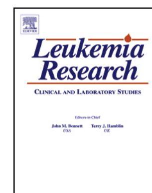
Leukemia Research 39 (2015) 355–360

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres



Drug transporters play a key role in the complex process of Imatinib resistance *in vitro*

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ARTICLE INFO

Article history:
Received 18 September 2014
Received in revised form 29 October 2014
Accepted 14 December 2014
Available online 23 December 2014

Keywords:
CML
Imatinib resistance
Therapeutic interruptions
Membrane drug transporters
P-glycoprotein
OCT1

ABSTRACT

Imatinib resistance has been associated with *BCR-ABL* alterations, but other mechanisms might be involved, like drug transporters. Additionally, the impact of poor adherence in resistance has been little explored. Using sensitive and resistance CML cell lines, we investigated the expression of influx/efflux transporters, like P-gP and OCT1. In the therapeutic interruption model, we observed decrease of influx and increase in efflux transporters combined with *BCR-ABL* over-expression. Comparatively, resistant cells obtained by continuous TKI exposure only demonstrated alterations in drug's transporters. By exploring P-gP expression of resistant cells, we observed the potential of P-gP inhibitor in circumventing Imatinib resistance. Our results revealed the importance of treatment interruptions for expected response levels and show the complexity of Imatinib resistant process. Efflux transports appear as not only relevant for acquisition of resistant phenotype, but also as valid therapeutic tool for managing resistance.

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1. Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder, and it was one of the first diseases to be associated with a chromosomal alteration, the Philadelphia (Ph) chromosome. From the reciprocally translocation between the chromosome 9 and 22, arises the *BCR-ABL* fusion gene, which encoded an oncoprotein that exhibits constitutively active tyrosine kinase activity. This molecular alteration becomes the most effective target for the therapy used against this disease, being the Imatinib the first-line treatment. This drug is a tyrosine kinase inhibitor (TKI) that binds to the *BCR-ABL* tyrosine kinase through the ATP binding domain, preventing tyrosine autophosphorylation and in turn, the phosphorylation of its substrates, blocking downstream signaling pathways that promote leukemogenesis [1,2].

Besides the good results obtained with Imatinib, the number of cases of resistance to this TKI has increased, which has several causes. The mechanisms involved in resistance to Imatinib are usually divided according to its dependence on *BCR-ABL* fusion gene. Point mutations, over-expression and gene amplifications are included in the group of *BCR-ABL* dependent mechanisms. The other mechanisms comprise those independents of fusion gene, in particular those factors associated to drug metabolism and drug transport to inward and outward of the target cells [3,4]. Particularly, the P-glycoprotein (P-gP, ABCB1 or MDR1) and the BCRP – breast cancer related protein (ABCG2) are the efflux drug transporters that extrude Imatinib from the interior of the cell, reducing

Abbreviations: BCRP, breast cancer related protein; CML, chronic myeloid leukemia; IMA, Imatinib; P-gP, P-glycoprotein; OCT, organic cation transporter; TKI, tirosine kinase inhibitor.

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http://dx.doi.org/10.1016/j.leukres.2014.12.008
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its concentration and consequently its activity against the *BCR-ABL* [5]. Additionally to drug efflux, the entrance of Imatinib into the cells is also a process dependent of membrane transporters, namely OCT1 (organic cation transporter 1) and OCTN2 (organic cation/carnitine transporter). Low expression of these transporters leads to a reduction in Imatinib concentration inside the cell [6]. Currently, mutations and other dependent mechanisms of resistance are the most referred as responsible for the loss of response. However, membrane transporters have also emerged as important players in therapeutic failure. Therefore, the knowledge of these changes would enable the use of new therapeutic approaches in CML and/or to circumvent Imatinib resistance.

On the other hand, the adherence to therapy is also an important factor, especially in cases of long-term treatment, as occurs with Imatinib. The incorrect take of prescribed medication affects the effectiveness of the treatment and could also contribute to the development of drug resistance [7].

The purpose of this study was to create an *in vitro* model that mimics the poor adherence to therapeutics and to evaluate the role of drug transporters (influx and efflux) in Imatinib resistance, using sensitive and Imatinib-resistant cell lines. It was also an aim to evaluate the ability of P-gP inhibitor in overcome the Imatinib resistance.

2. Materials and methods

2.1. Cell culture and generation of Imatinib-resistant cell lines

The CML cell line, K562, was obtained from the American Type Culture Collection (ATCC). Cells were maintained in Roswell Park Memorial Institute 1640 (RPMI 1640) medium supplemented with 10% fetal bovine serum (FBS), 2 mM of L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Gibco, Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO_2.

The generation of Imatinib-resistant cell lines was based on two strategies: continuous exposure to Imatinib to form the K562-RC cell line [8], and discontinuous exposure to get the K562-RD cell line. The starting dose of Imatinib (Selleck Chemicals) was in 0.1 ηM and the dose was duplicated every 10 days of culture, as describe by Mahon et al. [9]. For the discontinuous strategy, the K562-RD cells were cultured during successively cycles of 10 days in the presence of Imatinib, followed by 10 days without the TKI. After 6 months, the resistance phenotype was confirmed, and both cell lines were cultured with 250 ηM of Imatinib. The sensitive cell line was maintained in culture without Imatinib to be used as a reference.

2.2. Cell proliferation and viability studies

To evaluate the effect of Imatinib on cell viability, all the cell lines were cultured in the absence and the presence of increased concentrations of TKI and analyzed by the resazurin assay [10]. The cells were plated at a cell density of 0.5×10^6 cells/mL and the resazurin was added to a final concentration of 10 μg/mL (Sigma–Aldrich) in 24 h intervals during 3 days. By the same method, we evaluated the effect of P-gP inhibitor, Reversin 205 (Sigma–Aldrich), in circumvent Imatinib resistance. For that, the resistant cells were incubated with 5 μM of Reversin 205 plus increasing concentrations of Imatinib. The results of 3–6 independent experiences were expressed in terms of mean ± standard deviation.

2.3. Conventional cytogenetic analysis

High resolution GTG metaphase analysis was carried out in parental K562 cells. Metaphase spreads were prepared as described by Dracopoli et al. [11] and karyograms established according to the conventional cytogenetics nomenclature [12–14].

2.4. BCR-ABL characterization

2.4.1. BCR-ABL transcripts quantification and mutations profile

Total RNA was extracted from all cell lines using Illustra™ RNAspin Mini RNA Isolation Kit (GE Healthcare) and cDNA synthesis was performed using SuperScript™ III Reverse Transcriptase (Invitrogen). The real-time RT-PCR technique (qRT-PCR) for measurement of *BCR-ABL* fusion gene transcripts was performed as previously described [15]. The mutation's analysis was executed by high-resolution melt (HRM) curve analysis, with a sensitivity at 5% (maximal dilution with result was 5%), as previous defined by Poláková et al. [16].

2.4.2. Fluorescence in situ hybridization analysis

Interphase nuclei from each cell lines were hybridized with fluorescent-labeled probes for *BCR-ABL*, namely the LSI® *t*(9;22) BCR/ABL Dual Fusion Dual Color (Abbott Laboratories), following standard procedures. For each slide, >100 nuclei were analyzed.

2.5. Detection of cell surface drug transporters by flow cytometry

In all cell lines, the levels of influx (OCT1 and OCTN2) and efflux transporters (P-gP and BCRP) were evaluated by flow cytometry using specific monoclonal antibodies labeled with fluorescent probes. To analyze the efflux transporters, about 1×10^6 cells from each cell line were centrifuged and incubated for 15 min in the dark at room temperature with 1 μg of each monoclonal antibody, anti-P-gP (IgG_{2b}) FITC (BD Pharmigen) and anti-BCRP (IgG_{2b}) PE (Santa Cruz Biotechnology). Then, the cells were washed and resuspended in PBS as previously referred by our group [8]. To evaluate the influx transporters, the cells were incubated with 1 μg of the primary antibodies anti-OCT1 (IgG) (Santa Cruz Biotechnology) and anti-OCNT2 (IgG) (MBL International), washed in PBS and incubated with a secondary antibody anti-rabbit-PE (IgG) or anti-rabbit-FITC (IgG) (Santa Cruz Biotechnology & DAKO) during 20 min in the dark. The experiments were performed in triplicate and analyzed using a FACScalibur flow cytometer. The results were expressed in percentage of cells positive for each molecule and also expressed in mean fluorescence intensity (MFI). Negative controls were established using isotype immunoglobulin G (IgG) and IgG_{2b} submitted to the same procedures.

2.6. P-glycoprotein kinetic assay

To evaluate the P-gP function, we performed, in all cell lines, a kinetic study with ^{99m}Tc-Sestamibi, as described in Abrantes et al. [17]. Radioactivity of cell pellets and supernatants was measured separately with a well-type gamma counter (DPC Gamma C12) to determine tracers' uptake percentage in the cells. These experiments were performed in triplicate.

2.7. Data analysis

Statistical analysis was carried out using GraphPad Prism software, version 5.00 for Windows (GraphPad Software, USA). Comparison of groups was made by ANOVA and Tuckey post-test. Statistical significance was considered for differences with $p < 0.05$. For half maximal inhibitory concentration (IC_{50}) determination, non-linear curve fit dose–response was performed. The degree of resistance was determined by the ratio between the IC_{50} of K562-RC or K562-RD and the IC_{50} of K562 cells.

3. Results

3.1. Establishment and characterization of Imatinib resistant cell lines

The K562 cell line is a valid *in vitro* model of study CML, since it keeps the *BCR-ABL* gene fusion without any mutation and still sensitive to Imatinib. Based on these characteristics, we established two Imatinib-resistant cell lines differing in the exposition to this TKI. The resistance was defined as the capacity of cells to survive despite the presence of Imatinib, and the degree of resistance is determined by the ratio between the IC_{50} of resistant cells and the IC_{50} of sensitive K562 cells.

In Fig. 1, it is represented the IC_{50} to Imatinib of the different cell lines. For the sensitive cells was necessary an exposure to 75 ηM of Imatinib to achieve the IC_{50}. The K562-RC cells showed an IC_{50} of 605 ηM whereas this value increases to 1389 ηM for K562-RD cells. Comparing with the parental cells, the K562-RC and K562-RD cell lines present an IC_{50} values 8 times and 18 times higher, respectively. Additionally, we also observed significant differences in the IC_{50} between both resistant cell lines: the IC_{50} of Imatinib in K562-RD cells was 2.55 times greater than IC_{50} of K562-RC cells.

After having verified the resistant phenotype, we explored the mechanisms that might be involved. Firstly, we examined the presence of *Ph* chromosome and *BCR-ABL* gene by conventional cytogenetic and by FISH. As observed in Fig. 2(a), the resistant cells exhibit, by FISH analysis, the same number of *BCR-ABL* fusion genes as the parental cell line, K562. Since the resistance can also be associated with over-expression and/or mutations in *BCR-ABL*, we analyzed these parameters in our cells. In terms of expression, we observed a 1.55-fold increase of *BCR-ABL* mRNA levels only in the

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