Deregulation of signaling pathways as an imatinib resistance mechanism in a chronic myeloid leukemia cell line

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Chronic myeloid leukemia (CML) panorama of treatment response, disease progression and survival rates changed drastically with the introduction of tyrosine kinase inhibitor (TKI)-based therapy with imatinib (IM). Despite its success and the development of more potent TKIs, the issue of resistance to treatment remains. Although *BCR-ABL* kinase domain (KD) mutations still the most frequent cause of resistance, the number of patients whose resistance is unrelated to mutations denotes the need for studying other resistance mechanisms. In order to address this question, a CML cell line resistant to IM, called K-IM, was selected in our laboratory by culturing K562 cells at increasing concentrations of IM up to 1.0 µM. Since this cell line harbors no BCR-ABL KD mutations, it constitutes a good model for the study of other mechanisms of resistance to IM. K-IM cells presented an increase in *BCR-ABL* messenger RNA (mRNA) levels, but neither an increase in Bcr-Abl activity nor an impaired inhibition by IM were observed. The K-IM cell line was significantly more resistant to IM than the parental cell line. Although treatment with 1.0 µM of IM promoted cell accumulation in the G0/G1 phases of the cell cycle, it was not accompanied by induction of cell death nor prevented the increase in the number of cells in culture. Since ABC transporters are determinants for the multidrug resistance phenotype, P-glycoprotein (Pgp) expression as well as Pgp and breast cancer resistance protein activity was evaluated, however, K-IM cells showed neither Pgp expression nor transporter activity. Another important mechanism is the deregulation of apoptosis pathways and the inhibitor of apoptosis proteins XIAP and survivin have been extensively studied as putative targets for cancer treatment. K-IM cells presented mRNA levels of XIAP similar to its parental cell line K562 and higher mRNA levels of survivin. Protein analysis confirmed an increase in survivin content, however its protein content was decreased by IM treatment. The deregulation of signaling pathways, such as MAPK/ERK, PI3K/AKT and JAK/STAT has emerged as a major mechanism of IM resistance. K-IM cells presented an increase in ERK1/2 phosphorylation levels that persisted after IM treatment. The treatment with MEK inhibitors alone reduced ERK1/2 phosphorylation but did not induce cell death in K-IM cells. Nevertheless the treatment with an STAT3 inhibitor resulted in significant cell death induction, suggesting this pathway to be related to K-IM cells IM-resistant phenotype. Our data suggests that different resistance mechanisms that promote resistance may occur simultaneously and that K-IM cell line is an intriguing model for the study of resistance mechanisms and putative drug targets.

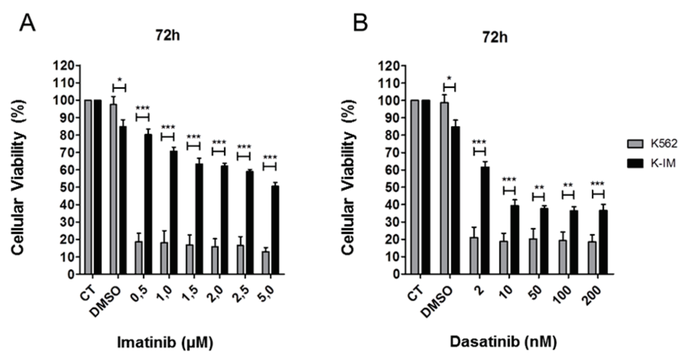

Figure 1: Cellular response to tyrosine kinase inhibitors. K562 and K-IM cells were incubated with different concentrations of imatinib (0.5 to 5.0 µM) (A) or dasatinib (2 to 200 nM) (B) for 72 hours and cell viability was assessed using the MTT assay. Data corresponds to 3 independent experiments bars represent standard error. Statistical analysis was performed using the two-way ANOVA test with Bonferroni post-test in which * corresponds to p<0,05; ** corresponds to p<0,01 and *** corresponds to p<0,001.

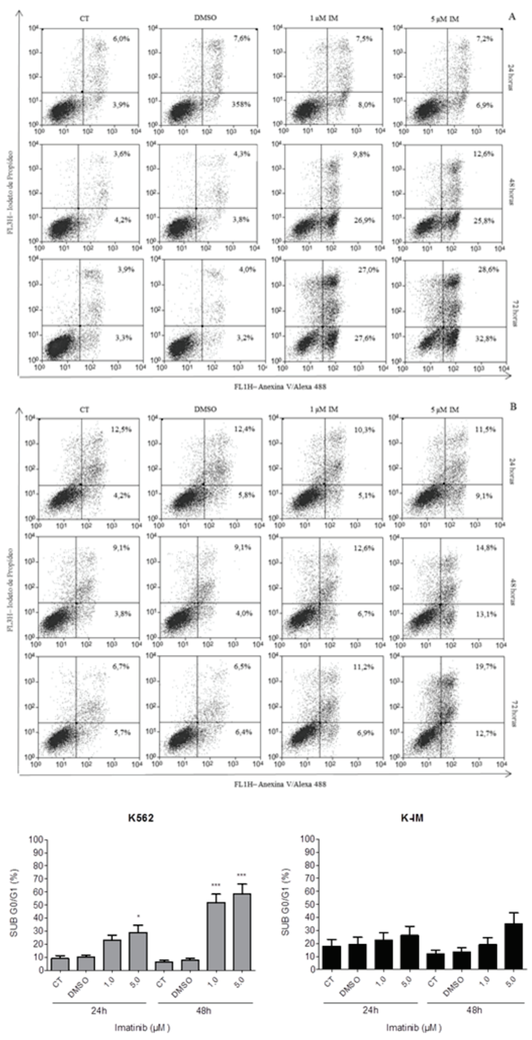

Figure 2: Cell death induction by imatinib. K562 (A) and K-IM (B) cells were treated with 1 or 5 µM imatinib or the vehicle DMSO for 24, 48 or 72 hours. Cell death induction was assessed by annexin V and propidium iodide double-staining. C) Statistical analysis was performed using the one-way ANOVA test with Dunnett post-test in which * corresponds to p<0,05; ** corresponds to p<0,01 and *** corresponds to p<0,001.

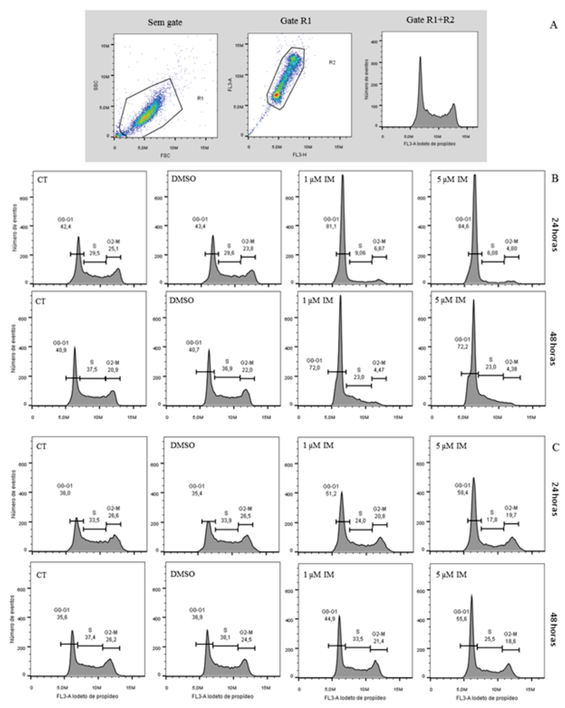

Figure 4: Cell cycle analysis after imatinib treatment. A) Exmaple of the selection strategies of the cells analysed for cell cycle distribution. K562 (A) and K-IM (B) cells were treated with 1 or 5 µM imatinib or the vehicle DMSO for 24 or 48 hours. Percentages of cells in each cell cycle phase is shown in the graphs.

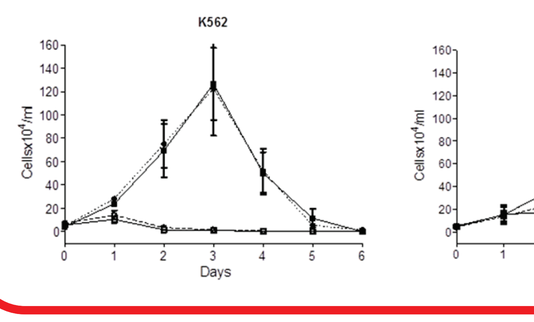

Figure 5: Cell growth evaluation. K562 (A) and K-IM (B) cells were treated with 1 or 5 µM imatinib or the vehicle DMSO and cells were counted using the tripan the tripean blue exclusion method every 24 hours for 6 days. Data corresponds to two independent experiments and bars represente standard deviation.

Figure 6: Evaluation of Bcr-Abl status: A) mRNA levels were analyzed using RT-qPCR in the K-IM cell line relative to K562 cell line. β-glucuronidase (GUSB) gene was used as endogenous control. B) Bcr-Abl activity was indirectly evaluated thourgh the CRKL phosphorylation levels, total CRKL and HSC70 protein levels were evaluated as control. C) For the assessment of Bcr-Abl inhibition by imatinib treatment, K562 and K-IM cells were treated with 1 or 5 µM of imatinib or the vehicle DMSO for 24 hours. The protein content and phosphorylation levels of CRKL protein were assessed using Western Blot. HSC70 protein was used as sample loading control.

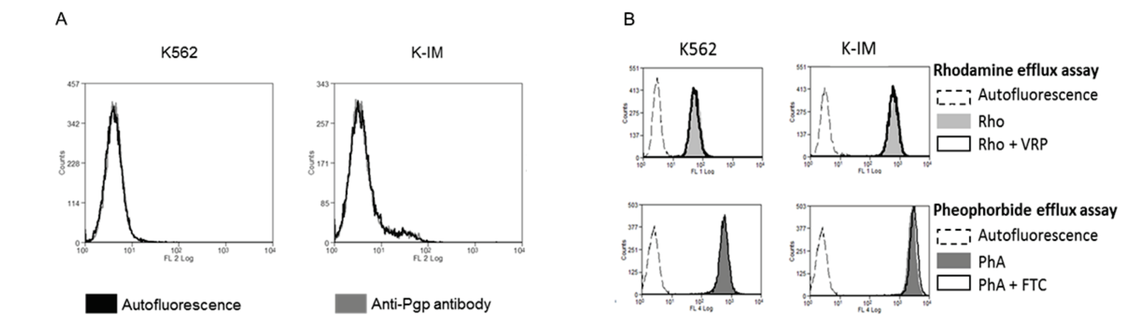

Figure 7: Evaluation of efflux transport proteins. A) K562 and K-IM cells were incubated with anti-P-glycoprotein (Pgp) antibody conjugated to ficoeritrin and analyzed in the flow cytometer. B) Pgp and breast cancer resistance protein (BCRP) activity was evaluated. Cells were incubated with the fluorescent substrate Rhodamine 123 (Rho) in the presence or absence of the Pgp modulator verapamil (VRP) and analized in the flow cytometer for Pgp activity. Cells were incubated with the fluorescent substrate Pheophorbide A (PhA) in the presence or absence of the BCRP modulator Fumitremorgin C (FTC) and analized in the flow cytometer for BCRP activity. Images are representative of 3 independent experiments.

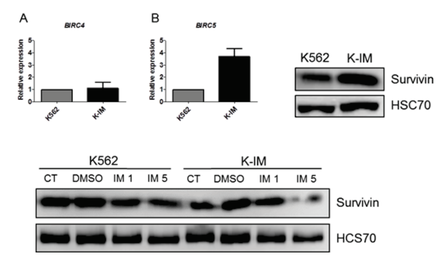

Figure 8: Evaluation of the inhibitor of apoptosis proteins. *BIRC 4* (XIAP) (A) and BIRC 5 (survivin) (B) mRNA levels were assessed using RT-qPCR. β-glucuronidase (GUSB) gene was used as endogenous control. C) Survivin protein levels were assessed by western blot and HSC70 protein was used as control. D) K562 and K-IM cells were treated with 1 or 5 µM imatinibe or the vehicle DMSO for 24 hours and survivin protein levels were assessed using western blot. HSC70 protein was used as control

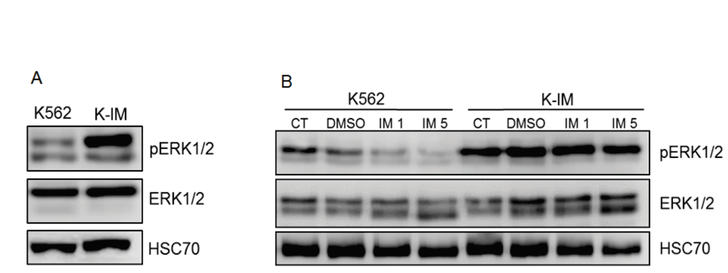

Figure 9: ERK1/2 proteins evaluation. A) ERK1/2 proteins total and phosphorylated levels were evaluated in K562 and K-IM cells. HSC70 protein was used as control. B) K562 and K-IM cells were treated with 1 or 5 µM imatinibe or the vehicle DMSO for 24 hours and ERK1/2 proteins total and phosphorylated levels were assessed using western blot. HSC70 protein was used as control.

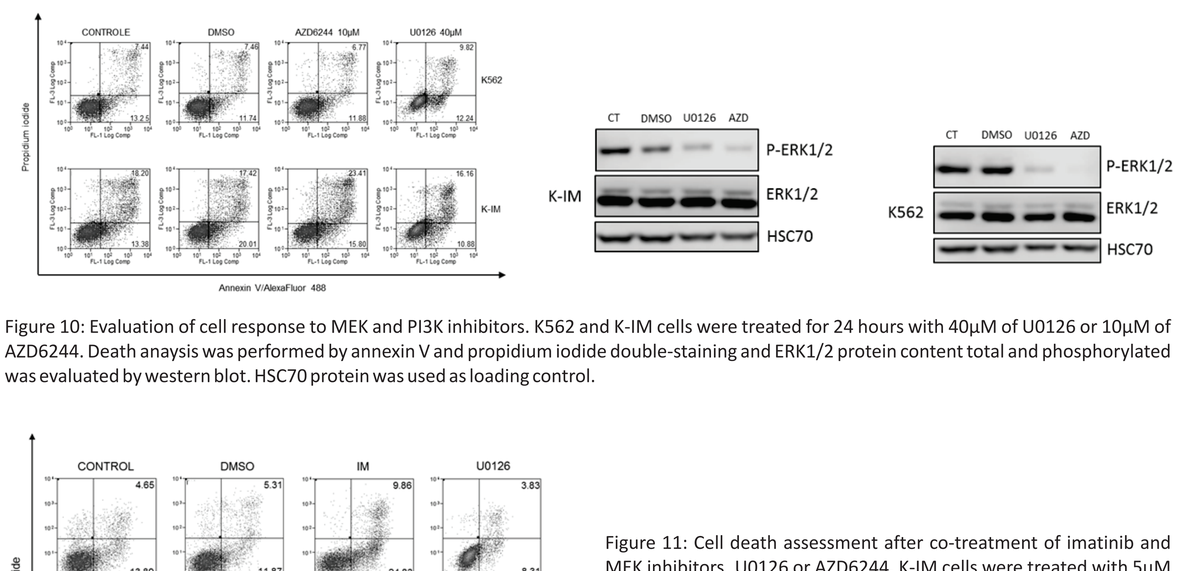

Figure 10: Evaluation of cell response to MEK and PI3K inhibitors. K562 and K-IM cells were treated for 24 hours with 40µM of U0126 or 10µM of AZD6244. Death anaysis was performed by annexin V and propidium iodide double-staining and ERK1/2 protein content total and phosphorylated was evaluated by western blot. HSC70 protein was used as loading control.

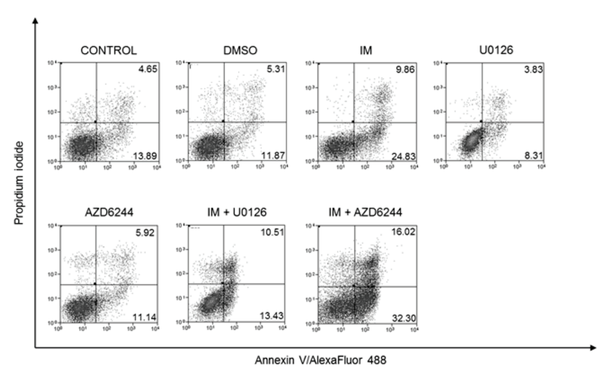

Figure 11: Cell death assessment after co-treatment of imatinib and MEK inhibitors U0126 or AZD6244. K-IM cells were treated with 5µM imatinib in the presence or absence of 40µM U0126 or 10µM AZD6244 for 48 hours and cell death was assessed by annexin V and propidium iodide double staining.

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