

# Loss of SYK and LYN Tyrosine Kinase Expression Impair Ponatinib-Induced Apoptosis in K562 Cells

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**Abstract:** Targeting the tyrosine kinase activity of BCR-ABL is the gold standard strategy in Chronic Myeloid Leukemia (CML) for the last decade. Whereas inhibitors of BCR-ABL tyrosine kinase are now used in frontline therapy for CML, third generation inhibitors of BCR-ABL tyrosine kinase such as ponatinib has been developed for the treatment of BCR-ABL resistant mutants. In the current study, we generated K562 ponatinib resistant cells (KRPO) and investigated its mechanism of resistance. No over expression of BCR-ABL or multidrug resistance gene (MDR-1) were found among the investigated mechanisms as verapamil did not overcome resistance. Downregulated expression of both p72 SYK and p53/56 LYN kinases was found in ponatinib K562 resistant cell line. Albeit both mRNA and protein level were decreased in KRPO for SYK only protein level was downregulated for LYN suggesting a posttranscriptional mechanism. This mechanism of resistance also prevents imatinib, nilotinib or dasatinib-induced CML cells apoptosis.

**Keywords:** Chronic myeloid leukemia, Drug resistance, BCR-ABL, Tyrosine kinase inhibitors, Kinase.

## INTRODUCTION

Chronic myeloid leukemia (CML) is characterized by the presence of the Philadelphia (Ph) chromosome that results from a t(9;22)(q34;q11) reciprocal translocation [1, 2]. The Ph chromosome contains a *BCR-ABL1* hybrid gene, the molecular hallmark of CML [3]. *BCR-ABL1* encodes a fusion oncoprotein with de-regulated protein tyrosine kinase (TK) activity, which drives leukemogenesis *in vitro* and *in vivo* [4, 5]. Imatinib, an anti-Abl tyrosine kinase inhibitor (TKI) that competes with ATP for binding to the Abl kinase domain, is until now the gold standard therapy of CML in chronic phase. However, resistance or intolerance to imatinib led to the development of second generation anti-Abl TKIs including nilotinib and dasatinib [6, 7]. Although these new drugs dramatically improved the clinical outcome of CML patients, few cases remain resistant to TKI treatment. Indeed, the main mechanism of resistance in CML patients is mutations in the BCR-ABL tyrosine kinase domain. One of them the T315I BCR-ABL1 mutation has been considered as the mutation from hell until now as any TKI was able to overcome it. Development of third generation TKI like ponatinib will encompass BCR-ABL T315I mutation [8].

In an attempt to study the potential mechanism of resistance to ponatinib, we generated ponatinib - resistant cells from the Ph-positive cell line K562 (KRPO). Characterization of ponatinib-resistance

mechanisms discarded BCR-ABL and/or multi drug resistance P-glycoprotein (Pgp) over expression while down regulation of two tyrosine kinases SYK and LYN was detected in ponatinib-resistant K562 cells. Albeit both mRNA and protein level were decreased in KRPO for SYK only protein level was downregulated for LYN suggesting a posttranscriptional mechanism.

Our data suggest that BCR-ABL positive cells can evade the inhibitory effect of ponatinib by a new mechanism involving downregulation of SYK and LYN tyrosine kinases.

## MATERIALS AND METHODS

### Reagents

RPMI 1640 medium, fetal calf serum (FCS), phosphate buffered saline (PBS), trypan blue and antibiotics were purchased from Invitrogen (Cergy Pontoise, France). Ponatinib, imatinib, nilotinib and dasatinib were purchased from (Euromedex, Mundolsheim, France). MTS was purchased from Promega (Charbonnières, France). Verapamil was purchased from Isoptine (Knoll Laboratory, Levallois-Perret, France). The following antibodies were used : 4G10 anti-phosphotyrosine (Euromedex, Mundolsheim, France), anti-Abl 8E9 (Pharmingen, Becton-Dickinson, Le Pont De Claix, France), anti-Hsp60 (K-19), anti-LYN (H-6), anti-SYK (4D10) (Santa Cruz, Heidelberg, Germany).

### Cell Lines

The BCR-ABL positive human cell line used in this study: K562 was from ATCC. Ponatinib-resistant cells

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were derived as previously described for imatinib and designated KRPO [9]. Cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin at 37°C in a humidified atmosphere containing 5 % CO<sub>2</sub>. Aliquots were taken at 24-h intervals for assessment of cell viability by Trypan blue exclusion (Sigma Aldrich, St Quentin Fallavier, France).

### Generation of Ponatinib-Resistant K562 Cells

K562 cells were maintained in culture and gradually exposed to increasing concentrations of ponatinib, at a rate of 10 pM increments every 10 days of culture. After approximately twelve months, sub-lines of cells growing in 10 nM ponatinib were maintained continuously in culture with the inhibitor and concentration was increased to 40 nM over six months. The parental, sensitive cell line was maintained in parallel cultures without ponatinib to be used as controls.

### Cell Proliferation Assay

Cell proliferation was measured by the number of viable cells using MTS tetrazolium (Cell Titer96 Aqueous, Promega, Charbonnières, France). Washed cells in RPMI 1640 medium were plated (10<sup>4</sup> cells) in quadruplicate into microtiter-plate wells plus various doses of inhibitors as indicated (imatinib, nilotinib, dasatinib or verapamil). Measure of viability was performed on a 4 day kinetic and each day 20 µl of MTS were added to the wells. After 2 hours incubation at 37°C, the plates were read in a microplate auto reader (Dynex Technologies, Billingshurst, UK) at the wavelength of 490 nm. The mean results of the 4-well set are standardized in comparison with the initial optical density at day 0. All the experiments were repeated at least three times.

### Western Blot Analysis

Protein lysates were prepared according to Kabarowski *et al.* [10]. Protein concentration was measured by the BCA<sup>TM</sup> Protein Assay (Pierce, Rockford IL, USA) and the lysates were stored at -80°C. Twenty five µg of protein were separated on 10 % SDS-PAGE gels, transferred onto PVDF membranes (BIO-RAD, Marnes-La-Coquette, France) by semi-dry electrophoretic transfer, probed with individual antibodies and visualised by the ECL system (Perkin Elmer, Courtaboeuf, France).

### Quantification by RT-QPCR

Total RNA was extracted from CML cell lines. Quantitative RT-PCR was performed using MX3005P from Stratagene (Nantes, France) according to the manufacturer's instructions. With a 10-fold serial dilution series for cDNA, the assay was found to be linear over at least 5 orders of magnitude (slope, -3.414; intercept, 35.81). Quantification of relative expression was performed using *GUS* as endogenous control, as previously described [11]. Analysis was done by comparative Ct method giving the amount of target normalized to the endogenous reference and relative to the same pool of mononuclear cells as the calibrator.

### Detection of Apoptosis by Flow Cytometry

K562 cells were incubated for 24 h with or without TKI. Upon 24h, response to TKI was detected by incubating cells (5.10<sup>4</sup>) with annexin V-APC and DAPI then analyzed by flow cytometry. Results are expressed as the percent of annexin V positive cells.

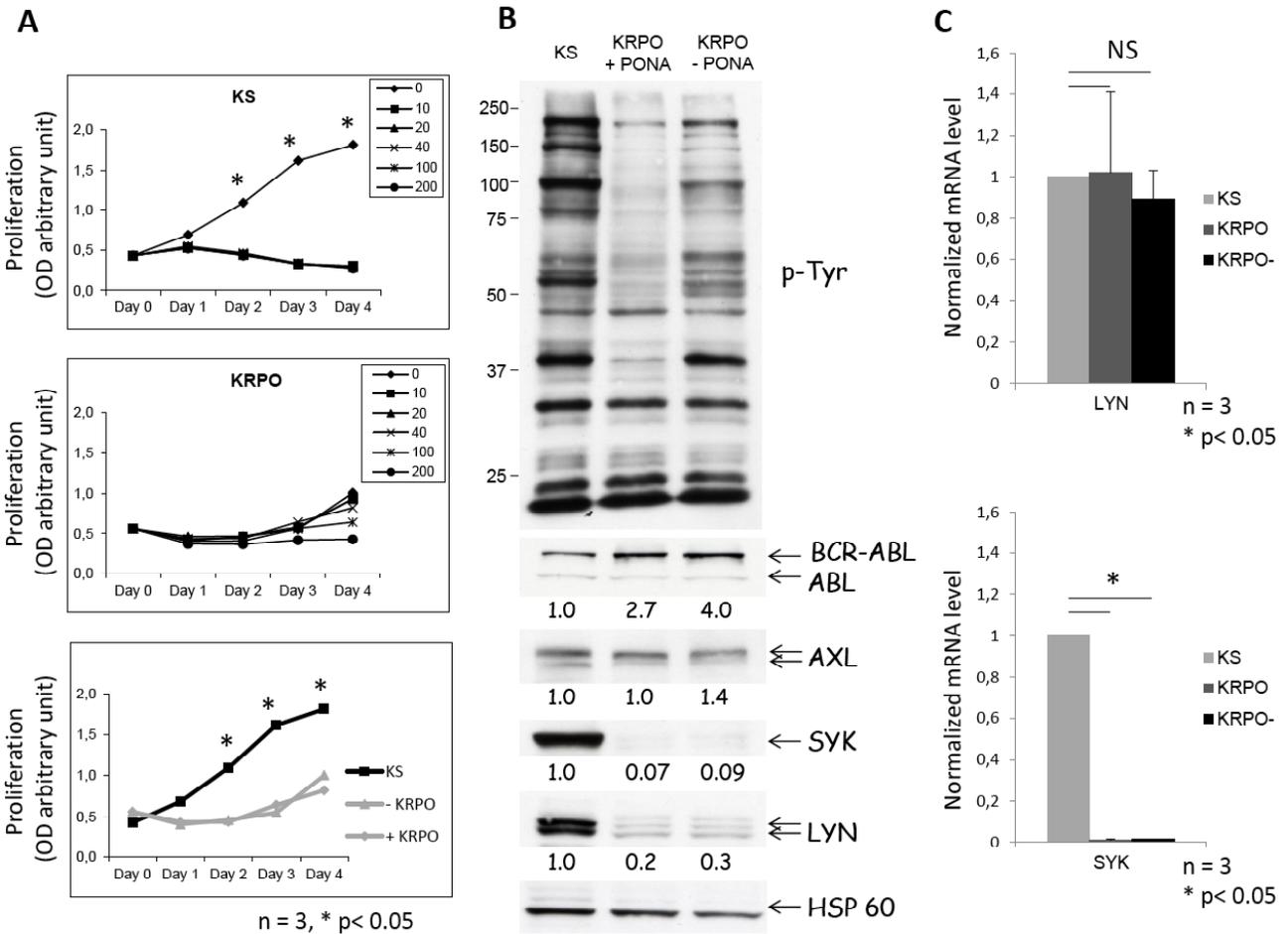
### Statistical Analysis

Mann-Whitney or Wilcoxon tests were used to calculate differences between means; differences were considered significant when  $p < 0.05$ . No significant difference was labelled not significant (NS).

## RESULTS

### Generation of Ponatinib-Resistant K562 Cells

K562 cells were grown in the presence of increasing concentration of ponatinib over a period of twelve months to generate resistant cells to 10 nM ponatinib. Resistance was defined as the capacity to survive in the continuous presence of the highest dose of ponatinib. Ponatinib concentration was increased to reach 40 nM. Indeed, the final concentration of 40 nM of ponatinib was chosen in relation to the dose able to overcome all BCR-ABL1 mutations and corresponding to the dose attained in CML patients plasma. Proliferation of the parental cell line KS was compared to the ponatinib-resistant one, called KRPO, which was able to grow in liquid culture until 40 nM of ponatinib (Figure 1A). For instance, KRPO was growing at 10, 20 and 40 nM of ponatinib whereas 10 nM was already killing the KS sensitive counterparts. Indeed, as low as 0.1 nM ponatinib is already inducing apoptosis of K562 cells (result not shown).



**Figure 1:** Ponatinib resistance of K562 cells correlates to down regulation of SYK and LYN tyrosine kinase expression.

Proliferation of the ponatinib-sensitive (KS) and resistant cell lines (KRPO) were tested in MTS assays in the presence of increasing concentrations of ponatinib (A). Results are expressed as mean of the optical density of the 4-well set standardized in comparison with the starting optical density at day 0, which is directly proportional to the number of viable cells. Results are from one experiment representative of 4.

Phosphotyrosine immunoblots were performed on protein lysates from KS, KRPO plus ponatinib and KRPO without ponatinib cells (B). BCR-ABL, AXL, SYK and LYN expression were detected using corresponding antibodies. Level of expression was normalized to Hsp60 as loading control. Results are representative of five experiments. Densitometry analysis results are indicated underneath each blot lane. Target proteins detected are indicated on the right by an arrow. Quantification of SYK and LYN mRNA expression was performed by RT-QPCR from KS and KRPO plus ponatinib (KRPO+) and KRPO without ponatinib (KRPO-) cells (C). Significance is indicated by an asterisk for p<0.05. No significant difference is indicated by NS.

We also tested the rate of proliferation of KRPO cells with or without ponatinib in comparison to KS cells. Ponatinib-resistant cells show a strongly reduced proliferation in comparison to KS cells which is not modified by ponatinib withdrawal over 4 days.

**KRPO Cells Harbours a Different Tyrosine Phosphorylated Pattern than K562 Cells.**

In order to investigate whether resistance to ponatinib may be associated to changes in tyrosine kinase activity, Western blot was performed using antiphosphotyrosine antibody. KRPO cells were growing with ponatinib 40 nM or starved for 24h. Ponatinib-resistant cells harboured a phosphotyrosine

pattern very different to their KS sensitive counterpart. The pattern of protein tyrosine phosphorylation in KRPO cells shows decreased phosphorylated bands at 39, 53, 56, 75, 100, 150 and 210 KDa. To exclude a decrease of tyrosine phosphorylation only linked to the inhibition by ponatinib, KRPO cells were starved for 24h. Tyrosine phosphorylation detection shows a similar although weaker pattern than parental K562 cells.

The level of BCR-ABL1 protein in ponatinib sensitive and resistant cell lines show that KRPO cells expressed a similar level of BCR-ABL1 than its parental counterpart (no significant difference in the

protein level of BCR-ABL1 was detected on four separate experiments). Even ponatinib withdrawal did not significantly modify BCR-ABL1 level of expression while it changes tyrosine phosphorylation of a 210 kDa band. To exclude the over expression of some other TK involved in resistance to other TKI, we detect the expression of SYK and LYN. Albeit Axl expression was similar in all tested condition, a strong decrease of SYK and LYN expression was detected in KRPO cells with or without ponatinib. Level of expression of these two TK was quantified at mRNA level by RT-QPCR. SYK mRNA was under the threshold of detection in KRPO cells suggesting a transcriptional regulation or RNA stability. In contrast, LYN mRNA was similar in KS and KRPO cells. Same difference was measured after 24h ponatinib withdrawal.

### **KRPO Cells are Resistant to Imatinib, Nilotinib and Dasatinib**

KRPO cells are resistant to 10, 20 and 40 nM ponatinib. This resistance is associated to a strong modification of tyrosine phosphorylated proteins. We investigate KRPO response to imatinib, nilotinib and dasatinib. KS cells incubated for 24h with ponatinib, imatinib, nilotinib or dasatinib have between 30 to 35% of annexin V positive cells. In contrast, KRPO cells were not responding to these TKI (supplementary data A)

Because over expression of Pgp (encoded by the *MDR-1* gene) can functionally modify the uptake of several drugs including TKI as it was already reported, we investigate if Pgp inhibition may restore sensitivity to ponatinib in KRPO cells [12]. Interestingly, incubation of KS cells with ponatinib and verapamil increases the induced-apoptotic response. In contrast, verapamil did not modify or did not sensitize KRPO cells to ponatinib suggesting that Pgp is not the main cause of resistance in KRPO cells (supplementary data B).

### **DISCUSSION**

In the present work, we have used the BCR-ABL positive cell line K562 to generate a ponatinib-resistant CML cell line. Ponatinib has been developed to overcome resistance to TKI such as those mediated through BCR-ABL mutations. This pan-BCR-ABL inhibitor is inducing K562 apoptosis with an IC50 between 0.1 and 0.5 nM which is in agreement to the literature [8]. Previous studies on CML resistant cell lines to other TKI have demonstrated that tyrosine

kinases may be mediating such resistance. For example we and other investigators have reported that AXL over expression may lead to resistance to imatinib or nilotinib [11, 13]. In addition such resistance to nilotinib in K562 cells have been associated to a complex mechanism involving the activation of the TK SYK and the over expression of LYN and AXL. Some SRC family TK has also been involved in imatinib resistance both *in vitro* and *in vivo* [14]. Indeed, the TK LYN was upregulated and associated to the over expression of the anti-apoptotic protein Bcl2.

Here, we generate KRPO cells, which are resistant to 40 nM of ponatinib. This concentration is the higher used to block the BCR-ABL T315I mutation. The first feature of KRPO cells is the reduced rate of proliferation in comparison to KS cells. The reduced proliferation is not dependent of kinase inhibition by ponatinib as harvesting KRPO for four days failed to restore proliferation. In contrast, phosphotyrosine content is clearly associated to inhibition by ponatinib. Ponatinib withdrawal is followed by reappearance of tyrosine phosphorylated proteins in KRPO cells. However, this did not lead to the re-expression of SYK and LYN. Because the decrease of SYK level of expression is correlated to both a decrease of mRNA and protein it will be interesting to investigate the methylation status of its gene promoter and/or to study KRPO response to demethylating agents as it was reported in breast or colon cancer cells [15, 16]. In addition, SYK may play a similar suppressor role than in breast cancer [17].

Amazingly, common TKI resistance mechanism was not detected in ponatinib-resistant K562 cells. For example, using a strategy similar to the one used previously to characterize imatinib or nilotinib-resistant cells we investigated BCR-ABL level of expression. No significant modification of BCR-ABL level of expression was detected in KRPO cells even if it is frequently over expressed in CML cell lines resistant to TKI. Yet, no BCR-ABL mutation in the TK domain was detected in KRPO cells. Regarding *MDR-1* gene function through Pgp protein expression, this mechanism has already been reported in imatinib-resistant cell lines [12, 18, 19]. In KRPO cells, inhibition of the Pgp protein function by verapamil did not change the resistance to ponatinib while a significant increase of ponatinib-induced apoptosis was detected in ponatinib K562 sensitive cells.

Currently, a label free proteomic analysis has been undertaken comparing KRPO cells with or without ponatinib to KS cells.

Overall, the results presented in this study show for the first time that CML cells can resist to ponatinib and this is associated to a strong down regulation of the expression of two TK, SYK and LYN. From a clinical point of view, ponatinib was the last therapeutic strategy for T315I mutated or TKI intolerant CML patients. The different pattern of side effects reported upon ponatinib treatment requires managing a decrease of the dose. Associated to the possibility of emerging resistance mechanism to ponatinib, this should be taken into account to optimize the dose still allowing strong BCR-ABL1 TK inhibition and rapid response.

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