Erk 1/2 and survivin could contribute to imatinib resistance in CML cell line independent of Bcr-Abl

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About 25% of Chronic Myeloid Leukemia (CML) patients develop resistance to tyrosine kinase inhibitor (TKI) treatment. Amidst several molecular mechanisms, Bcr-Abl-independent activation of MAPK/Erk signaling and survivin overexpression were shown to be important mechanisms of imatinib (IM) resistance. Still, the need to better comprehend the mechanisms that underlie resistance and develop new therapeutic strategies lingers. Toward studying aspects of resistance, the CML cell line resistant to IM, K-IM, was developed in our laboratory and this study aims at unraveling the resistance mechanisms present in K-IM cell line. K-IM cells showed neither mutations in BCR-ABL gene nor efflux protein expression or activity. There was an increase in BCR-ABL and BIRC5 mRNA levels. K-IM was more resistant to the TKIs IM and dasatinib compared to K562 and K562-Lucena cell lines. Cells were arrested in G0/G1 phase after treatment with IM in all cell lines, however, in K-IM cells, apoptosis may not have been triggered since there was no increase in DNA fragmentation or annexin V/propidium iodide staining. Bcr-Abl IM-induced inhibition drastically reduced Erk 1/2 phosphorylation and survivin protein levels in K562 and K562-Lucena but not in K-IM suggesting that they may contribute to resistance independent of Bcr-Abl. The new cell line K-IM could be used as a model to study Bcr-Abl-independent molecular resistance mechanisms and also as a tool for drug development targeting resistant patients.

INTRODUCTION

Chronic myeloid leukemia is a myeloproliferative disorder characterized by the constitutively active Bcr-Abl tyrosine kinase protein. Treatment with the tyrosine kinase inhibitor imatinib (IM) represents a enormous success in targeted therapy. However, about 25% of patients develop intolerance or resistance to treatment. BCR-ABL mutation, gene amplification and overexpression are very frequent resistance mechanisms, but do not account for all resistant patients. Bcr-Abl independent mechanisms such as efflux transporters overexpression, inhibitor of apoptosis proteins (IAPs) overexpression or activation of proliferative and survival signaling pathways like MAPK/Erk could contribute to IM resistance. In order to better comprehend IM resistance mechanisms, the K-IM cell line was developed in our laboratory through continue exposure to increasing IM concentrations up to 1μM.

METHODS

The CML cell lines K562, K562-Lucena (a MDR cell line) and K-IM were used in this study. Cell viability was assessed using the MTT assay. Mutation was evaluated through direct sequencing. Transcriptional levels were assessed using RT-qPCR. Efflux transporters expression and activity were analyzed by flow cytometry. Cell cycle, DNA fragmentation and annexin V/propidium iodide (PI) incorporation were assessed by flow cytometry. Cell proliferation was assessed through cell counting using the trypan blue exclusion method. Protein levels and phosphorylation status were assessed by Western Blotting.

RESULTS AND CONCLUSION

Cell viability reduction after IM treatment was significantly lower in K-IM cells than in K562 and K562-Lucena (Fig. 1A). When treated with dasatinib, K-IM and K562-Lucena cell viability was significantly higher than K562 (Fig 1B). We sought to investigate the mechanisms of resistance present in the K-IM cell line and found that there was neither efflux transporters expression (Fig 2) nor activity (Fig 3). Mutations in the BCR-ABL kinase domain were not observed (data not shown). Also, BCR-ABL mRNA levels (Fig 4A) and Crkl phosphorylation levels (Fig 5) were increased, suggesting Bcr-Abl overexpression could play a role in K-IM cells resistance. Since IAPs overexpression has been implicated in IM resistance, we analyzed survivin and XIAP levels. Survivin mRNA levels (Fig 4B) and protein expression (Fig 5) were increased, nonetheless XIAP mRNA levels were similar between K-IM and K562 (Fig 4C). MAPK/Erk, a well-established signaling pathway downstream Bcr-Abl that promotes proliferation and survival, seems to be more active in K-IM cells as Erk 1/2 phosphorylation levels were increased in K-IM compared to the other cell lines (Fig 5). IM treatment induced cell cycle arrest at G0/G1 phase in all cell lines (Fig 6). There was no significant induced cell death in K-IM cells treated with 1 μM of IM, differently than K562 and K562-Lucena (Fig 7 and 8). Cells were counted to determine whether they continue to proliferate despite the cell cycle arrest. K-IM cell number continued to increase at 1μM of IM until the 4th day, although K562 and K562-Lucena cell number drastically reduced after 48 hours of treatment (Fig 9). Reduction in Crkl phosphorylation was greater in 5 μM of IM (Fig 10). IM treatment reduced Erk 1/2 phosphorylation in K562 and K562-Lucena cells, but not in K-IM cells (Fig 10), suggesting that Erk could be activated independently of Bcr-Abl. Survivin protein levels were decreased when cells were treated with IM in all cell lines (Fig 10). Together our results suggest K-IM cells resistance to be associated with increased Bcr-Abl levels and increased activation of MAPK/Erk

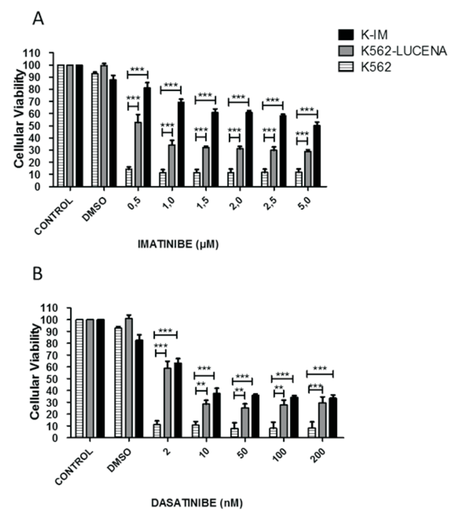

Figure 1: Cellular response to tyrosine kinase inhibitors. Viability of K562, K562-Lucena and K-IM cells was assessed through the MTT assay, after 72 hours of treatment with imatinib (A) or dasatinib (B). Statistical analysis was made using the two-way ANOVA with Bonferroni post-test. * corresponds to p<0,05, ** corresponds to p<0,01 and *** corresponds to p<0,001

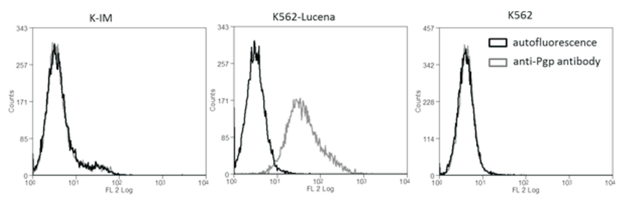

Figure 2: P-glycoprotein (Pgp) expression. Cells of K562, K562-Lucena and K-IM cell lines were incubated with anti-Pgp antibody and then analyzed through flow cytometry. Representative image of 3 independent experiments.

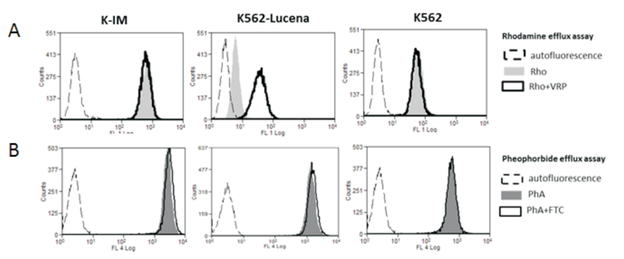

Figure 3: Evaluation of efflux transporters activity. Cells were incubated with the fluorescent substrate rhodamine 123 (Rho) in the presence or abscence of the Pgp inhibitor verapamil (VRP) and analyzed in the flow cytometer for the analysis of Pgp activity (A). Cells were incubated with the BCRP fluorescent substrate pheophorbide A (PhA) in the presence or absence of the BCRP inhibitor Fumitremorgin C (FTC) for the analysis of BCRP activity (B). Images representative of 3 independent experiments.

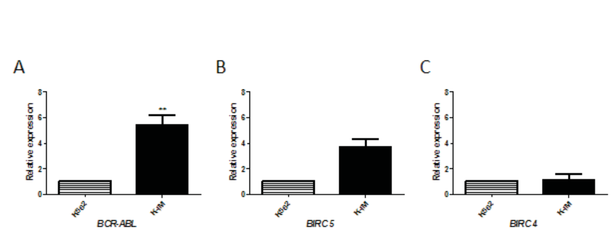

Figure 4: Quantification of mRNA levels. mRNA content of *BCR-ABL*, *BIRC 4* (encoding XIAP) and *BIRC 5* (encoding survivin) was determined in the K562 and in K-IM cell lines through quantitative real-time PCR. Statistical analysis was done using the one-way ANOVA test. ** corresponds to p<0,01.

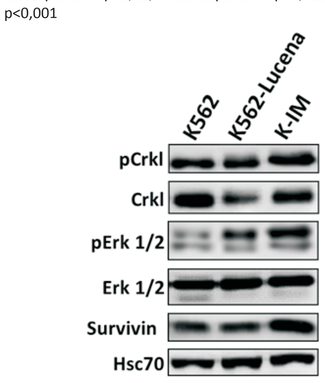

Figure 5: Protein evaluation. Basal levels of survivin, Crkl and Erk 1/2 protein and phosphorylated Crkl (pCrkl) and Erk 1/2 (pErk 1/2) were assessed through western blot. The Hsc70 protein was used as loading control. Image representative of 3 independent experiments.

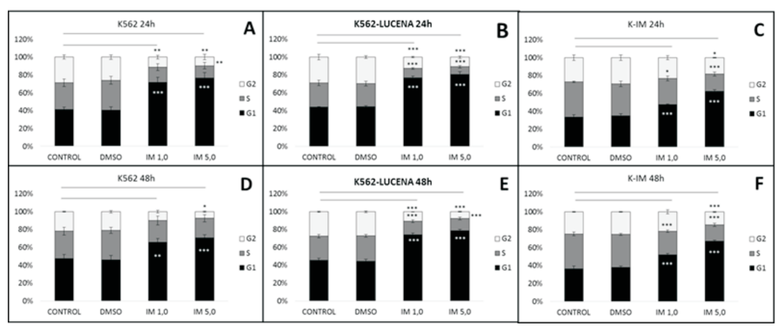

Figure 6: Cell cycle analysis. Cells were treated with imatinib for 24 (A,B,C) or 48 hours (D,E,F) before being stained with propidium iodide (PI). PI fluorescence was detected by a flow cytometer and cells were stratified in the cell cycle phases according to DNA content. Representative of 3 independent experiments. Statistical analysis was done using one-way ANOVA with Bonferroni post-test. * corresponds to p<0,05, ** corresponds to p<0,01 and *** corresponds to p<0,001.

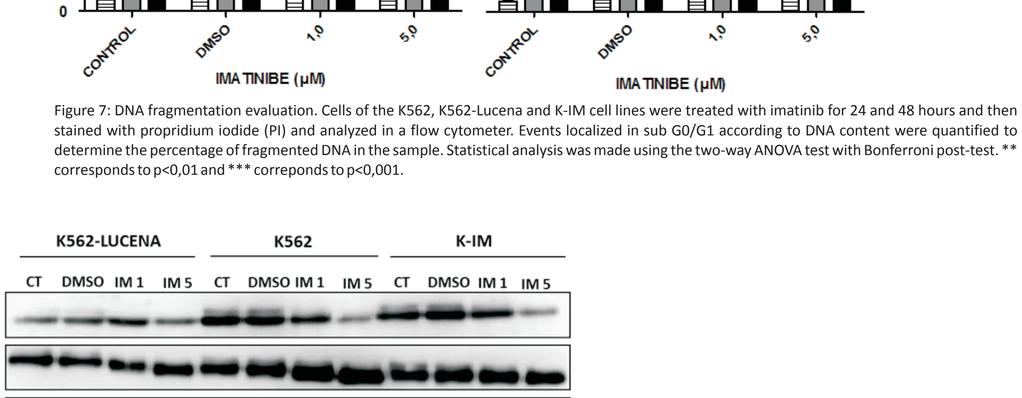

Figure 7: DNA fragmentation evaluation. Cells of the K562, K562-Lucena and K-IM cell lines were treated with imatinib for 24 and 48 hours and then stained with propridium iodide (PI) and analyzed in a flow cytometer. Events localized in sub G0/G1 according to DNA content were quantified to determine the percentage of fragmented DNA in the sample. Statistical analysis was made using the two-way ANOVA test with Bonferroni post-test. ** corresponds to p<0,01 and *** correponds to p<0,001.

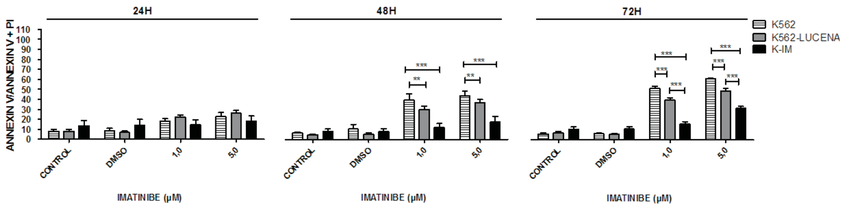

Figure 8: Death induction evaluation. Cells of the K562, K562-Lucena and K-IM cell lines were treated with imatinib for 24, 48 or 72 hours and death induction was evaluated in a flow cytometer according to annexin V and propidium iodide stainning. Representative of 3 independent experiments. Statistical analysis was performed using the two-way ANOVA test with Bonferroni post-test. ** corresponds to p<0,01 and *** corresponds to p<0,001.

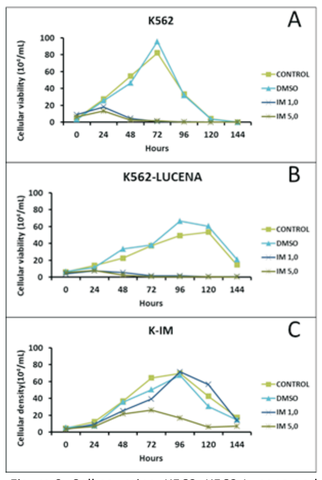

Figure 9: Cell counting. K562, K562-Lucena and K-IM were treated with imatinib and living cells were quantified every 24 hours through the trypan blue exclusion method.

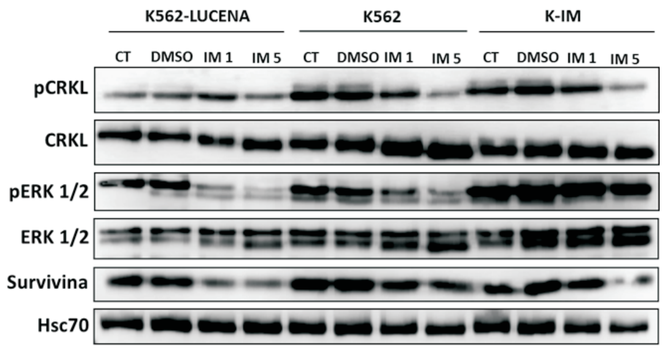

Figure 10: Protein evaluation after imatinib treatment. Cells of the K562, K562-Lucena and K-IM cell lines were treated with imatinib for 24 hours and protein content and phosphorylation status of Crkl, Erk and survivin were evaluated by the western blot method. Hsc70 was used as loading control.9

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