinib-resistant K562 cells (K562_IM and K562_DA), derived from human CML, by culturing parent K562 cells with gradually increasing concentrations of imatinib and dasatinib. The continuous exposure to imatinib (over 253 days) and dasatinib (over 160 days) allowed us to obtain several cell lines resistant to different concentrations of TKIs: a valuable experimental system that mimics the acquired resistance. We then analyzed the main mechanisms responsible for the development of resistance in these cells. In addition, to determine maintenance of the resistant phenotype, we cultured two resistant cell lines (K562_1.0 μM IM and K562_5.0 μM IM) in the continuous presence of the corresponding concentration of imatinib for more than 100 days after the acquisition of resistance. Specifically, we focused on the mRNA expression of genes that code for proteins potentially involved in transport or redistribution of imatinib and dasatinib anticancer agents (*ABCB1, ABCC1, ABCC3, ABCG2, MVP, SLC22A1*). Protein expression of ABCB1 was further confirmed by immunofluorescence staining. Simultaneously we measured *BCR–ABL1* mRNA transcripts and sequenced the kinase domain of the *ABL1* coding region to evaluate the role of *BCR–ABL1* overexpression and/or mutations in the development of the resistant phenotype.

Materials and methods

Cell lines

The human CML K562 cell line expressing *BCR–ABL1* (DSMZ; German National Resource Center for Biological Material) was cultured in RPMI 1640 medium (Sigma) supplemented with 10% fetal

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bovine serum (Sigma) under an atmosphere of 5% CO_2 at 37°C. Cells were passed twice weekly.

Stock dilutions of both imatinib and dasatinib were prepared in dimethyl sulfoxide at a concentration of 10 mM and 20 mM, respectively, and stored at −20°C.

Resistant K562 cells were generated through incubation with increasing concentrations of imatinib, starting with a concentration of 0.05 μM, to establish five different IM-resistant cell lines. After acquiring the ability to divide in the presence of a specific concentration of the drug, a proportion of cells were frozen, and the remaining cells were grown at the next highest drug level. In this way, subpopulations of cells that were able to grow in the presence of 0.25, 0.5, 1.0, 2.0, and 5.0 μM imatinib were then selected for further studies, and referred to as K562_0.25 μM IM, K562_0.5 μM IM, K562_1.0 μM IM, K562_2.0 μM IM, and K562_5.0 μM IM, respectively. The same methodology was applied to develop the cell lines resistant to 0.5, 0.75, and 1.5 nM of the more potent inhibitor dasatinib. To determine maintenance of the resistant phenotype, we cultured K562_1.0 μM IM and K562_5.0 μM IM cell lines in the continuous presence of the corresponding concentration of IM for more than 230 and 140 days, respectively, after the acquisition of resistance, until day 393. All resistant cell lines had their respective passage control, i.e. K562 wild type cells (K562_wt) that were grown in parallel, in the same conditions, except that they were not exposed to the TKI.

Cell proliferation assay: MTS

Cell viability was determined using the CellTiter96®-AQueous One Solution Cell Proliferation Assay (Promega). Viable cells reduce MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)] to a purple formazan compound with an absorbance at 490 nm. K562_wt and K562_5.0 μM IM cell lines were diluted to a density of 1.0×10^4 per 100 μL, plated in each well of a 96-well plate, and incubated with several IM concentrations up to 10 μM, for 48 h under an atmosphere of 5% CO_2 at 37°C. Negative and DMSO controls were also performed. After that, cells were incubated with 20 μL of CellTiter96®AQueous One Solution reagent per well, for an additional period of 3 h, and absorbance was detected with a ZenYth 3100 (Anthos) spectrofluorimeter and Multimode Detection Software (Beckman Coulter). The absorbance is directly correlated with cell survival. For each MTS assay, samples were analyzed in triplicate, and the MTS assays were repeated in at least three independent experiments.

Immunofluorescence staining

IM resistant K562_1.0 μM IM and K562_5.0 μM IM and K562_wt cells were distributed in poly-D-lysine coated culture slides (Santa Cruz) and allowed to adhere for approximately 20 h. Cells were fixed with formaldehyde (4%) and permeabilized with triton X-100 (0.5%), and unspecific sites blocked with bovine serum albumin (4%). Then cells were incubated with the primary antibody ABCB1 (Santa Cruz-55510) followed by secondary antibody goat anti-mouse immunoglobulin G–fluorescein isothiocyanate (IgG–FITC) (Santa Cruz-2010). After washing, counter-staining of the nucleus was done with Hoescht and slides were mounted with an anti-fading medium (Vectashield; Vectorlabs). Cells were analyzed at ×1000 amplification by fluorescence microscopy (Leica DMLB, Germany). Images of randomly selected cells were captured from each slide, using Cytovision (v3.0) capture software (Genetix). Images captured at ×200 were analyzed using the CellProfiler image analysis software package (www.cellprofiler.org) [20,21]. Integrated fluorescence of FITC coupled anti-ABCB1 antibody was calculated for each cell and mean values plotted with 95% confidence intervals. At least two independent experiments were performed, and a minimum of 100 cells per cell line were analyzed in each experiment.

RNA isolation and cDNA synthesis

For RNA extraction the AllPrep DNA/RNA/Protein Kit (Qiagen) was used. The concentration and purity of resulting RNA were estimated at 260 and 280 nm using the Nanodrop spectrophotometer Nd-1000 (Thermo Scientific), and only those samples with A_{260}-to-A_{280} ratios between 1.9 and 2.1 were considered further. Afterward, 2 μg of total RNA was reverse transcribed with the High Capacity RNA-to-cDNA Kit (Applied Biosystems) in a final volume of 20 μL.

Quantitative real time PCR

The relative expression level of transporter genes and also of the *BCR–ABL1* transcript were evaluated by quantitative real time polymerase chain reaction (qRT-PCR) in a 7300 qRT-PCR system using TaqMan probes and TaqMan Universal PCR Master Mix, following the manufacturer's instructions (Applied Biosystems). mRNA levels were measured using available Assays-on-Demand Products from Applied Biosystems: *ABCB1*, Hs00184491_m1; *ABCC1*, Hs00219905_m1; *ABCC3*, Hs00358656_m1; *ABCG2*, Hs00184979_m1; *LRP/MVP*, Hs00245438_m1; *SLC22A1* (*SLC22A1*), Hs00427550_m1; *BCR–ABL1*, Hs03024784_ft; *GAPDH*, 4352934E; and *GusB*, 4333767F.

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We included no-template controls (NTCs) and no-reverse-transcriptase controls (RT negative) for each cDNA synthesis. Only those that did not amplify, showing that primer–dimer formation and genomic DNA contamination were negligible, were considered further.

The relative gene expression levels were calculated by the $2^{-\#\#Ct}$ method [22] using a sample of K562_wt as calibrator for both resistant cell lines and their passage controls. The threshold cycle (Ct) was defined as the actual PCR cycle when the fluorescence signal increased above the background threshold. Average Ct values from duplicate or triplicate qRT-PCR reactions were normalized to average Ct values for endogenous housekeeping genes *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) for transporters and *GusB* (β-glucuronidase*)* for *BCR–ABL1*, from the same cDNA preparations, and then were compared to the normalized value of the calibrator sample. Values are reported as the average of triplicate analyses.

The validity of the comparison between mRNA expressions was ensured by checking the efficiency of the PCR. It was over 95% and similar for all qRT-PCR assays.

Data analysis

All qRT-PCR reactions were repeated at least three times using cDNA from two independent syntheses. Results are shown as the mean with standard deviation (SD). The statistical significance of differences between means for resistant cells and their passage controls was calculated by Student's *t*-test, with 95% confidence interval.

Mutation analysis

An 863 bp fragment containing the *BCR–ABL1* kinase domain was amplified from cDNA in a semi-nested PCR and sequenced in the forward and reverse directions as described by Branford and Hughes [23]. Briefly, a first-stage PCR used forward primer BCRF (5' TGACCAACTCGTGTGT GAA ACTC) and reverse primer ABL1KinaseR (5' TCC ACTTCGTCTGAGATACTGGATT) and a second-stage PCR used forward primer ABL1kinaseF (5' CGCAACAAGCCCACTGTCT) and reverse primer ABL1kinaseR. The direct sequences of the K562 cell line and the GeneBank NM_005157.3 *ABL1* wild type cDNA reference sequence were compared using BioEdit Sequence Alignment Editor software (Department of Microbiology, North Carolina State University).

Results

Generation of cell lines resistant to IM and DA

We established an *in vitro* experimental system that mimics the acquired resistance, developed by the continuous exposure of human CML derived cell line K562 expressing *BCR–ABL1* gene to increasing concentrations of imatinib (over 253 days) and dasatinib (over 160 days), as illustrated in Figure 1. In this way, subpopulations of cells that were able to grow in the presence of 0.25, 0.5, 1.0, 2.0, and 5.0 μM imatinib and 0.5, 0.75, and 1.5 nM dasatinib were acquired for further studies. The level of resistance was defined by the imatinib or dasatinib concentration at which the cell replication rate was comparable to that of untreated parental cells. Cells

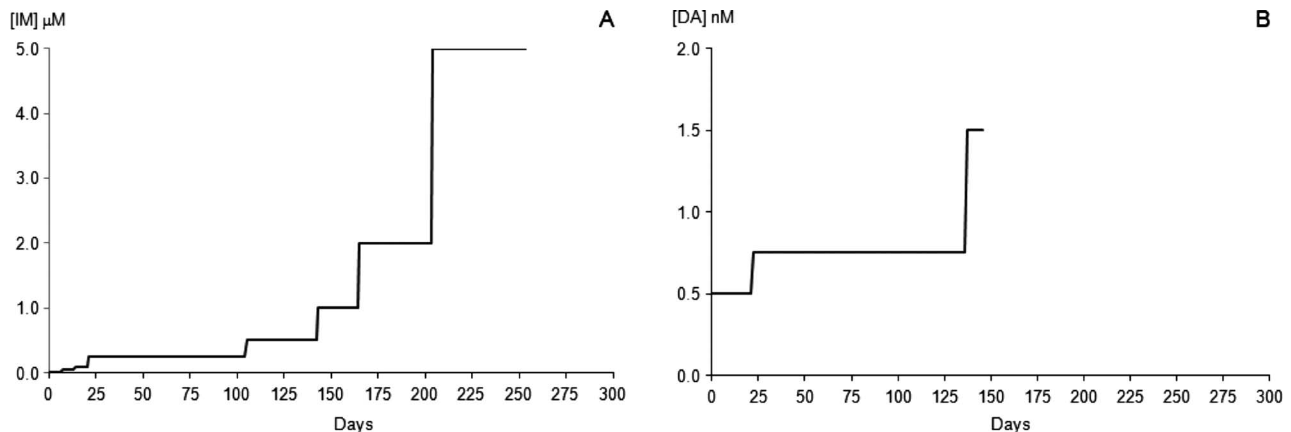

Figure 1. Development of resistance to imatinib (A) and dasatinib (B) in BCR–ABL1-positive cell line K562, generated through incubation with increasing concentrations of TKI, starting with a concentration of 0.05 μM imatinib and 0.5 nM dasatinib, respectively. The subpopulations of cells that were able to grow in the presence of 0.25, 0.5, 1.0, 2.0, and 5.0 μM imatinib and 0.5, 0.75, and 1.5 nM dasatinib were then selected for analysis. Parental, sensitive cell lines were maintained in parallel cultures without TKI to be used as passage controls.

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were regularly counted using a hemocytometer to follow the replication rate. Passage controls were established by growing K562 cells in the absence of the TKI inhibitor. Additionally, we verified that after being frozen for several months, K562_5.0 μM IM cells maintained resistance to increasing concentrations of imatinib (Figure 2).

BCR–ABL1 *associated resistance mechanisms*

To evaluate potential resistance mechanisms related to the *BCR–ABL1* gene in K562_IM and K562_DA cells, we examined *BCR–ABL1* gene expression by real-time PCR and *ABL1* kinase domain mutations of the *BCR–ABL1* gene by direct sequencing.

The overall levels of *BCR–ABL1* transcripts in the different resistant cell lines as compared with each corresponding control and with the starting population showed a small, although statistically significant, increase. However, this was maintained similar in all resistant cell lines and stable throughout the time after the acquisition of resistance, except for the cell line resistant to the highest concentration of dasatinib, where the difference of expression level compared with control was not significant (Figure 3).

PCR amplification of the 863 bp region encompassing the entire *ABL1* kinase domain followed by direct sequencing of the products did not allow the identification of any mutation, either in K562_wt or in imatinib- or dasatinib-resistant cell lines. These results were maintained for 40 passages (more than 100 days) of the time after cells acquired resistance.

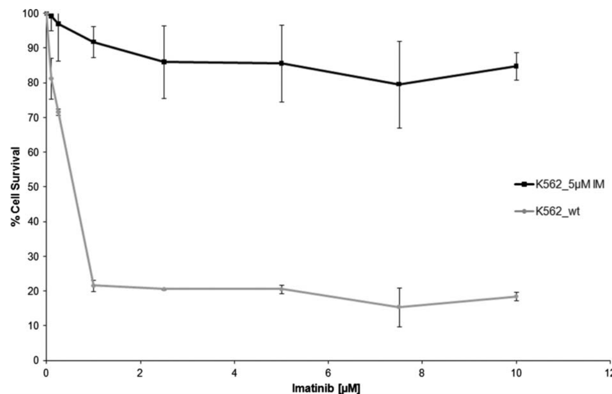

Figure 2. Cell survival of K562_5 μM IM and K562_wt cell lines, evaluated with MTS assay, exposed to increasing doses of imatinib.

Expression of transporter genes

No mRNA expression was detected for *ABCC3* (no amplification over 35 cycles), and *ABCC1* seemed to have a limited role in both imatinib and dasatinib resistance. All remaining studied transporters were overexpressed in the majority of resistant cell lines, and all resistant cell lines had increased expression of more than one drug transporter.

In particular, all imatinib resistant cell lines showed significantly high levels of *ABCB1* and *MVP*, compared with the control sensitive parental cell line, while the other transporter genes were found to be significantly overexpressed only in some resistant cell lines. For all the studied genes that code for the efflux proteins, it was observed that the level of overexpression did not increase linearly with the degree of imatinib resistance (Figure 4). The highest overexpression was observed for the *ABCB1* gene in the K562_1.0 μM IM, which expressed on average 92 times more *ABCB1* mRNA than the K562_wt calibrator sample, whereas in K562_2.0 μM IM the gene was 32-fold overexpressed and 18-fold in K562_5.0 μM IM. Albeit with much lower levels of expression, also *MVP* presented the same trend as

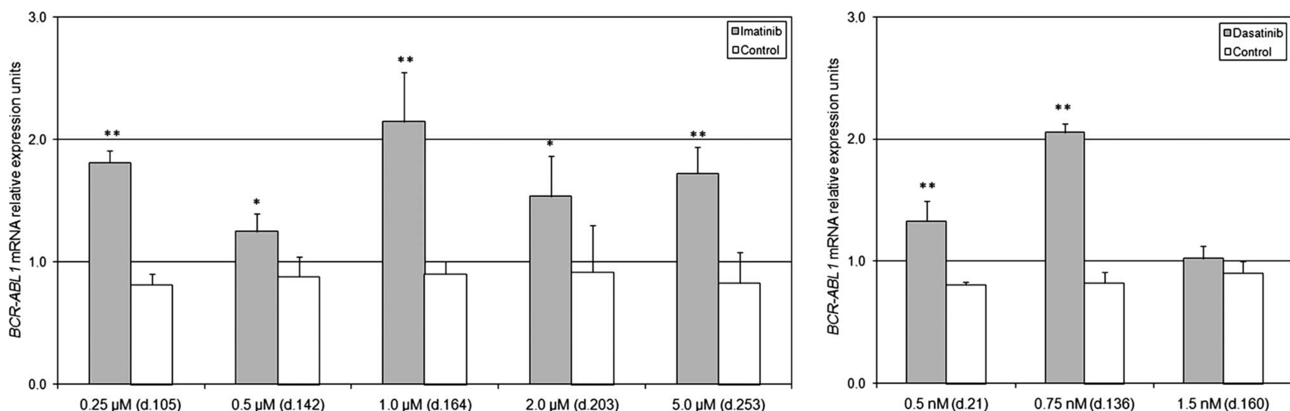

Figure 3. BCR–ABL1 mRNA expression in K562_IM cells (left) and K562_DA cells (right) and in their corresponding controls (K562 cells grown in parallel in the absence of TKI). Statistically significant differences $P < 0.05$ compared to control using Student's t-test are indicated with one asterisk and double asterisks indicate $P < 0.01$.

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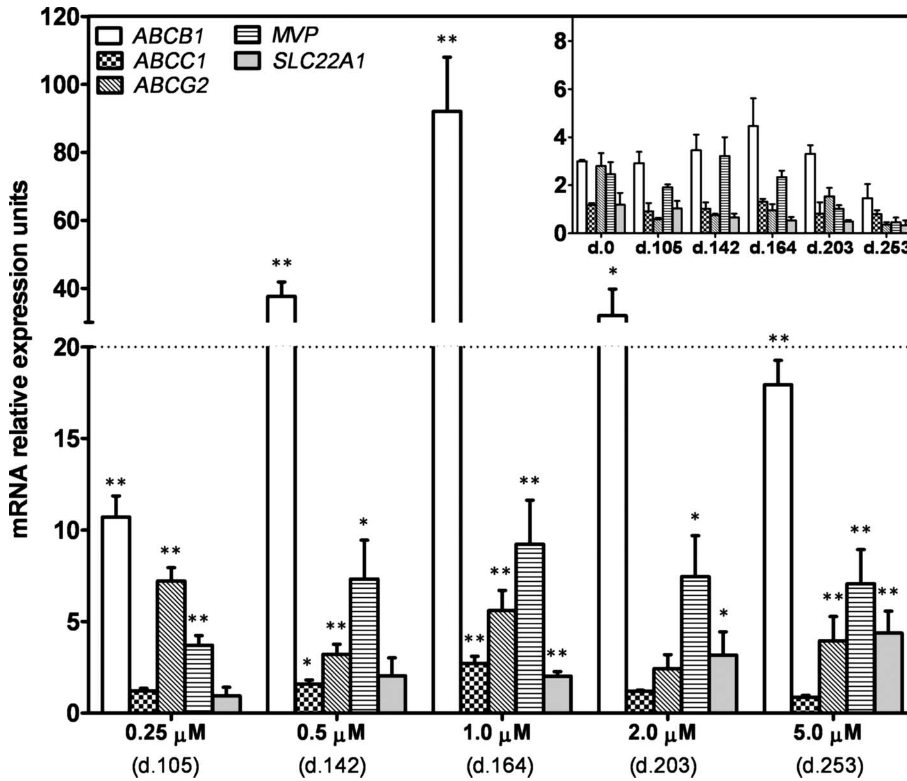

Figure 4. Relative mRNA expression levels of ABCB1, ABCC1, ABCG2, MVP, and SLC22A1, measured by real time RT-PCR for all imatinib-resistant K562 cells and their respective controls (inset). Transporters for which differential expression reached statistical significance $P < 0.05$ compared to control using Student's t-test are indicated with one asterisk and double asterisks indicate $P < 0.01$.

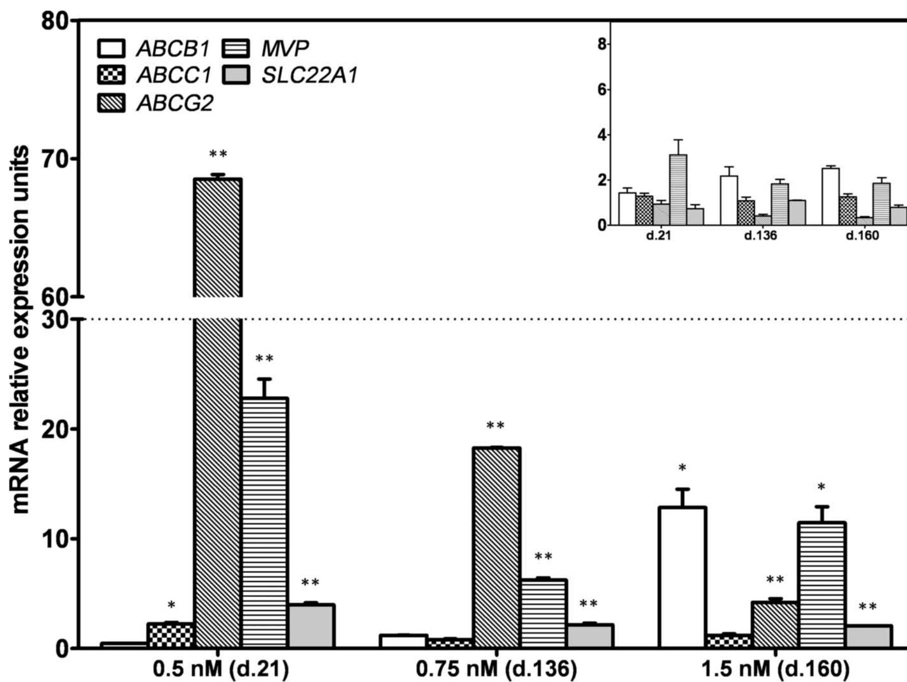

Figure 5. Relative mRNA expression levels of ABCB1, ABCC1, ABCG2, MVP, and SLC22A1, measured by real-time RT-PCR for all dasatinib-resistant K562 cells and their respective controls (inset). Transporters for which differential expression reached statistical significance $P < 0.05$ compared to control using Student's t-test are indicated with one asterisk and double asterisks indicate $P < 0.01$.

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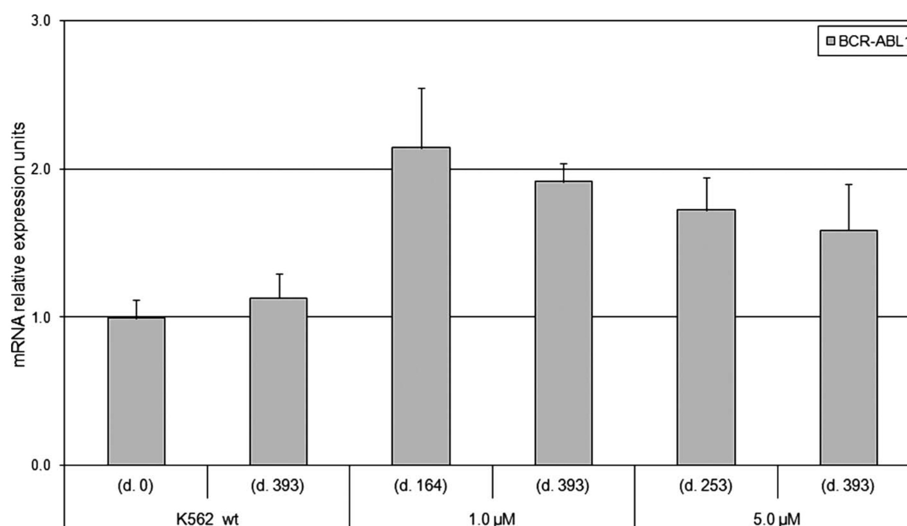

Figure 6. Relative mRNA expression levels of *BCR–ABL1* in K562_IM cells resistant to 1 and 5.0 μM imatinib at the time they acquired resistance (day 164 and day 253, respectively) and after chronic exposure to the same drug concentration (day 393) and in passage control cells (K562_wt) at the beginning of the experience (day 0) and at the end (day 393).

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ABCB1: both increased their expression up to the K562_1.0 μM IM cell line and then decreased in cell lines resistant to higher doses of IM. Conversely, *ABCG2* attained its higher expression (average seven times) in the cell line resistant to the lowest concentration of the drug (K562_0.25 μM IM). The influx transporter *SLC22A1* was the only gene whose expression levels increased with increasing doses of imatinib (Figure 4).

In the dasatinib resistant K562 cells, *ABCG2* showed the highest levels of mRNA relative expression; i.e. 68, 18, and 13 times more expressed in K562_0.5 nM DA, K562_0.75 nM DA, and K562_1.5 nM DA, respectively, than in the K562_wt calibrator sample (Figure 5). Also *MVP* and *SLC22A1* were overexpressed in the three resistant cell lines. Conversely to what we observed in imatinib resistant cell lines, *ABCB1* was only overexpressed (13-fold) in one cell line, the K562_1.5 nM DA.

The development of resistance to both TKIs was characterized by a similar trend: the highest mRNA expression of a transporter, observed for *ABCB1* in the K562_1.0 μM IM and for *ABCG2* in K562_0.5 nM DA, was not attained in cell lines resistant to higher doses of the corresponding drug (Figures 4 and 5) but at lower concentrations, and thereafter their expression was diminished in cell lines resistant to higher doses.

Dynamics of resistant phenotype

In addition, resistance mechanisms were assessed in K562_1.0 μM IM and in K562_5.0 μM IM in culture in the presence of 1.0 and 5.0 μM imatinib after 230 and 140 days, respectively, until day 393. Again, after that time, no mutations in the kinase domain of BCR–ABL1 were detected. Although maintaining a two-fold overexpression when compared to the control, the expression level of *BCR–ABL1* did not vary significantly with time (Figure 6). In contrast, transporter genes presented a significant variation in expression levels over time of exposure to 1.0 and 5.0 μM imatinib (Figure 7). These results were corroborated by ABCB1 antibody immunofluorescence staining (Figures 8 and 9). The level of *SLC22A1* increased during that period: in K562_1.0 μM IM it increased from twice the expression to be four-fold overexpressed after chronic exposure, and in K562_5.0 μM IM increased from four- to seven-fold overexpression. For all other transporter genes, their expression decreased significantly after imatinib chronic exposure. The most relevant change in expression was observed for *ABCB1* in K562_5.0 μM IM, which was no longer overexpressed at day 393. All others, although with significantly decreasing relative expression values, maintained a significant overexpression when compared to the respective passage control (Figure 7).

Discussion

Determination of the expression profiles of ABC transporter genes through quantitative real-time PCR, a fast and sensitive detection method that allows reproducible quantification of very low amounts of total RNA, can be a valuable approach for the diagnosis and monitoring of MDR in patient samples and toward adequate clinical treatment [11].

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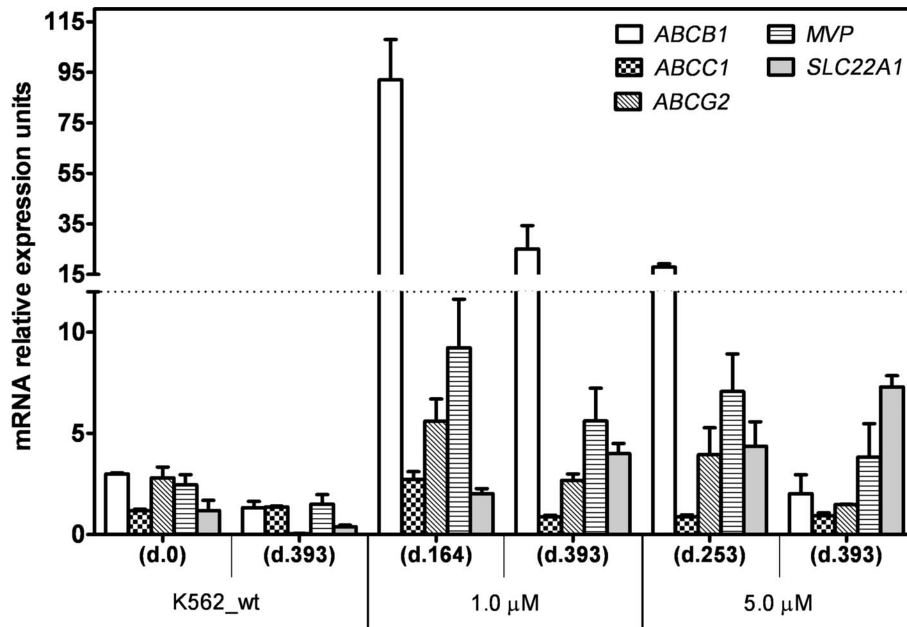

Figure 7. Relative mRNA expression levels of ABCB1, ABCC1, ABCG2, MVP, and SLC22A1 in K562_IM cells resistant to 1 and 5.0 μM imatinib at the time they acquired resistance (day 164 and day 253, respectively) and after chronic exposure to the same drug concentration (day 393) and in passage control cells (K562_wt) at the beginning of the experience (day 0) and at the end (day 393).

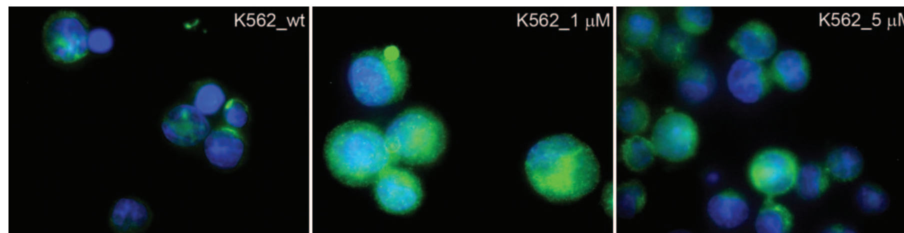

Figure 8. ABCB1 detected by immunofluorescence staining with primary antibody ABCB1 (Santa Cruz-55510) followed by secondary antibody goat anti-mouse IgG–FITC (green staining; Santa Cruz-2010) in K562_wt and K562_1 μM IM and K562_5 μM IM. After washing, counter-staining of the nucleus was done with Hoescht (blue staining). Images were analyzed with $\times 1000$ amplification in a fluorescence microscope (Leica) equipped with the software Cytovision v3.0 (Genetix).

To perform molecular and dynamic studies of CML drug resistance, we established several cell lines with different resistance levels that mimic the acquired resistance, through continuous exposure of a human CML derived cell line expressing *BCR–ABL1* oncogene (K562) to increasing concentrations of imatinib and dasatinib. Such a methodology has only been rarely applied [6,24–28], probably because, due to imatinib and dasatinib efficacy and specificity, the assessment of resistance is difficult and time consuming (Figure 1). This methodology allowed us to analyze many derived resistant cell lines and characterize the development of a resistant phenotype. In fact, while a lot of effort has gone into understanding specific targets and cellular pathways underlying TKI resistance, relatively few studies have focused on the dynamic cellular

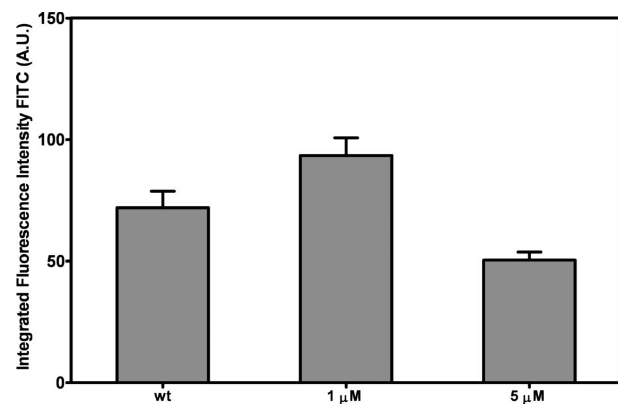

Figure 9. Mean values with 95% confidence intervals of integrated fluorescence intensity for FITC coupled anti-ABCB1 antibody calculated for K562_wt and K562_1 μM IM and K562_5 μM IM resistant cell lines.

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responses allied with resistance evolution [29]. Therefore, our goal was to mimic as closely as possible the development of resistance *in vivo* using this *in vitro* cell model.

The two main mechanisms involved in the development of resistance to TKIs is the presence of mutations in the *BCR–ABL1* tyrosine kinase domain [2] and its overexpression/amplification [24,26–28]. In this regard, we evaluated the involvement of *BCR–ABL1* overexpression and acquisition of mutations in the *BCR–ABL1* kinase domain in the development of resistance. In this work, however, we were not able to detect any mutations in the *BCR–ABL1* kinase domain, and we observed only a small increase in *BCR–ABL1* expression throughout resistance (results not shown and Figures 3 and 6). Indeed the acquisition of various degrees of imatinib or dasatinib resistance was not followed by relevant changes in the expression level of *BCR–ABL1* (Figures 3 and 6). These results excluded *BCR–ABL1* up-regulation and point mutations as being relevant for the acquired resistant phenotype of all our cell lines (results not shown and Figures 3 and 6).

We also evaluated the expression profile of *ABCB1, ABCC1, ABCC3, ABCG2, MVP, and SLC22A1* genes, in order to clarify their role as molecular determinants of imatinib and dasatinib drug resistance. Since no mRNA expression was detected for *ABCC3* and *ABCC1* presented only a few small expression variations, they both seem to have a limited role in imatinib and dasatinib resistance. The four remaining studied transporters (*ABCB1*, *ABCG2*, *MVP*, and *SLC22A1*) were overexpressed in the majority of resistant cell lines and all resistant cell lines had increased expression of at least three of these drug transporter genes (Figures 4 and 5). Thus, it seems likely that different transporter expression patterns play an important role in interindividual differences in drug sensitivity [8]. However, the mRNA levels of the efflux transporter genes studied did not follow a linear relationship with the resistance level (Figure 4).

In fact, a linear relationship between mRNA expression and increasing imatinib concentrations was only observed for the influx transporter gene *SLC22A1*. Also, variation in expression of these genes occurred over the time of exposure to the same concentration of imatinib while maintaining resistance (Figures 7, 8, and 9), suggesting that resistance mechanisms can vary dynamically.

The overexpression of *ABCB1* (P-glycoprotein) is probably a major cause of acquisition of the initial resistant phenotype in imatinib resistant cell lines, a finding consistent with many previous studies that have already demonstrated that P-glycoprotein (P-gp) confers resistance to imatinib [e.g. 25,30,31]. However, the highest *ABCB1* mRNA levels were detected in K562_1.0 μM IM, in contrast to the results of Mahon *et al.* [26], while studying P-gp expression by fluorocytometry. These authors found that K562 cells resistant to 1.0 μM IM showed negligible baseline levels of P-gp that overlapped the profiles of their sensitive counterparts. Those authors also did not find mutations in the *BCR–ABL1* kinase domain nor overexpression of BCR–ABL1 protein, indicating that other mechanisms may be underlying imatinib resistance in their cell line.

The highest *ABCB1* mRNA expression observed in the K562_1.0 μM IM cell line was followed by a significant decrease in expression ($P < 0.01$) in cell lines resistant to higher doses of the drug (Figures 4 and 5). Such a discrepancy between the level of resistance and the expression of ABCB1 has been described before, but its cause remains unexplained [32]. A possible explanation can be given by microRNAs whose regulation of drug resistance mediated by *ABCB1* has already been demonstrated [33], indicating them as potential targets for modulating MDR in cancer cells as a therapeutic strategy.

A similar trend was observed for *ABCG2* in dasatinib resistant cells. After initial dasatinib exposure K562 cells induced an overexpression of *ABCG2*, whose mRNA levels were significantly decreased at higher dasatinib concentrations ($p < 0.01$). However, the highest decrease occurrred once *ABCB1* expression was induced, suggesting that both pumps cooperate in the extrusion of dasatinib and are coordinately regulated (Figure 5). The same trend was observed in Caco2 cells exposed to imatinib [34].

While some studies found imatinib to be a potent inhibitor of *ABCG2* [14,18,25], others found imatinib to be a substrate of BCRP [13,17,34]. Also, *BCR–ABL1* itself may regulate ABCG2 protein expression via AKT activity [19], and besides, there is some evidence to suggest that *ABCG2*-mediated resistance to imatinib is only effective at imatinib concentrations below 1 μM [13]. Despite the fact that the highest *ABCG2* mRNA expression (seven-fold) was observed for the lowest imatinib concentration (K562_0.25 μM IM), we found that *ABCG2* mRNA in K562_1.0 μM IM cells was six-fold and in K562_5.0 μM IM cells was four-fold higher than in the K562_wt calibrator sample. Therefore, *ABCG2* may also contribute to imatinib resistance in K562 cells.

The influx of imatinib into the cell is an active process initially mediated by *SLC22A1* [16], whereas effective uptake of dasatinib is likely to occur even at very low levels of expression of *SLC22A1*, at which imatinib is ineffective [35]. Additionally, it was observed that mRNA and protein expression of

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SLC22A1 are not affected by imatinib exposure in K562 cells [25]. Nevertheless, our results show quite the opposite: *SLC22A1* increased its mRNA expression along with increasing imatinib concentrations.

At therapeutic doses, the imatinib maximum concentration is typically 3–6000 ng/mL, with trough levels near 1000 ng/mL [36]. Thus, we selected K562_1.0 μM IM and K562_5.0 μM IM to evaluate the dynamics of the resistant phenotype during chronic exposure to 1.0 μM and 5.0 μM imatinib, respectively. To our knowledge, the only study that investigated whether prolonged imatinib treatment affects the expression pattern of genes potentially involved in drug transport was performed on Caco2 cells cultured up to 100 days in the presence of 10 μM imatinib [34]. In that study, imatinib exposure specifically induced expression of *ABCB1* and *ABCG2*, while the basal mRNA levels of *ABCC1* were almost not affected by chronic imatinib treatment. These authors also observed that the induction of both genes stabilized after approximately 50 days (five-fold induction) and lasted throughout the complete exposure time, indicating that this drug-induced overexpression is a steady phenomenon and stable over time [34]. Conversely, our data after prolonged exposure to imatinib (230 and 140 days in 1 μM and 5 μM, respectively) showed a significantly different mRNA expression profile for *ABCB1* (Figure 7). Its expression levels decreased at both drug concentrations and most notably K562_5 μM IM, returning to basal expression levels (no different from control, $P > 0.05$). Although presenting lower levels of mRNA expression, *ABCG2* and *MVP* maintained their overexpression relative to control. This is in agreement with other *in vitro* and *in vivo* data showing that a drug-resistant phenotype may be primarily mediated by a single dominant transporter, although it may also be facilitated by the activity of additional ABC transporters expressed in tumors, which have not yet been linked to drug resistance, but may have prognostic relevance [8,11]. The only transporter that presented increasing expression levels throughout time was the *SLC22A1* influx transporter, and this deserves to be taken into consideration: why is the expression level of *SLC22A1* increasing over time of exposure to the same concentration of imatinib while all the studied efflux ABC transporters and *MVP* decrease? If this occurs in patients through chronic treatment, perhaps the pretreatment expression level of *SLC22A1* is not really critical in determining treatment outcome as predicted by some studies [37–40].

All in all, we can conclude that in our cellular models the resistant phenotype is only partly stable. The results we obtained indicate that the role of the studied efflux drug transporter genes may be important at the initial stages of resistance but after prolonged exposure and for higher doses of TKI, other mechanisms take place. We have focused on the role of drug transporters; however, development of the drug-resistant phenotype is a multifactorial process rather than the result of a single mechanism. It is the consequence of a complex network of various cellular pathways and molecular mechanisms that are commonly up-regulated in tandem in many MDR cells. Therefore, the problem of drug resistance cannot be solved solely by circumventing the expression of drug transporters. Altered DNA repair capacity was also found by our group in these same resistant cells [41]. In addition, the elevated expression of antiapoptotic proteins was also evaluated but, to date, it does not seem to entirely explain the observed resistant phenotype.

The distinct cell lines resistant to several concentrations of imatinib and dasatinib represent a valuable model to identify drug resistance-associated genes. Further studies, both *in vitro* and *in vivo*, are needed, and will hopefully establish the possible mechanisms of resistance in CML and open the way for specific pharmacological intervention so that the prevention of resistance by combined therapies will be more effective.

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