Can P-glycoprotein mediate resistance to nilotinib in human leukaemia cells?

Petr Koszytu, Petr Dolezel, Petr Mlejnek*

Department of Biology, Faculty of Medicine and Dentistry, Palacky University Olomouc, Hnevotinska 3, Olomouc 77515, Czech Republic

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The effect of P-glycoprotein (P-gp, ABCB1, MDR1) expression on cell resistance to nilotinib was studied in human leukaemia cells. We used K562/Dox cells overexpressing P-gp and their variants (subclones) with a gradually decreased P-gp expression. These subclones were established by stable transfection of K562/Dox cells with a plasmid vector expressing shRNA targeting the ABCBI gene. Functional analysis of P-gp using a specific fluorescent probe indicated gradually decreased dye efflux which was proportional to the P-gp expression. We observed that K562/Dox cells overexpressing P-gp contained a significantly reduced intracellular level of nilotinib when compared to their counterpartner K562 cells, which do not express P-gp. This effect was accompanied by a decreased sensitivity of the K562/Dox cells to nilotinib. Importantly, cells with downregulated expression of P-gp gradually lost their ability to decrease the intracellular level of nilotinib although they still significantly decreased the intracellular level of daunorubicin (DNR). Accordingly, cells with the reduced expression of P-gp concomitantly failed to provide resistance to nilotinib, however, they exhibited a significant resistance to DNR. Taken together, we demonstrated that the conclusion as to whether P-gp is involved in nilotinib resistance or not strongly depends on its expression at protein level.

1. Introduction

Nilotinib (Tasigna, AMN107), a second-generation tyrosine kinase inhibitor (TKI), is approved for the treatment of chronic phase and accelerated phase of Philadelphia chromosome-positive (Ph+ chronic myeloid leukaemia (CML)) in adults intolerant or resistant to imatinib (Glivec, STI571). It acts as a competitive ATP inhibitor of fusion Bcr-Abl kinase by stabilising its inactive configuration [1]. In addition to Bcr-Abl, nilotinib also targets other tyrosin kinases, including platelet-derived growth factor receptor (PDGFR) α and β, and c-Kit [2]. Preclinical studies indicated that nilotinib is effective against several Bcr-Abl mutant forms that are found in patients with a diagnosis of Ph+ CML, such as E255V, M351T, Q252H/R, Y253F, F317L, and E355G. However, it fails to inhibit the T315I mutant [3]. Although nilotinib has shown a significant advancement in the treatment options available in clinical practice, recent studies suggest that its efficiency might be significantly reduced due to the overexpression of ATP-binding cassette (ABC) transporters [4].

So far, 48 genes coding for ABC transporters in human genome have been identified. ABC transporters are mostly unidirectional and translocate diverse substrates from the cytoplasm to the outside of the cell or into intracellular compartments [5]. Three members of ABC transporters, namely P-glycoprotein (MDR1, ABCB1), multidrug resistance-associated protein 1 (MRP1, ABCC1), and breast cancer resistance protein (BCRP, ABCC2), are likely involved in clinical drug resistance. In addition, several members of the ABC family such as ABCB2, ABCC3, ABCC4, ABCC5, ABCC6, and ABCC7, have been shown to confer resistance to some anticancer drugs. However, their clinical significance is uncertain because they have not been sufficiently studied [6].

P-gp was originally described as the first membrane transporter participating in the development of the drug resistance in animals [7]. Early studies suggested that P-gp functions as an ATP-dependent efflux pump for diverse naturally occurring hydrophobic anticancer drugs such as colchicine or doxorubicin [8]. Since then, number of P-gp substrates has increased rapidly and comprises a variety of structurally distinct hydrophobic compounds including natural products (colchicine, flavonoids), steroids (aldosteron), anticancer drugs (anthracyclines, vinca alkaloids, and taxanes), cardiac drugs (digoxin), antibiotics (actinomycine D), protease inhibitors (pepatstine A), immunosuppressive agents (cyclosporine A), fluorescent dyes (rhodamine123), and many others [9,10].

Not surprisingly, some TKIs were also identified as substrates of P-gp [11]. Mahon et al. were first who demonstrated that resistance to nilotinib may be due to the P-gp overexpression [12]. Reduced intracellular accumulation of nilotinib in K562 cells

Abbreviations: DNR, Daunorubicin; Ph+, Philadelphia chromosome-positive; CsA, cyclosporin A; MFI, mean fluorescent intensity.
* Corresponding author. Tel.: +420 585632168; fax: +420 585632966.
E-mail address: mlejnek.petr@volny.cz (P. Mlejnek).
overexpressing P-gp was reported by an independent research group [13]. However, some authors contradicted these findings. For example, no effect on intracellular levels of nilotinib in cell lines transfected with ABCB1 gene or in primary CD34+ cells was observed by Davies et al. [14]. In addition, nilotinib was identified as a high affinity substrate of ABCG2 but not ABCB1 [15].

Unfortunately, such contradictory results are not exceptional in this research field, although they are conducted in relatively well defined in vitro systems. In addition, there exists a large discrepancy between the results obtained in the in vitro experiments and the outcome of clinical trials [16,17]. Although many factors may have an impact on disparate results, we believe that more relevant experimental arrangement used in in vitro experiments might lead to more consistent results among different laboratories and that these results will enable to predict outcomes in clinical practise.

For example, most of the in vitro experiments are conducted with cells overexpressing P-gp, which are obtained either by the selection for a long period in the presence of cytotoxic drugs [18] or by the cell transfection with human ABCB1 cDNA [19]. However, it might be difficult to compare results in experiments where P-gp expression level is a critical parameter since the P-gp expression is usually extremely high in drug selected cells but relatively low in transfected cells [19]. In addition, in vitro experiments carried out with huge expression levels of P-gp are hardly relevant to the clinical samples.

Here we addressed the question how the P-gp expression affects the cell resistance to nilotinib in comparison to daunorubicin (DNR), which represents its well characterised substrate [10,20]. We observed that overexpressed P-gp mediated distinct resistance to DNR and lower but significant resistance to nilotinib. In contrast, cells with the lowest expression of P-gp, which still mediate significant resistance to DNR, completely lost their resistance to nilotinib.

2. Materials and methods

2.1. Chemicals

Nilotinib hydrochloride (Tasigna, AMN107) was kindly provided by Novartis (Basel, Switzerland). Daunorubicin hydrochloride, (DNR; purity ≥ 95%, HPLC grade) was obtained from Sigma (Sigma, Saint Louis, Missouri, USA). Zosuquidar trihydrochloride (ZSQ; LY335979) was purchased from Selleckchem (Huston, TX, USA). Cyclosporin A (CsA) was obtained from Enzo Life Sciences AG (Lausen, Switzerland).

2.2. Cell culture

Human chronic myelogenous leukaemia K562 cells, obtained from ECACC, were cultured in the RPMI-1640 medium supplemented with a 10% calf foetal serum and antibiotics in 5% CO2 atmosphere at 37 °C. K562/Dox cells, which overexpress P-gp (ABCB1, MDR1), were kindly provided by Prof. J.P. Marie (University of Paris 6, France). K562/Dox cells were cultured under the same conditions. More detailed characterisation of K562/Dox cell line is given elsewhere [21]. K562/DoxDR1-3 cells with down-regulated expression of P-gp were established by stable transfection of K562/Dox cells with a plasmid vector expressing shRNA targeting the ABCB1 gene [22].

2.3. P-gp expression analysis using flow cytometry

P-gp expression was studied by using UIC2 (Beckman Coulter, USA) monoclonal antibody conjugated with phycoerythrin (UIC2-PE) according to the manufacturer’s instruction. Phycoerythrin conjugated isotype IgG2a was used as a control. The fluorescence of the cells was analysed by flow cytometry (Cyromics FC500, Beckman Coulter, USA). P-gp expression was determined by the ratio of the mean fluorescence intensity (MFI) shift of UIC2-PE antibody to isotype control (UIC2-PE/IgG2-PE). For each sample, 10,000 events were collected. All the experiments were performed in triplicate.

2.4. Functional assay of P-glycoprotein

Calcein acetoxyethyl ester (calcein AM) accumulation was used as functional assay of P-gp [23,24]. Cells (2.5 × 10^6 cells/ml) were incubated in a growth medium containing 0.25 μM calcein AM (Molecular Probes, Eugene, OR, USA) with or without 5 μM CsA for 20 min at 37 °C. Cells were immediately analysed by flow cytometry (Cyromics FC500, Beckman Coulter, USA) at excitation and emission wavelength of 488 and 525 nm, respectively. Dye uptake was expressed as the MFI in the presence and absence of P-gp inhibitor. For each sample, 10,000 events were collected. All the experiments were performed in triplicate.

2.5. Determination of cell survival and proliferation

The MTT assay was used for estimation of cell viability and growth as originally described by Mosman [25].

2.6. Assay for determination of intracellular DNR level

Cell pellets were extracted using 1% (v/v) formic acid in 50% (v/v) ethanol in water. The cell extracts were subsequently analysed by liquid chromatography coupled a low-energy collision tandem mass spectrometer (LC/MS/MS) as described previously [26].

2.7. Assay for determination of intracellular nilotinib level

2.7.1. Extraction of nilotinib from cells

Cells at density 5 × 10^5 cells/ml were incubated in growth medium with various concentration of nilotinib for 4 h (to achieve steady-state level) at 37 °C. Afterwards cells were centrifugated through silicone oil and resulting pellet was extracted with 1% formic acid (w/v) in 50% methanol/water (v/v)). Nilotinib content in cell extract was quantified using HPLC/MS/MS.

2.7.2. Quantitation of nilotinib by LC/MS/MS analysis

The HPLC system consisted of UltimaMate 3000 RS pump, degasser, autosampler and column compartment (Dionex, Germering, Germany). Separations were performed at ambient temperature on a Polaris C18 A 250 mm x 2.0 mm (i.d.), 5 μm particle size column (Varian Inc., Lake Forest, CA, USA) connected with a guard C18 4.0 mm x 2.0 mm (i.d.) precolumn (Phenomenex, Torrance, CA, USA). Solvents used for separation were A (90% MetOH in 0.25% FA, v/v) and B (0.25% formic acid, v/v). The flow rate was 300 μl/min with linear gradient elution from 0 to 3 min (65–95% of solvent A), from 3 to 4 min (95% of solvent A), from 4 to 5 min (95–65% of solvent A) and from 5 to 8 min (65% of solvent A). Sample injection volume was set at 20 μl. The effluent was introduced into the API 3200 triple quadrupole mass spectrometer (MDS SCIEX, Ontario, Canada) and electrospray ionisation in positive ion mode was used for detection. The mass spectrometer was operated in the multiple-reaction-monitoring (MRM) mode. Nilotinib was monitored by MRM transition 530 > 289 (dwell-time = 150 ms). Ion spray probe parameters were set to the following values: needle voltage 5500 V, declustering potential 71 V, temperature 400 °C, curtain gas (nitrogen) 1.38 bar, nebuliser gas (zero air quality) 3.45 bar, turbo V gases (zero air quality) 3.45 bar. The nitrogen pressure in the second quadrupole was measured at 3.7 × 10^-8 bar, the collision energy and entrance potential was set at 37.0 eV and 9.0 V, respectively. The instrument was operated in unit resolution.
2.8. Statistical analysis

Data are reported as the mean ± S.D. Statistical significance of differences was determined by Student’s t-test. Only the P values less than 0.05 were considered significant.

3. Results

3.1. Characterisation of P-gp expressing cells

To study the relationship between the P-gp expression and the cell resistance, K562/Dox cells were stably transfectected with a plasmid targeting the ABCB1 gene and then cells with decreased expression of P-gp were selected. Using this approach we established subclones of K562/Dox cells (K562/DoxDR1, K562/DoxDR2, and K562/DoxDR3) with a gradually decreased expression of P-gp (Fig. 1a and b). Expression of P-gp in K562 cells, which were used as a control, was indistinguishable from the isotype control (Fig. 1). Differences in P-gp expression were confirm also by western blot analysis (not shown).

The function of P-gp was verified using calcein accumulation assay [23,24]. As shown in the Fig. 2, the cell fluorescence was inversely related to the P-gp expression and this effect was reverted by CsA, a potent P-gp inhibitor. Importantly, K562/DoxDR1 cells with the lowest P-gp expression exhibited a significant decrease in calcein accumulation when compared to K562 cells (Fig. 2).

3.2. The effect of P-gp expression on intracellular drug level

Owing to the fact that the P-gp mediated resistance is due to the efflux of a drug out of cells, we analysed extracts from cells exposed to DNR and nilotinib. DNR, which belongs to well characterised P-gp substrates [10,20], was used as a reference drug. As expected, a dramatic decrease in intracellular level of DNR was observed in K562/Dox cells with the highest P-gp expression (Fig. 3a). Decreased expression of P-gp led to an increased accumulation of DNR, however, even K562/DoxDR1 cells with the lowest expression of P-gp contained a significantly reduced intracellular level of DNR (Fig. 3a). Similarly, the intracellular content of nilotinib increased with the decreasing P-gp expression (Fig. 3b). Importantly, the intracellular nilotinib levels were much higher when compared to DNR (Fig. 3). Even the highest P-gp expression in K562/Dox cells reduced the steady-state intracellular level of nilotinib only approximately 3 times. Interestingly, no significantly reduced intracellular nilotinib level was found in K562/DoxDR1 cells with the lowest P-gp expression (Fig. 3b).

3.3. The effect of P-gp expression on cell resistance

Cytoxic studies revealed that cells expressing P-gp confer a significant resistance to DNR, although it declined in cells with a decreasing P-gp expression (Table 1). Importantly, even the lowest P-gp expression in K562/DoxDR1 cells mediated a significant resistance to DNR (Table 1). Application of P-gp inhibitors such as ZSQ completely reversed the resistance to DNR (Table 1). These results indicated that the observed resistance to DNR was mediated by the P-gp function. Not surprisingly resistance to nilotinib was relatively weak but significant in cells with a high expression of P-gp (Table 2). Similarly to DNR, cell resistance to nilotinib was inversely related to the P-gp expression, however, it was completely lost in

![Fig. 1. Analysis of P-gp expression. P-gp expression was detected using specific UIC2-PE monclonal antibody and analysed by flow cytometry. Panel (a): Flow cytometric analysis. Isotype control (grey histogram); K562 cells (dash line); K562/DoxDR1 cells (dash-dot line); K562/DoxDR2 cells (solid line); K562/DoxDR3 cells (dash-dot-dot-dot line); K562/Dox cells (dotted line). Histograms represent a typical result. Panel (b): Quantitative analysis of P-gp expression. P-gp expression was quantified as the mean fluorescence intensity (MFI) shift (ratio of MFI of UIC2-PE antibody and isotype control). The experimental points represent mean values from three replicate experiments, with standard deviations. * denotes significant change in P-gp expression (P<0.05) between K562 cells and cells expressing various levels of P-gp (K562/Dox, K562/cellsDOX1-3).](image1)

![Fig. 2. Analysis of P-gp function using calcein accumulation assay. Cells were incubated with calcein AM in the absence or presence of P-gp inhibitor for 20 min. Calcein uptake, expressed as the mean fluorescence intensity (MFI), was analysed using flow cytometry. Cells incubated with 0.25 μM calcein AM alone (dark grey columns); cells incubated with 0.25 μM calcein AM+ 5 μM CsA (white columns). The experimental points represent mean values from three replicate experiments, with standard deviations. * denotes significant change in MFI of calcein (P<0.05) between K562 cells and cells expressing various levels of P-gp (K562/Dox, K562/cellsDOX1-3).](image2)
K562/DoxDR1 cells with the lowest P-gp expression (Table 2). Also, the observed resistance to nilotinib was fully reversible by ZSQ, suggesting involvement of P-gp (Table 2).

### 4. Discussion

P-gp is considered a nonspecific membrane transporter because it interacts with hundreds of structurally diverse substrates [27]. However, the broad substrate specificity of P-gp does not mean that all interacting molecules are effluxed at the same rate. The efflux rate affects the intracellular level of P-gp interacting molecule. When the transported molecule is an anticancer drug then its intracellular level, or more precisely its steady-state intracellular level is an important parameter which may limit its pharmacological effects. The steady-state intracellular level of an anticancer drug is a complex parameter, which is affected by other factors such as extracellular drug concentration, intracellular binding capacity, drug influx rate, and the drug metabolism to name the most important ones, in addition to the drug efflux rate. The steady-state intracellular drug level depends also on its chemical structure and even a small structure difference can change it dramatically. Imatinib and its main metabolite N-desmethylinatinib can be given as an example [28]. Not surprisingly, the steady-state intracellular drug levels for nilotinib and DNR also substantially differ in K562/Dox cells with the highest P-gp expression (Fig. 3a and b). Differences in the steady-state intracellular levels of nilotinib and DNR (Fig. 3a and b) correspond to the different sensitivity of K562/Dox cells to these drugs (Tables 1 and 2).

However, the efflux rate depends mainly on the P-gp activity, which is related to its expression. Indeed, the expression level of the drug transporter is one of the important factors that must be taken into account when we consider the possible involvement of the particular ABC transporter in resistance to the particular anticancer drug [6]. Our results clearly indicate that the P-gp expression is a crucial factor that affects the cell resistance (compare Figs. 1–3, and Tables 1 and 2). Decreased expression of P-gp leads to a lower efflux rate (Fig. 2) which in turn results in the increased intracellular DNR and nilotinib accumulation (Fig. 3a and b). These effects are manifested by the decreased resistance against both drugs (Tables 1 and 2). Despite the increased sensitivity to DNR due to the decreased expression of P-gp, even K562/DoxDR1 cells with the lowest P-gp expression still exhibit significant resistance to this drug (Table 1). Similar results were obtained for colchicine (not shown), another well characterised substrate of P-gp [10,20]. In contrast, the relatively low but significant decrease in nilotinib intracellular level accompanied by weak but significant protection from this drug found in the K562/Dox cells was gradually diminished with decreasing P-gp expression (Fig. 3b, Table 2). Importantly, the ability to decrease the nilotinib intracellular level below control level as well as the cell resistance were completely lost in K562/DoxDR1 cells with the lowest P-gp expression (Fig. 3b, Table 2).

The results presented in this paper unambiguously suggest that the expression level of P-gp may significantly affect the conclusion of whether P-gp mediates resistance to nilotinib or not. Unfortunately, neither of authors who addressed the question whether P-gp may mediate resistance to nilotinib directly examined the effect of the transporter expression level on drug sensitivity [12–15]. Despite the fact that it is difficult to compare expression levels of P-gp used in experiments conducted by the above mentioned research groups, we can speculate that their conclusions also depended substantially on this parameter. A pioneering work reporting that the resistance to nilotinib may be due to the P-gp overexpression used doxorubicin selected K562/Dox cells with a high P-gp expression [12,29]. Essentially the same cells
were used in our experiments reported here (cells with highest P-gp expression, see Fig. 1). In contrast, Hegedus et al. [15] concluded that P-gp does not provide resistance to nilotinib. Interestingly, supplementary data in this paper indicate that the expression of P-gp was very low [15]. These results clearly support our idea that the decision whether P-gp can mediate the resistance to nilotinib strongly depends on its expression level. Unfortunately, we were unable to compare the P-gp expression levels in two other reports since these data are not available [13,14].

To summarise our data, we clearly demonstrated that the conclusion as to whether the P-gp is involved in nilotinib resistance or not strongly depends on its expression level. However, the significance of the P-gp contribution for clinical resistance to nilotinib depends on whether resistant leukaemia cells exhibit high or low P-gp expression level.

Conflict of interest

The authors declare that they are not engaged in any conflict of interest.

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