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Reversal of ABCB1 mediated efflux by imatinib and nilotinib in cells expressing various transporter levels

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ABSTRACT

Recently, it has been suggested that imatinib (IM) and nilotinib (NIL) could be studied beyond their original application, as inhibitors of the drug efflux pump ABCB1 (P-glycoprotein, MDR1). Since the reversal of ABCB1-mediated resistance has never been successfully demonstrated in the clinic, we addressed the question of whether IM and NIL may actually serve as efficient inhibitors of ABCB1. Here we define an efficient inhibitor as a compound that achieves full (90–100%) reversal of drug efflux at a concentration that does not exhibit significant off-target toxicity *in vitro*. In this study, human leukemia K562 cells expressing various levels of ABCB1 were used. We observed that cells expressing higher ABCB1 levels required higher concentrations of IM and NIL to achieve full reversal of drug efflux. Among the well-known ABCB1 inhibitors, a similar effect was found for cyclosporin A (CsA) but not for zosuquidar. IM was efficient only in cells with the low and moderate ABCB1 expression at high concentrations that were cytotoxic in the absence of Bcr-Abl. In contrast, NIL was as efficient an inhibitor of ABCB1 as CsA. Low and moderate expression levels of ABCB1 could be efficiently inhibited by NIL concentrations without cytotoxic effects in the absence of Bcr-Abl. However, high expression levels of ABCB1 required higher NIL concentrations with off-target cytotoxic effects. In conclusion, application of NIL, but not of IM, in clinics is promising, however, only in cells with low ABCB1 expression levels. We hypothesize that some patients may benefit from an inhibitor exhibiting an ABCB1 expression-dependent effect.

1. Introduction

The expression of a constitutively active BCR–ABL tyrosine kinase, as a result of t (9; 22) (q34; q11) translocation, is the hallmark of chronic myeloid leukemia (CML) and is found in more than 90% of the cases. BCR-ABL tyrosine kinase thus became a legitimate target for pharmacological inhibition [1,2], and led to the development of tyrosine kinase inhibitors (TKIs), with imatinib (IM, Gleevec) the first in class [3]. Despite the very significant therapeutic effects of IM, it fails in approximately 25% of patients due to the development of resistance [4]. Diverse mechanisms may be responsible for IM failures. In the majority of patients, resistance coincides with the reactivation of the BCR-ABL tyrosine kinase activity. This mostly results from point mutations that disrupt the binding of IM to BCR-ABL tyrosine kinase. Alternatively, gene amplification can reactivate the activity of BCR-ABL tyrosine kinase [5]. IM resistance stimulated the development of novel inhibitors of BCR-ABL tyrosine kinase designed to overcome its resistance. Rational design of novel inhibitors exhibiting effectiveness against IM-resistant mutants of BCR-ABL was carried out, based on the crystal structure of the IM-ABL complex. This led to the development of a high-affinity ATP-competitive inhibitor nilotinib (NIL, Tasigna), approximately 20 times more potent than IM, which inhibits both wild type and IM-resistant BCR-ABL with significant clinical activity across the entire spectrum of BCR-ABL mutants with the exception of T315I [6]. Last, a novel mechanism of resistance that coincides with the decreased intracellular accumulation of TKIs due to the overexpression of ABC (ATP-binding cassette) transporters has been reported [7,8].

The ABC transporters comprise a large and functionally diverse family of membrane transporters. There have been identified 48 genes coding for ABC transporters in the human genome. ABC

Abbreviations: IM, imatinib; NIL, nilotinib; CsA, cyclosporin A; ZSQ, zosuquidar; DRN, daunorubicin; ECACC, European Collection of Authenticated Cell Cultures; P-gp, P-glycoprotein; CML, chronic myeloid leukemia; MFI, mean fluorescence intensity.

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transporters are mostly unidirectional; they utilize the energy of ATP binding and hydrolysis to translocate diverse substrates from the cytoplasm to the outside of the cell or into intracellular compartments [9]. Importantly, two ABC transporter family members, ABCB1 (MDR1, P-glycoprotein (P-gp)) and ABCG2 (BCRP) have been studied for their role in the transport of TKIs in cancer cells. Results of these studies are somewhat controversial. For example, many authors demonstrated that resistance to IM may be due to the ABCB1 [7,10] and/or ABCG2 overexpression [8,11]. However, some authors contradicted these findings [12–15]. Similarly, cytotoxic effects of NIL were shown to be reduced in cells overexpressing ABCB1 or ABCG2 [11,16]. Again, other studies reported contradictory findings for NIL [17,18]. Recently, we reported that the expression level of ABCB1 or ABCG2 is a critical factor that always affects the intracellular drug level and thus the cell resistance to this drug [19,20]. Our finding offers a consistent explanation for the contradictory results in the literature. Indeed, different laboratories use cells with expression levels of ABC transporters that vary from low [13] to very high levels [7]. Unfortunately, such results are not only difficult to compare among themselves, but more importantly, they may generate contradictory data when the effect of transporter expression level is not taken into account.

Recent reports suggested that interaction between TKIs and ABC transporters is more complex. Indeed, some TKIs, including IM and NIL may serve as substrates of ABCB1 at low concentrations, and as its inhibitors at high concentrations [16]. For example, IM reversed the ABCB1-mediated resistance in different cancer cell lines [21,22]. And, NIL potentiates the efficacy of conventional chemotherapeutic drugs in cancer cells overexpressing the ABCB1 and/or ABCG2 transporter [23,24]. While such results are encouraging, the experience from previous years warn us against excessive enthusiasm. Indeed, in the last decades a number of clinical trials with inhibitors of ABC transporters were carried out with overall disappointing results [25,26]. Most trials showed more toxicity when the inhibitors were added; study in unselected populations exacerbated the problem of toxicity; and toxicity led to dose reductions that impaired efficacy.

Here we demonstrate that inhibition efficiency of IM, NIL, and CsA depends on the ABCB1 expression level: cells expressing high ABCB1 levels require higher concentrations of IM, NIL, or CsA to achieve the full reversal of the drug efflux. We found that IM was a weak inhibitor of ABCB1 that exhibited off-target cytotoxic effects at doses required to inhibit ABCB1. In contrast, NIL was an efficient inhibitor of ABCB1 that could be considered for clinical study since human cancers do not commonly bear the high levels of ABCB1 seen *in vitro*.

2. Materials and methods

2.1. Chemicals

Imatinib mesylate (STI571, Gleevec; IM, purity $\geq$ 98%) and nilotinib hydrochloride (Tasigna, AMN107, NIL) were kindly provided by Novartis (Basel, Switzerland). Daunorubicin hydrochloride, (DRN; purity $\geq$ 95%, HPLC grade) was obtained from Sigma-Aldrich (Saint Louis, Missouri, USA). Zosuquidar trihydrochloride (ZSQ; LY335979) was purchased from Selleckchem (Houston, TX, USA). Cyclosporin A (CsA) was obtained from Enzo Life Sciences AG (Lausen, Switzerland).

2.2. Cell culture

Human chronic myelogenous leukemia K562 cells, obtained from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK), were cultured in the RPMI-1640 medium supplemented with a 10% calf foetal serum and antibiotics in 5% CO_2 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 [27].

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 [19,28].

Human promyelocytic HL-60 and histiocytic lymphoma U937 cell lines were cultured in the RPMI-1640 medium supplemented with a 10% calf foetal serum and antibiotics in 5% CO_2 atmosphere at 37 °C. Cells were obtained from ECACC.

Human embryonic kidney HEK-293, immortalised keratinocyte HaCaT, and skin fibroblast BJ cell lines were cultured as monolayers in DMEM medium supplemented with a 10% calf foetal serum and antibiotics in 5% CO_2 atmosphere at 37 °C. Cells were propagated every 2–3 days when they reach 70–80% of confluence using Accutase (Sigma-Aldrich, USA). Cells were obtained from ECACC.

Human neuroblastoma SH-SY5Y cell line was cultured in DMEM medium supplemented with a 10% calf foetal serum and antibiotics in 5% CO_2 atmosphere at 37 °C. Cells were propagated every 3–4 days when they reach 70–80% of confluence using Accutase (Sigma-Aldrich, USA). Cells were obtained from ECACC.

2.3. ABCB1 expression analysis using western blot analysis

Cells (approximately 2×10^6 cells) were washed in ice cold PBS and then extracted by lysis buffer (50 mM Tris/HCl buffer pH 8.1 containing 1% NP-40, 150 mM NaCl, 50 mM NaF, 5 mM EDTA and 5 mM sodium pyrophosphate, supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich; Saint Louis, Missouri, USA). Cell extracts were mixed with 5× Loading buffer (0.3 M Tris-HCl (pH = 6.8), 0.5 M dithiothreitol, 10% SDS, 50% glycerol, 0.05% bromophenol blue), and the samples were denatured by heating at 96 °C for 5 min. Samples equivalent to 30 mg protein were analysed by Western blot analysis using monoclonal anti-P-gp (ABCB1) antibody produced in mouse, clone F4 (1:1000) and monoclonal anti-actin antibody produced in mouse, clone AC-40 (1:2000). All primary antibodies were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). The signal was detected using a horseradish peroxidase–conjugated secondary antibody (1:3000; Dako, Glostrup, Denmark). Products were visualized using an enhanced chemiluminescence (ECL; Amersham, Little Chalfont, UK).

2.4. Functional assay of ABCB1

Calcein acetoxymethyl ester (calcein AM) accumulation was used as functional assay of ABCB1 [29]. Cells were loaded with calcein AM (Molecular Probes, Eugene, OR, USA) and then analysed by flow cytometry (Cytomics FC500, Beckman Coulter, USA), as described elsewhere [19]. Dye uptake was expressed as the mean fluorescence intensity (MFI) in the presence and absence of ABCB1 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 previously [30].

2.6. Assay for determination of intracellular DRN level

Cells at the density of 4×10^5/ml were incubated in the growth